Selective inhibition of acetylcholinesterase 1 from disease-transmitting mosquitoes

Design and development of new insecticides for vector control

Cecilia Engdahl
“If you think you’re too small to make a difference, try going to bed with a mosquito in the room”

old African proverb
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Abstract

Acetylcholinesterase (AChE) is an essential enzyme with an evolutionary conserved function: to terminate nerve signaling by rapid hydrolysis of the neurotransmitter acetylcholine. AChE is an important target for insecticides. Vector control by the use of insecticide-based interventions is today the main strategy for controlling mosquito-borne diseases that affect millions of people each year. However, the efficiency of many insecticides is challenged by resistant mosquito populations, lack of selectivity and off-target toxicity of currently used compounds. New selective and resistance-breaking insecticides are needed for an efficient vector control also in the future.

In the work presented in this thesis, we have combined structural biology, biochemistry and medicinal chemistry to characterize mosquito AChEs and to develop selective and resistance-breaking inhibitors of this essential enzyme from two disease-transmitting mosquitoes.

We have identified small but important structural and functional differences between AChE from mosquitoes and AChE from vertebrates. The significance of these differences was emphasized by a high throughput screening campaign, which made it evident that the evolutionary distant AChEs display significant differences in their molecular recognition. These findings were exploited in the design of new inhibitors. Rationally designed and developed thiourea- and phenoxycetamide-based non-covalent inhibitors displayed high potency on both wild type and insecticide-insensitive AChE from mosquitoes. The best inhibitors showed over 100-fold stronger inhibition of mosquito than human AChE, and proved insecticide-potential as they killed both adult and larvae mosquitoes.

We show that mosquito and human AChE have different molecular recognition and that non-covalent selective inhibition of AChE from mosquitoes is possible. We also demonstrate that inhibitors can combine selectivity with sub-micromolar potency for insecticide resistant AChE.
Swedish summary


För att ta reda på om det är möjligt att skapa selektiva insekticider har vi studerat egenskaperna hos enzymet AChE från mygga och jämfört med egenskaperna hos det mänskliga enzymet. Vi identifierade viktiga funktionella och strukturella skillnader att ta fasta på i vår design av nya insekticider. Genom att experimentellt utvärdera ett substansbibliotek identifierade vi ett antal substanser som blockerade aktiviteten hos myggans AChE men som inte lika effektivt påverkade det mänskliga enzymet. De selektiva substanser som identifierades har vi därefter haft som kemisk utgångspunkt i vår design av nya substanser. Med hjälp av skillnaderna vi identifierat mellan enzymerna och de kemiska utgångspunktarna från utvärderingen så skapade vi ett stort antal nya substanser. Dessa användes för att kartlägga vilka av substansernas egenskaper som bidrar till önskad effekt på enzymen. Här presenterar vi de bästa substanserna för ändamålet, som dessutom visade sig blockera AChEs aktivitet även i mer komplexa sammanhang då de dödade de myggor och mygglarver som utsattes för dem.

I den här avhandlingen visar vi att det med noggrant designade substanser är möjligt att selektivt blockera myggans AChE utan att i någon större grad påverka det mänskliga enzymet. Vi har dessutom utvecklat ett stort antal nya substanser som uppvisar insekticidal effekt på sjukdomsspridande myggor. På sikt tror vi att forskningen som presenteras här kan bidra till att minska den globala bördan av myggburna sjukdomar.
List of publications

This thesis is based on the following publications, which are attached in the end and referred to in the text as paper I-IV.


*Authors contributed equally to the work.
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Comments on contribution

I. Performed most experimental work. Took part in all data analysis and was one of the main contributors to writing the manuscript.

II. Adapted the assay to a high throughput format and run the screens on both targets. Performed all follow up experiments and took part in the X-ray data collection and structure refinement. Contributed to the data analysis and to writing the manuscript.

III. Performed some and analysed all of the IC$_{50}$ determinations. Participated in the design and analysis of, and performed the *ex vivo* experiments. Performed cell toxicity testing of compounds and took part in the initial *in vivo* experiments. Contributed to writing the manuscript.
IV. Performed some and analysed all of the IC\textsubscript{50} determinations. Collected X-ray diffraction data and refined the structure. Participated in the experimental design and analysis of \textit{in vivo} experiments and contributed to writing the manuscript.

Publications not included in the thesis


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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>AaAChE1</td>
<td><em>Aedes aegypti</em> acetylcholinesterase 1</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>ADE</td>
<td>antibody-dependent enhancement</td>
</tr>
<tr>
<td>Ae. aegypti</td>
<td><em>Aedes aegypti</em></td>
</tr>
<tr>
<td>AgAChE1</td>
<td><em>Anopheles gambiae</em> acetylcholinesterase 1</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>An. gambiae</td>
<td><em>Anopheles gambiae</em></td>
</tr>
<tr>
<td>ATCh</td>
<td>acetylthiocholine</td>
</tr>
<tr>
<td>BTCh</td>
<td>butyrylthiocholine</td>
</tr>
<tr>
<td>CAS</td>
<td>catalytic site</td>
</tr>
<tr>
<td>cf.</td>
<td>conferre (&quot;compare&quot;)</td>
</tr>
<tr>
<td>ChOx</td>
<td>choline oxidase</td>
</tr>
<tr>
<td>Cx pipiens</td>
<td><em>Culex pipiens</em></td>
</tr>
<tr>
<td>DDT</td>
<td>dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DF</td>
<td>dengue fever</td>
</tr>
<tr>
<td>DHF</td>
<td>dengue hemorrhagic fever</td>
</tr>
<tr>
<td>DmAChE</td>
<td><em>Drosophila melanogaster</em> acetylcholinesterase</td>
</tr>
<tr>
<td>DSS</td>
<td>dengue shock syndrome</td>
</tr>
<tr>
<td>DTNB</td>
<td>dithiobisnitrobenzoate</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (&quot;for example&quot;)</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (&quot;and others&quot;)</td>
</tr>
<tr>
<td>etc</td>
<td>et cetera (&quot;and so forth&quot;)</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>hAChE</td>
<td><em>Homo sapiens</em> acetylcholinesterase</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HTS</td>
<td>high throughput screening</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est (&quot;that is&quot;)</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IRS</td>
<td>indoor residual spraying (of insecticides)</td>
</tr>
<tr>
<td>ITN</td>
<td>insecticide-treated bed-net</td>
</tr>
<tr>
<td>JE</td>
<td>Japanese encephalitis</td>
</tr>
<tr>
<td>k₉₉</td>
<td>turnover number</td>
</tr>
<tr>
<td>kᵢ</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>KM</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>mAChE</td>
<td><em>Mus musculus</em> acetylcholinesterase</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>OP</td>
<td>organophosphate</td>
</tr>
<tr>
<td>PAS</td>
<td>peripheral anionic site</td>
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<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Pdb</td>
<td>protein data bank</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>PTCh</td>
<td>propylthiocholine</td>
</tr>
<tr>
<td>RMSD</td>
<td>root-mean-square deviations</td>
</tr>
<tr>
<td>RVF</td>
<td>Rift valley fever</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SDM</td>
<td>site-directed mutagenesis</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>Sf</td>
<td><em>Spodoptera frugiperda</em></td>
</tr>
<tr>
<td>SR</td>
<td>selectivity ratio</td>
</tr>
<tr>
<td>SSR</td>
<td>structure-selectivity relationship</td>
</tr>
<tr>
<td>TcAChE</td>
<td><em>Torpedo californica</em> acetylcholinesterase</td>
</tr>
<tr>
<td>TPSA</td>
<td>total polar surface area</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>w/o</td>
<td>without</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum rate of reaction</td>
</tr>
<tr>
<td>YF</td>
<td>yellow fever</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström</td>
</tr>
</tbody>
</table>
1. Introduction

1.1. Infectious diseases

Infectious diseases are caused by pathogens such as bacteria, viruses, fungi, and other infectious agents.1 Humans can get infected by touching, eating, drinking or breathing something that contains an infectious pathogen. Some pathogens can also be transmitted by sexual contact or via the bite of an infected organism, i.e. a disease-vector such as mosquitoes. Historically, before the causative agents of these diseases were known and treatment became available, outbreaks were devastating and could wipe out whole communities. Two of the deadliest outbreaks in history are the outbreak of bubonic plague referred to as the Black Death that during the 14th century killed up to 60% of Europe’s population2 and the influenza-outbreak called the Spanish flu that killed approximately 50 million people by the ending of World War 1.3

Today, thanks to increased hygiene standards, scientific progress and the development of vaccines and antibiotics, mortality and morbidity caused by infectious diseases have decreased significantly. Still, 15% of the global total deaths are estimated to be caused by infectious diseases such as lower respiratory infections, tuberculosis, diarrheal diseases (cholera), AIDS/HIV, and malaria (Figure 1.1).4

![Figure 1.1. Number of global deaths from infectious diseases in 2000 and 2015. Data from reference 4.](image-url)
1.2. Vector-borne infectious diseases

Vector-borne infectious diseases refer to infections that are transmitted via a disease-vector. A disease-vector is a living organism, usually a bloodsucking insect or tick, with the ability to transmit pathogens between vertebrate hosts, including humans.\(^5\) Vector-borne infections are considered a significant global threat to public health as they cause over one million deaths annually and more than one billion people get infected each year.\(^6\) Malaria alone was the top seventh cause of death in low-income countries in 2015.\(^7\) It is estimated that half of the world’s population is currently living at risk of acquiring vector-borne diseases.\(^8\) Worst affected are tropical and subtropical regions, and concerned areas are often burdened with extreme poverty, lack of access to clean drinking water and proper sanitation.\(^6\) The two most serious mosquito-borne diseases, malaria and dengue, are introduced in the following section and additional important mosquito-borne diseases are mentioned briefly.

1.2.1. Malaria

Malaria is the vector-borne disease causing most deaths in the world, and was responsible for an estimated number of 429 000 deaths in 2015.\(^8\) As much as 70% of the affected were children under the age of six. Through a massive global effort, the burden of malaria has decreased between 2000 and 2015.\(^9\) An encouraging number of 17 countries eliminated malaria during that period, still over 200 million cases occurred worldwide in 2015.\(^8\)

Malaria is caused by infection of *Plasmodium* protozoan parasites that are transmitted to humans by *Anopheles* mosquitoes. *Plasmodium falciparum* is the most virulent species and responsible for 99% of the severe cases with a fatal outcome in humans.\(^8\) *Plasmodium falciparum* is the most common malaria-agent in Africa while *Plasmodium vivax* is the more abundant parasite in Asia and South and Central America.\(^10\)

Typical symptoms of uncomplicated malaria include a cyclic three-stage paroxysm of febrile episodes.\(^10,11\) Additional symptoms such as headache, nausea, vomiting, and general weakness are common for many other febrile diseases as well, making it difficult to distinguish malaria from other infections without laboratory-based diagnostics. Untreated uncomplicated infections can develop into severe malaria with life-threatening dysfunction of vital organs due to systemic infection and anemia.\(^10\)

Malaria is treatable if it is correctly diagnosed and treatment is initiated at an early stage. In 2015, Youyou Tu was awarded the Nobel Prize for her discovery of the drug artemisinin which is used to treat malaria (Figure 1.2). There is also chemoprophylaxis available for travelers going to endemic countries. The world’s first malaria vaccine will be taken into use in 2018, initially as a pilot implementation programme in Ghana, Kenya, and Malawi.
The phase III clinical trials of this vaccine (RTS,S/AS01) showed a vaccine efficacy of 36% in the age group of five to 17 months, after four doses.\textsuperscript{12}

![Chemical structure of artemisinin](image)

**Figure 1.2.** The chemical structure of the malaria drug artemisinin (left) that is isolated from the plant *Artemisia annua* (right), commonly used in Chinese traditional medicine.

### 1.2.2. Dengue

Dengue is an emerging mosquito-borne viral disease that caused an estimated number of 390 million mild or asymptomatic infections and 96 million apparent dengue cases in 2010.\textsuperscript{13} 70\% of the apparent dengue cases occurred in Asia where India alone bore 34\% of the global burden.\textsuperscript{13} Dengue is the fastest growing vector-borne disease in the world and can be caused by four virus serotypes.\textsuperscript{14} The virus is transmitted by *Aedes* mosquitoes such as *Aedes aegypti* and *Aedes albopictus*.\textsuperscript{15}

The clinical presentation of dengue range from asymptomatic or mild illness to severe disease with potential fatal outcome, reviewed in reference 16.\textsuperscript{16} The mildest form, called dengue fever (DF), is a self-limiting fever lasting for up to one week often in combination with severe headache, muscle ache, joint pain, nausea, and vomiting. DF can turn into the severe and life-threatening condition called dengue hemorrhagic fever (DHF) of which patients display thrombocytopenia (decreased level of thrombocytes in the blood), hemorrhagic manifestations, and plasma leakage. Critical levels of plasma leakage cause dengue shock syndrome (DSS) that is characterized by rapid, weak pulse and may result in death within 24 hours. Dengue infection generates long-lasting immunity to that particular virus serotype, but a secondary infection of another serotype may be associated with increased severity of the disease, a complex phenomenon called antibody-dependent enhancement (ADE).\textsuperscript{17}

Since 2016, there is one licensed vaccine available on the market, CYD-TDV (trade name Dengvaxia), which is a tetravalent live-attenuated virus vaccine based on the related yellow fever (YF) virus vaccine.\textsuperscript{18-20} Although research is intense and several compounds are patented,\textsuperscript{21} there is yet no licensed antiviral drug to treat dengue.
Other severe mosquito-borne diseases

Several other severe viral diseases are transmitted by *Aedes* mosquitoes, e.g. Chikungunya, Zika, YF, and Rift valley fever (RVF). Infections of these viruses usually cause a mild febrile illness that in some cases develop into severe clinical manifestations such as hemorrhagic fever (YF, RVF), infant microcephaly (Zika), encephalitis (YF), neurological disorders (RVF), or chronic joint pain (chikungunya). Japanese encephalitis (JE) is a viral illness spread by *Culex* mosquitoes in Asia. The acute symptoms include fever, headache, vomiting, confusion, and difficulty moving, while a later phase of the disease cause severe encephalitis with a case fatality rate at 30%. Lymphatic filariasis is a tropical neglected disease caused by nematode parasites that upon infection damage the lymphatic system, which can go unseen for years and finally result in lymphoedema. It is transmitted by *Culex, Aedes* and *Anopheles* mosquitoes. The socioeconomic burdens of isolation and poverty as a direct effect of these diseases are immense.

Vectors

Mosquitoes, ticks and flies are the most common disease-vectors. A vector's capacity to become infected and to transmit the pathogen to a susceptible host is defined as the vectors competence for that particular pathogen. Some important vectors, vector-borne pathogens, and the diseases they cause are listed in Table 1.1.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Pathogen</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mosquitoes</td>
<td><em>Anopheles</em> parasite</td>
<td>Malaria, Lymphatic filariasis*</td>
</tr>
<tr>
<td></td>
<td><em>Aedes</em> virus</td>
<td>Dengue, YF, Chikungunya, Zika, RVF</td>
</tr>
<tr>
<td></td>
<td><em>Culex</em> virus</td>
<td>JE, West Nile fever</td>
</tr>
<tr>
<td>Ticks</td>
<td><em>Hyalomma</em> virus</td>
<td>Crim-Congo hemorrhagic fever</td>
</tr>
<tr>
<td></td>
<td><em>Ixodes</em> bacteria</td>
<td>Lyme disease</td>
</tr>
<tr>
<td></td>
<td><em>Ixodes</em> virus</td>
<td>Tick-borne encephalitis</td>
</tr>
<tr>
<td></td>
<td><em>Dermacentor</em> bacteria</td>
<td>Tularaemia</td>
</tr>
<tr>
<td></td>
<td><em>Ornithodoros</em> bacteria</td>
<td>Relapsing fever (borreliosis)</td>
</tr>
<tr>
<td></td>
<td><em>Amblyomma</em> bacteria</td>
<td>Rickettsial fevers</td>
</tr>
<tr>
<td>Flies</td>
<td><em>Phlebotomus</em> parasite</td>
<td>Leishmaniasis</td>
</tr>
<tr>
<td></td>
<td><em>Simulium</em> parasite</td>
<td>Onchocerciasis (river blindness)</td>
</tr>
<tr>
<td></td>
<td><em>Glossina</em> parasite</td>
<td>African trypanosomiasis (sleeping sickness)</td>
</tr>
<tr>
<td>Aquatic snails</td>
<td>Various parasite</td>
<td>Schistosomiasis</td>
</tr>
<tr>
<td>Bugs</td>
<td><em>Triatoma</em> parasite</td>
<td>Chagas disease</td>
</tr>
<tr>
<td>Fleas</td>
<td><em>Xenopsylla</em> bacteria</td>
<td>Plague</td>
</tr>
</tbody>
</table>

*Also transmitted by *Aedes* and *Culex* mosquitoes.*
1.3.1. Mosquitoes as disease-vectors
The mosquito is sometimes considered to be the most dangerous animal in the world as it causes death and severe morbidity to the human population. About 100 of the approximately 3000 identified mosquito species are known to transmit human diseases. Epidemiology, field surveillance, as well as knowledge on mosquito biology and physiology are very important aspects for monitoring and controlling the spread of mosquito-borne diseases. Mosquitoes belong to the Culisidae family in the two-wing flying insect order called Diptera. They have a life cycle consisting of four life stages: egg, larva, pupa, and adult (Figure 1.3), where the larval stage itself consists of four distinct growth stages called 1st to 4th instar. Mosquitoes obtain sugar-rich nutrients by feeding on plant-nectar. Apart from this energy source, females also require a blood-meal to obtain proteins that are needed for egg production. The behaviour of mosquito species varies in many aspects, for example preferred breeding sites, host-specificity, pathogen-specificity, and oviposition. The behaviour of the mosquitoes influences the choice of vector control intervention (introduced later in this chapter).

Figure 1.3. The life cycle of a mosquito.

1.3.2. Anopheles mosquitoes
Mosquitoes of the Anopheles genera transmit malaria and have a large geographical distribution. Anopheles gambiae is the dominant vector species in sub-Saharan Africa, together with Anopheles arabiensis and Anopheles funestus. Anopheles stephensi is an important malaria vector in Middle East and Asia while Anopheles albimanus is more common in South and Central America.

Anopheles mosquitoes live close to humans; they bite and rest both in- and outdoors and are mostly active between sunset and sunrise. Therefore sleeping under a protective bed-net is recommended for humans. The
disease-transmitting species within the *Anopheles* genus are commonly described as highly anthropophilic (i.e. it prefers a human host over animals). *Anopheles* mosquitoes can be recognised by the presence of black dots on their wings and their typical resting position with the abdomen sticking up in the air (Figure 1.4). Breeding sites and larval habitat are typically located in sunlit, shallow, naturally occurring fresh water bodies, for example rice fields, in ditches or rain water puddles. Females lay their eggs singly on the water surface where they float until hatching.27

![Figure 1.4. Anopheles (left) and Aedes (right) mosquitoes. Photos kindly provided with courtesy of Anders Lindström.](image)

### 1.3.3. Aedes mosquitoes

Mosquitoes of the *Aedes* genera transmit a number of viruses capable of causing disease in humans (Table 1.1).29 *Aedes aegypti* and *Aedes albopictus* are the two most important vector species within this genus, and are recognised by their black and white striped legs (Figure 1.4).30 They are highly domesticated and are found in and around human habitats. *Ae. aegypti* originate from Africa and *Ae. albopictus* from Asia, but both are invasive species emerging to new areas of the world, bringing with them viral diseases to previously uninfected regions. Both the adaptive (becoming domesticated) and invasive (geographic spread) behaviour of these species make them dangerous from a public health perspective.31 In addition, their day-active behaviour is a challenge for vector control.

In contrast to *Anopheles*, *Aedes* mosquitoes lay the eggs on damp or moist ground where the eggs lay dormant until the area gets flooded, which can take up to months.30 *Ae. aegypti* populations that live in urban environments commonly place their eggs in non-natural containers, jars, cups, *etc.* inside and outside of houses.30
1.4. Vector control

Vector control has a central role in reducing the number of parasitic and viral infections, especially for the many mosquito-borne diseases where there is a lack of antivirals, vaccines, or treatment options. A good example of the huge impact that vector control has had during the last two decades is the large estimated number of avoided clinical cases of malaria. It was estimated that 663 million cases of malaria were avoided in 2000-2015, and that 81% of these could be attributed insecticide-based vector control interventions.\(^9\)

1.4.1. Insecticide independent vector control strategies

Personal protection is important to avoid mosquito bites and includes the use of repellents, protective clothing, and coverage with mosquito net at night. Also, manual removal of possible larval habitats in and around houses aid in the population reduction of disease vectors. Implemented biological vector control strategies primarily target the aqueous life stage of mosquitoes. Such strategies include the introduction of predators such as larvivorous fish, larvae of the mosquito genus *Toxorhynchites*, and water-living copepods, or the use of microorganisms such as *Bacillus thuringiensis israelensis* and several species of *Wolbachia* bacteria to cause infections in the mosquito.\(^{32}\) Ongoing research explores the possibilities to genetically modify mosquitoes to generate sterile males or mosquitoes with dominant lethal alleles.\(^{32}\)

1.4.2. Insecticide-based vector control interventions

A pesticide is a chemical substance used to kill, repel or control plants or animals considered as pests; insecticides specifically target insects. Choice of vector control intervention relates to e.g. the ecology and behavior of the mosquito-vector, aspects of human and environmental safety, vector resistance, cost, and social factors.\(^{33}\) The different vector control interventions all aim to lessen disease burden by reducing human-vector contact, vector survival or vector density, to suppress or halt pathogen transmission.

- **Insecticide-treated bed-nets (ITN)** primarily protect humans from mosquitoes that are active indoors at night (*Anopheles*). ITNs need to be re-impregnated regularly and be checked for holes to maintain their efficacy.
- **For indoor residual spraying (IRS)** all surfaces inside human habitats are sprayed with insecticides with the aim to kill mosquitoes if they land upon the surface. The spraying should be done regularly for continuous efficacy.
Larvicides are chemicals, surface oils or films administered to larval habitats to target the aquatic stage of mosquitoes with the aim to reduce vector density.

Space spraying is a method where an insecticide-containing fog is sprayed over a certain area using airplane, vehicle, or hand-held equipment. Efficacy of application depends on several factors such as method of release, weather, terrain, and target area size.

1.4.3. **The limited number of recommended insecticides**

There are four chemical compound classes of insecticides that are recommended by the World Health Organization (WHO) for controlling mosquito-vectors: chlorinated hydrocarbons, organophosphates (OPs), carbamates, and pyrethroids (Figure 1.5). These insecticides prevent the action of different proteins involved in nerve signaling, but give similar effect on the organism leading to paralysis and death. Chlorinated hydrocarbons and pyrethroids target voltage-gated ion channels in neurons, while OPs and carbamates inhibit the activity of acetylcholinesterase (AChE), a crucial enzyme in eukaryotic nerve signaling. Pyrethroids are the only approved compounds for ITN while all four classes are used for IRS. Mainly OPs, but also pyrethroids, are used for space spraying and larviciding.

A consequence of the limited number of insecticide classes and insecticide targets is the development of insecticide-related resistance, which highlights a vulnerability of current vector control strategies. On top of this, no new classes of insecticides have been recommended by WHO during the last decades, which is alarming.

**Figure 1.5.** The chemical structures of the main insecticide compounds used for control of malaria and dengue between 2000-2009, from the four recommended compound classes; chlorinated hydrocarbon (DDT), OP (malathion), carbamate (bendiocarb), and pyrethroid (cypermethrin).

1.4.4. **Insecticide resistance – a true setback**

Resistance to all four commonly used chemical classes of insecticides is emerging in mosquito populations, and thereby threatening the effectiveness of these compound classes. Of 75 malaria endemic countries, as many as 60 countries has identified mosquito strains being resistant to one, and 50 countries to two or more, insecticide classes as of 2015.
The mechanism of resistance is commonly grouped into three categories; metabolic resistance, target site resistance, and behavioral resistance. Metabolic insecticide resistance is linked to altered metabolic pathways or enzyme levels leading to a more rapid degradation or detoxification of the insecticide, often related to overproduction of detoxification enzymes such as cytochrome p450 monooxygenases. Target site resistance is a modification of the molecular target, causing a change in the target that reduce or abolish the potency and toxicity of the insecticide. A behavioral resistant mosquito has adapted its behavior to avoid contact with insecticides, often related to feeding and resting habits.

Strategies to slow down the emergence of resistant mosquito strains include rotation of the insecticides of choice over the years, the use of different interventions at the same location, mixture of compounds from different classes for the same intervention, and geographically mosaic spraying or use.

1.4.5. Additional concerns with current insecticides

The main environmental impact from insecticides can be related to the persistence of the hydrochloride DDT in soil and sediment, having serious long-term toxic effects on aquatic and bird wildlife. DDT was banned for use in America in 1972 but is still an important insecticide for malaria vector control in Africa. Another disadvantage of currently used pesticides is their toxic effect on non-target organisms, which causes both accidental and intentional intoxications of e.g. pets, birds, wildlife, important pollinators, and humans. Accidental and unintended intoxication in humans is commonly related to occupational exposure while intentional pesticide poisoning often relates to suicide attempts. In 2007 there was an estimated number of 258,000 deaths from pesticide self-poisoning globally and it was the most common method of suicide in China between 2006-2013.

1.5. Acetylcholinesterase

The evolutionary conserved, superefficient, and essential enzyme AChE terminates cholinergic signaling at synaptic clefts and neuromuscular junctions by hydrolyzing the neurotransmitter acetylcholine (ACh) (Figure 1.6). During neurotransmission, ACh is released into the synaptic cleft where it binds to ACh receptors at the post-synaptic membrane, transferring the signal to downstream nerve cells. Inhibition of this critical process by blocking the activity of AChE leads to a fast and complete disruption of cholinergic signaling, causing paralysis and eventually death. The important physiological role of AChE is one of the reasons why this enzyme has gained so much attention and has been studied for decades. AChE is the target for
several drugs, warfare nerve agents, and natural toxins, such as the extremely potent snake venom fasciculin. AChE is also the target of two of the four recommended insecticide classes; the OPs and the carbamates.

Figure 1.6. A schematic illustration of the biological role of AChE; hydrolysis of ACh in the synaptic cleft.

1.5.1. The structure of AChE
AChE (E.C. 3.1.1.7) is a serine hydrolase that belongs to the cholinesterase family of proteins. In vertebrates, it exist both in soluble and as cell surface-anchored forms.

The catalytic site (CAS) of AChE is located close to the bottom of a 20 Å deep and sterically confined gorge (Figure 1.7 A). The gorge is lined with aromatic amino acids and the entrance to the gorge is called the peripheral anionic site (PAS). Both the PAS and the aromatic lining of the gorge are involved in transient binding of ACh during its transport through the gorge. As for other serine hydrolases, the CAS contains a catalytic triad, in AChE constituted by Ser203, His447 and Glu334 (Homo sapiens AChE (hAChE) numbering used throughout the thesis, unless otherwise stated). Upon inhibition by OPs and carbamates, a covalent bond is formed with Ser203 thereby blocking the natural hydrolysis of ACh, resulting in accumulation of ACh and subsequent overstimulation of the nervous system.

The CAS can be described as several overlapping subsites (Figure 1.7 B). The anionic (choline) binding site includes Trp86 that interacts with the ammonium cation of ACh. This interaction positions the acyl-part of ACh towards the catalytic triad where hydrolysis is initiated by a nucleophilic
attack of Ser203 on the carbonyl carbon of ACh. The highly aromatic acyl pocket (formed by Trp236, Phe295, Phe297 and Phe338) has a shape complementarity to the methyl group in the acetyl moiety of ACh and stabilize the substrate during catalysis.\textsuperscript{57,68} In addition to these subsites, the oxyanion hole made up of residues Gly121, Gly122 and Ala204 stabilize the carbonyl oxygen of ACh during the transition state of the reaction.\textsuperscript{63} The aromatic phenols of Tyr124 and Tyr337 constitute the most narrow section of the active site gorge, referred to as the bottle neck or the waist of the gorge.

![Figure 1.7](image)

**Figure 1.7.** A) The 3D structure of hAChE with the active site gorge displayed as a surface, B) schematic figure of AChE indicating the subsites of the active site gorge.

### 1.5.2. AChE1 of mosquitoes

Most insects, including mosquitoes, carry two genes encoding AChE; ace-1 and ace-2, probably due to an old duplication.\textsuperscript{69} In true flies, \textit{e.g.} the well-studied fruit fly \textit{Drosophila melanogaster},\textsuperscript{70} there is only one ace gene present and it is suggested that the duplicated gene has been lost through evolution.\textsuperscript{71,72} A similar event has probably occurred during evolution of vertebrate AChE as \textit{e.g.} humans also carry one ace gene. It has been experimentally established that both AChE1 and AChE2 (from the ace-1 and ace-2 genes) are expressed in mosquitoes although AChE1 appear to have the central catalytic function.\textsuperscript{71}

The amino acid sequence identity of \textit{An. gambiae} AChE1 (AgAChE1) and \textit{Ae. aegypti} AChE1 (AaAChE1) is 93%. AChE1 has 48-49% amino acid sequence identity to hAChE and less to AChE of \textit{D. melanogaster} (DmAChE). The first crystal structure of any mosquito AChE was deposited to the PDB in March 2017 (pdb code: 5X61) showing the typical alpha/beta hydrolase fold and a high structural agreement with \textit{Torpedo californica} (Tc) AChE when superposed.\textsuperscript{73}
1.5.3. Insecticide insensitive AChE1

A naturally occurring target site mutation in AChE1 that mediates insecticide resistance is a glycine to serine conversion at position 122 (G122S, corresponding to G119S in TcAChE). The G122S mutation has been identified at least four times independently of each other in An. gambiae and the JE vector Culex pipiens. No equivalent mutation seems to occur in Ae. aegypti ace-1, probably due to the different codon for glycine that requires two point mutations for the conversion to serine. The G122S mutation affects the active site sterically and causes a fitness cost for the mosquito. The mutated form of AChE1 is insensitive to both OPs and carbamates. The mosquitoes carrying the mutation thereby pose a serious and acute threat to vector control and public health. Additional target site mutations in AChE1 causing insensitive mosquito populations are the F338W (F331W in TcAChE) in Culex triaeniorhynchus and the F297V (F290V in TcAChE) in Cx. pipiens, although none of these are (yet) as abundant as G122S.

1.5.4. Current development of AChE1 inhibitors

The majority of the research towards new AChE1 inhibitors focuses on the development of covalent inhibitors where two strategies have been explored. One approach is to re-design existing carbamate insecticides targeting the conserved catalytic Ser203, using the pharmacophore of propoxur as a chemical starting point. This has proven promising and some compounds display high selectivity ratio for AgAChE1 over hAChE. Also, carbamate-derivatives and difluoromethyl ketones showed inhibition of both AgAChE1-G122S and recombinant AgAChE1. Although promising, none of these compounds display the desired profile combining selectivity and G122S-potency.

Another approach is based on the presence of a unique cysteine in the active site gorge of AChE1 which is not present in vertebrate AChE. Spatially this cysteine corresponds to Phe295 in vertebrate AChE. Potent and selective inhibitors forming a covalent bond to the cysteine residue have been developed, but whether these compounds also inhibit the AgAChE1-G122S mutant has not been demonstrated to date.

To the best of our knowledge, before the work presented in this thesis started, non-covalent inhibitors have not been explored for their potential as selective inhibitors of AChE1. Alout et al. tested a non-covalent, reversible class of pyrimidinetron furan-substituted compounds that showed promising potency on AgAChE1 and AgAChE1-G122S both in vitro and on mosquito larvae, however, the selectivity profile of these compounds was not reported.
2. Objectives

The overall objective of our work is to contribute to decrease the global burden of mosquito-borne infectious diseases. Challenges for current vector control strategies include off-target toxicity, lack of selectivity of the current insecticides and the spread of insecticide resistant mosquito populations. To approach these challenges we will combine structural, biochemical and chemical approaches to develop selective and resistance-breaking non-covalent inhibitors of the essential enzyme AChE1 from two disease-transmitting mosquitoes.

Specifically, we aimed to:

1. Express and characterize AChE1 from *An. gambiae* and *Ae. aegypti* and compare structural and functional properties to vertebrate AChEs.

2. Discover chemical starting points for insecticide-development and investigate the molecular recognition of AChE1 and hAChE.

3. Exploit findings from 1 and 2 into the design and development of potent, selective and resistance-breaking non-covalent inhibitors of AChE1.

4. Explore the insecticidal potential of newly developed inhibitors by investigating their efficacy in *ex vivo* and *in vivo* systems.
3. Methods and assays

3.1. Production of AChE1

Traditionally, proteins to be studied were isolated from natural sources; from tissues, blood, plants, etc. For example, the initial characterization studies on AChE used the electric organs of electric eels (Electrophorus) and electric rays (Torpedo) as enzyme source.93,94 The studies presented in this thesis have mainly used recombinant enzymes, which often is advantageous with respect to yield and efficient purification methods. Production of AChE1 is presented in paper I.

3.1.1. AChE1 constructs

We have produced two protein constructs: full-length AChE1 proteins were used for all biochemical studies, while C-terminally truncated proteins (at ...VAAT536) were used in crystallization trials. In addition to these, the insecticide resistance conferring G122S point mutation in AgAChE1 was introduced. AChE1’s innate N-terminal signal sequence was kept in all protein constructs.

Modifications to the full-length construct (i.e. the truncated version and the mutant) were introduced by site directed mutagenesis (SDM). SDM is a molecular genetic technique based on polymerase chain reaction (PCR) that enables the introduction of site-specific mutations in a gene. Briefly, primers carrying the desired mutation were designed and used for amplification of the ace-1 gene. The modified gene was in excess after the PCR reaction, the “old gene” was enzymatically degraded. After purification of the plasmid the correct sequence was confirmed by sequencing and the construct was used to express the modified protein.

3.1.2. AChE1 expression

We have used the well-established baculoviral protein expression system for production of AChE1 from mosquitoes.95 Briefly, the ace-1 gene was incorporated into the baculovirus chromosome that, in turn, was transfected into the expression Spodoptera frugiperda-9 (Sf9) cell line, where it recruits the endogenous cellular polymerase to transcribe its own genes. In the very late phase of viral gene expression, the polyhedrin promoter is activated and ace-1, which is under control of this promoter, is over-expressed. The viral proteins assemble into virus particles, lyse the cells, and continue to invade nearby cells, increasing protein production exponentially. AChE1’s innate signal sequence directs it out of the cell; protein expression and secretion
was verified by AChE1 activity measurements of supernatants of infected Sf9 cells.

### 3.1.3. AChE1 purification

The purification of AChE1 was based on a purification protocol established for other AChEs. Chromatographic techniques used for protein purification involve a stationary and a mobile phase and separates proteins based on different properties e.g. size, charge, or specific binding affinity. Briefly, purification of AChE1 was performed in two chromatography steps. First the supernatant of the cell cultures was centrifuged and loaded on an affinity column with the ligand procainamide hydrochloride linked to the stationary phase. Following elution and concentration, the sample was loaded on a gel filtration column to separate the molecules according to size. The sample was thereafter concentrated or dialyzed depending on the subsequent use. The purification process was monitored by measuring the enzymatic activity before and after each purification step using the Ellman assay (introduced in section 3.2.1). Gel electrophoresis was used to visualize the purification progress and the purity of the final sample.

### 3.2. Assays to monitor AChE activity

AChE has been thoroughly studied in numerous laboratories worldwide since it was discovered as the target for nerve agents. Herein we have used functional assays to monitor the activity and inhibition of AChE in paper I-IV.

#### 3.2.1. Ellman assay

We have used a widely applied colorimetric assay to monitor the enzymatic activity of AChE (Figure 3.1). The activity-based assay was developed by George I. Ellman in 1961 and is called the Ellman assay. Briefly, enzyme catalyzed hydrolysis of the substrate analog acetylthiocholine (ATCh) is quantified in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Cleavage of DTNB (sometimes called the Ellmans reagent) by the hydrolyzing product thiocholine generates a yellow colored product. The change in absorbance at 412 nm is monitored over time and is directly proportional to the enzyme catalyzed hydrolysis of ATCh. In this work, the assay was typically performed at 30 °C using a substrate concentration of 1 mM in sodium phosphate buffer at pH 7.4 on secreted non-purified AChE1.
3.2.2. Choline oxidase assay

To investigate the potency of inhibitors (and other kinetic properties of AChE) with a method that is independent from the Ellman assay, we have evaluated another functional assay. This chemiluminescence assay has been suggested as a rapid and successful method to measure AChE activity\textsuperscript{99,100} and is herein named after the second enzyme in the assay reaction, choline oxidase (ChOx). Briefly, AChE catalyzed hydrolysis of the natural substrate ACh generates the products choline and acetate (Figure 3.2). Choline is subsequently oxidized by ChOx to betaine and hydrogen peroxide. Luminol is then reacted with the hydrogen peroxide by horseradish peroxidase (HRP) catalysis. This last step is a chemiluminescent reaction allowing a quantitative measurement of the reaction. The time dependent emission of chemiluminescence is monitored and is directly proportional to the AChE catalyzed hydrolysis of ACh.

Figure 3.2. Schematic illustration of the ChOx assay reaction.
3.2.3. Enzyme kinetics

We have used kinetic parameters to describe and categorize the function of AChE1. The kinetics of the enzymatic reaction mechanism catalyzed by AChE can be described by the Michaelis-Menten model below:

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P
\]

The enzyme (E) catalyzes the chemical reaction that transforms the substrate (S) into a product (P) via the formation of an enzyme-substrate complex (ES). \(k_1\) is the rate constant of the formation and \(k_1\) the dissociation of the ES complex. \(k_2\) is the rate constant for ES to EP conversion and EP dissociation (here shown as one step). \(k_2\) is the same as \(k_{\text{cat}}\), commonly called the enzymatic turnover number and is defined as the maximum number of substrate molecules that is converted to product per enzyme molecule and unit time. For non-allosteric enzymes following the Michaelis-Menten model, like AChE, the initial velocity \(V_0\) of a reaction depends on substrate concentration \([S]\) and thus \(V_0\) will increase as \([S]\) increases, until the system is saturated and the enzyme is working at maximal speed, \(V_{\text{max}}\). Once \(V_{\text{max}}\) is reached, all catalytic sites are occupied at any given time and small fluctuations in the concentration of the substrate will not affect \(V_0\).

The Michaelis constant \(K_M\) is the \([S]\) needed to reach half \(V_{\text{max}}\) and is an inverse indicator of the enzymes affinity for the substrate; the lower \(K_M\) the higher affinity for the substrate. The relation between reaction rate and substrate concentration is given by the Michelis-Menten (Equation 1) below:

\[
V_0 = \frac{V_{\text{max}}[S]}{K_M+[S]} \quad \text{(Eq. 1)}
\]

Using the Ellman assay, \(K_M\) was determined by measuring the \(V_0\) at different substrate concentrations. In many of our studies (e.g. determination of half-maximal inhibitory concentration (IC\(_{50}\), see below) we have assured that \(V_0\) is measured using a high substrate concentration \([S]>>K_M\) and that the formation of the product proceeds at a rate that is linear with time. However, \([S]\) has been below concentrations causing substrate inhibition in AChE1.

To determine \(k_{\text{cat}}\), the protein concentration \([E]\) must be known. In our case, we titrated the number of AChE1 using the OP ethyl ((2-[bis(propan-2-yl)amino]ethyl)sulfanyl)(methyl)phosphinate (VX) to determine the protein concentration. Once the protein concentration was determined, \(k_{\text{cat}}\) was calculated using Equation 2. Titration of the G122S mutant was not possible since it was resistant to OP compounds and thus \(k_{\text{cat}}\) was not determined.

\[
k_{\text{cat}} = \frac{V_{\text{max}}}{[E]_{\text{tot}}} \quad \text{(Eq. 2)}
\]
### 3.2.4. Inhibition studies

The potency of non-covalent inhibitors of AChE was investigated by determining their IC\(_{50}\) values, which is the concentration of the inhibitor needed to block 50% of the maximal enzymatic activity (Figure 3.3 A); the lower the value the better inhibitor potency. For IC\(_{50}\) determinations, \(V_0\) was determined immediately after addition of inhibitor solutions of different concentrations up to a maximum of 1 mM. Dose-response curves were generated by plotting the relative enzymatic activity against the logarithmic concentrations of the inhibitor.\(^{10}\) Covalent inhibitors were investigated by determination of their inhibition constants (\(k_i\)) to account for the time-dependent manner of a covalent inhibition (Figure 3.3 B). The higher the \(k_i\) value, the higher is the potency of the inhibitor. For \(k_i\) determinations, samples were pre-incubated in the presence of the inhibitor at specified concentrations and \(V_0\) was measured at several time-points until no further decrease in activity could be observed. \(k_{obs}\) values were obtained from a linear regression of curves plotted from the logarithmized relative activity and time. When plotting \(k_{obs}\) as a function of inhibitor concentration, \(k_i\) can be obtained from the resulting slope.

![Figure 3.3](image.png)

**Figure 3.3.** Example figures of A) a dose-response curve used to determine an IC\(_{50}\) value, B) a plot of \(k_{obs}\) at different concentrations of the inhibitor, used to obtain \(k_i\).

### 3.3. Small organic molecules as chemical tools

Our approach to find new compounds that target insect AChE1 resembles the general approach of a drug discovery process. The steps include target selection and characterization (**paper I**), hit compound discovery and validation (**paper II**), hit to lead development, *in vitro* and *in vivo* studies (**paper III** and **IV**). For all steps, small organic molecules have been used as chemical tools.

#### 3.3.1. Hit discovery by high throughput screening

High throughput screening (HTS) of a chemical library is a common method to identify chemical starting points and often used in the early phases of a
drug discovery campaign. In this context, we screened a compound library consisting of 17,500 synthetic, drug-like molecules searching for inhibition of Ag- and AaAChE1. The activity-based Ellman assay (described in section 3.2.1) was semi-automated and adapted to a 96-well plate format. The average slope of negative control-wells was set to 100% activity and all compounds ability to inhibit AChE1 was expressed in relation to this. A reference plate consisting of eight unique compounds with known AChE1-inhibition profiles and eight replicates per compound per plate was run as every 10-20th plate in order to monitor the stability of the system. The reference plates were used to evaluate the robustness of the screening campaign.

3.3.2. Design and use of synthesized analogues
Our approach to study structure-activity relationships (SAR) and structure-selectivity relationships (SSR) between inhibitors and AChE was to first identify the core chemical structure of an interesting hit compound. Secondly, the core structure was altered with regards to substituents, linkers, functional groups and other features to generate sets of analogues that span the chemical space. Third, the newly synthesized analogues were biochemically evaluated, usually by IC50 determinations and X-ray crystallography. This data was analyzed for SAR or SSR to identify structural features or properties of the inhibitors that contribute to potency and/or selectivity. This was commonly an iterative process where the SAR/SSR analyses lead us back to the design of more analogues.

3.3.3. Evaluation of unwanted toxicity of compounds
An important step when transferring compounds tested in vitro to the more complex system of a living mosquito (in vivo) is to investigate unwanted toxicity of the compound. Here, we used the compound resazurin as an oxidation-reduction indicator. Resazurin is a blue and weakly fluorescent molecule that turns to the pink fluorescent resorufin upon reduction. Briefly, exponentially growing insect cells (Sf9) were exposed to newly synthesized AChE1 inhibitors of various concentrations for 24 hours. Thereafter, the media of the treated cells were replaced with fresh serum-free growth medium mixed with a solution of resazurin. After 3-4 h incubation at optimal growth conditions, the metabolic reduction of resazurin (blue) to resorufin (pink) was measured by visual inspection and by fluorimetry. The irreversible reaction of resazurin to resorufin is proportional to aerobic respiration and thus can be used to determine cell viability and cell toxicity of the compounds. To evaluate the safety of handling new compounds by workers a human cell line is preferred.
3.4. Methods to study AChE structure

Structure determination by X-ray crystallography was used to determine *Mus musculus* AChE (mAChE)-inhibitor complexes in paper II, IV and chapter 6. These structures were useful when investigating binding modes, interactions and dynamics.

3.4.1. Protein crystallization

Once a pure protein sample of AChE1 had been obtained we screened for crystallization conditions using commercially available crystallization screens, at two protein concentrations. We used sitting drops and the vapor diffusion technique where a drop containing a mixture of the protein and the precipitant solution is in a closed system with the precipitant solution in a reservoir (Figure 3.4). The drop has a lower concentration of precipitant and will evaporate to reach equilibrium. During this process, the concentration of the protein and precipitant will increase and under favorable conditions the proteins will crystallize. Protein crystallization is commonly the rate limiting step in the structure determination of proteins.

Several parameters were further explored to improve the size and quality of AChE1 crystals; *e.g.* protein concentration, buffer pH, addition of small organic molecules, and the hanging drop method. Surrounding environmental factors like temperature, darkness, and humidity may also influence crystallization although it was not investigated herein: crystallization trials were performed at 4 °C.

![Figure 3.4.](image)

**Figure 3.4.** The vapor diffusion technique can use either sitting or hanging drops.

3.4.2. X-ray data collection and structure refinement

The crystal structures reported in this thesis are all protein-inhibitor complexes, and were used to study binding poses of the ligands and identify important non-covalent interactions between the inhibitor and AChE. To generate a complex, a protein crystal was soaked with a solution saturated with the compound before it was flash-frozen in liquid nitrogen. During data collection, the crystal was exposed to X-rays that interact with the electron cloud of the protein atoms in the crystal. If the initial quality of the crystal is
good and the crystal survives soaking and flash freezing, a diffraction pattern of scattered waves can be recorded (Figure 3.5). The reason for using crystals is that scattered waves from electrons of a single protein are not intense enough to detect, but waves amplified by the millions of molecules that are ordered in a crystal lattice reinforce each other and amplifies the signal. The raw data (i.e. diffraction pattern and intensities of the recorded reflections) collected at the synchrotron together with information from a previously determined structure of mAChE (pdb code: 1J06) was used to calculate an initial electron density map (Figure 3.5). Ideally, the electron density map describes the electron cloud of all atoms in the complex. Using molecular graphics, an atomic model of the protein and the inhibitor that is consistent with the electron density map was built. The model was refined in a number of cycles alternating between computational refinement and manual rebuilding of the atomic coordinates. X-ray diffraction data presented in this thesis were collected at the MAX-lab synchrotron (Lund, Sweden) and the BESSY synchrotron (Berlin, Germany).

Figure 3.5. Examples of a diffraction pattern (left) and an electron density map of mAChE in complex with an inhibitor (right).

3.5. In vivo studies

The development process of new insecticides includes three phases that an optimized lead compound needs to pass on its way to a final product. Compounds developed in this thesis have so far been tested in phase one trials (paper III and IV). Phase one is the laboratory studies of the compounds insecticidal effect, where the compounds are investigated for whether they have the ability to kill mosquitoes or not. Phase two involves small-scale and phase three large-scale field trials.102
3.5.1. **Mosquito rearing**
Investigation of inhibitors insecticidal effect in vivo was performed at the Kenya Medical Research Institute (KEMRI) in Nairobi, Kenya. Continuous rearing of *Ae. aegypti* Mombasa strain and *An. gambiae* Kisumu strain were done in species-separate trays (for larvae) and cages (for adults) in an insectary that mimics their natural climate factors. Larvae were kept in dechlorinated water, fed on finely ground larval fish food, and maintained at optimal larval concentrations to avoid possible effects of competition. Pupae were picked daily from the larval trays and transferred to water-filled cups that were placed in cages to allow emergence of adult mosquitoes. Cotton balls soaked in glucose solution were provided as an energy source. The mosquito life cycle was kept continuous by allowing adult mosquitoes to blood feed on anesthetized mice or hamsters after six hours of starvation. Female mosquitoes then lay their eggs on moist filter paper or water filled petri dishes placed at the bottom of the cage that were subsequently transferred to shallow water buckets for hatching.

3.5.2. **Insecticidal effect of compounds**
Phase one insecticidal activity tests of the compounds were carried out based on the WHO's guidelines for testing adulticides and larvicides. Topical application tests were performed on adult mosquitoes. Briefly, female mosquitoes were anesthetized by being placed in the freezer and thereafter the compound or control solution in 0.1 µL acetone was deposited on the upper part of the pronotum (the mosquitoes back). After the topical application, the mosquitoes were returned to the insectary, where they were supplied with a glucose meal and maintained under standard conditions. Mortality rates were recorded 24 and 48 h post application. For the larvicidal test, larvae at third instar were introduced into a cup containing compound dissolved in de-chlorinated water. The larvae were then fed and maintained in the insectary under standard conditions. Mortality rates were recorded 24 and 48 h post exposure.
4. Functional characterization of AChE1 and comparisons with vertebrate orthologues

The aim of the work presented in this thesis was to design and develop potent and selective inhibitors of the pesticide target AChE1. In a rational design, fundamental characteristics of the target protein direct the design of potent inhibitors. In this chapter and in paper I, functional, structural and physical properties of the essential enzyme AChE1 was characterized. By including vertebrate AChEs from mouse and human in our studies, we were able to compare evolutionary distant AChEs, to gain insight into their catalytic function, molecular recognition, and to investigate the potential to design selective inhibitors of AChE1.

4.1. Expression of catalytically active AChE1

Recombinant AChE1 of the disease-transmitting mosquitoes An. gambiae (Ag) and Ae. aegypti (Aa) were produced by the baculovirus expression system and the insect cell line Sf9. In contrast to bacterial expression, the Sf9 cell line enables post-translational modifications of eukaryotic proteins. Sf-based cells together with baculoviral expression of proteins are widely used and may produce high yields of soluble proteins. In addition, this system has been successfully used by others to express insect AChEs.

Full-length constructs of AgAChE1 and AaAChE1, covering 737 and 702 amino acids, respectively, with the innate secretion signal included in the amino-terminus of 161 and 128 amino acids, were produced and used for kinetic studies. The expression from adherent Sf9 cells resulted in approximately 10 mg/L of secreted enzyme into the growth medium. However, expression from suspension cultures of Sf9 increased the level of expression about threefold due to the possibility to keep a higher density of the cells. The enzymes were produced into the serum-free growth medium and all following kinetic studies were performed without any further purification of the secreted enzyme.

4.2. Kinetic characterization of AChE1

In paper I, some basic catalytic parameters including kinetic constants, substrate preferences and substrate inhibition of AaAChE1, AgAChE1 and AgAChE1-G122S were investigated and compared with the properties of the corresponding vertebrate AChEs. The recombinant enzymes AgAChE1 and AaAChE1 showed similar enzyme kinetics with some minor but important differences from m- and hAChE. Both AChE1 enzymes preferred small
substrates (ATCh) over larger substrate analogues (Figure 4.1 and 4.2 A), as was seen by $V_{\text{max}}$ and $k_{\text{cat}}$ (Figure 4.2 A and Table 4.1). Also, a clear substrate inhibition was observed for both $Ag$AChE1 and $Aa$AChE1 at substrate concentrations exceeding 0.5-1 mM (Figure 4.2 B). These findings agree with earlier observations for vertebrate AChEs$^{67,68}$ and invertebrate AChEs.$^{105,109,111}$ Similar substrate preference and substrate inhibition was also found for the $Ag$AChE1-G122S mutant (Figure 4.2 B and Table 4.1).

The relative catalytic efficiency given by the ratio of $V_{\text{max}}/K_M$ indicated that the mosquito enzymes may hydrolyze larger substrates more efficiently than the corresponding vertebrate enzymes (Table 4.1). No catalysis of BTCh was observed for $m$- or $h$AChE. This finding suggests a larger gorge for AChE1 as it accommodated and hydrolyzed larger substrates in comparison to the vertebrate AChEs.

![Figure 4.1. The chemical structures of the substrate analogues used to investigate substrate preferences.](image)

![Figure 4.2. A) Michaelis-Menten graph of AaAChE1 using the three different substrates, B) substrate inhibition of AaAChE1, AgAChE1 and AgAChE1-G122S.](image)

The recombinant AChE1 enzymes displayed low $K_M$ values for the preferred substrate ATCh with values of 27 µM for $Ag$AChE1 and 25 µM for $Aa$AChE1. As a reference, the $K_M$ value for $h$AChE was determined to 146 µM. Interestingly, the $Ag$AChE1-G122S mutant enzyme displayed an intermediate $K_M$ value of 58 µM. The higher affinity for the substrate of the mosquito enzymes may contribute to the observed lower rate of hydrolysis for AChE1; the $k_{\text{cat}}$ values of 124-140 s$^{-1}$ was about 100-fold lower than what has been reported for $h$AChE.$^{68}$ The $k_{\text{cat}}$ values reported here and in paper I were also lower than previously reported turnover numbers for $Ag$AChE1 (650 and 3000 s$^{-1}$).$^{105,84}$ The differences might be related to the use of different experimental methods, assay conditions and/or different protein constructs.
Table 4.1. Kinetic parameters of Aa-, AgAChE1, AgAChE1-G122S, h- and mAChE.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$V_{max}$ (mA/min)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$V_{max}/K_M$</th>
<th>$K_M$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCh</td>
<td>406 (389–422)</td>
<td>140</td>
<td>16.2</td>
<td>25 (20–30)</td>
</tr>
<tr>
<td></td>
<td>PTCh</td>
<td>266 (258–275)</td>
<td>92</td>
<td>13.3</td>
<td>20 (17–23)</td>
</tr>
<tr>
<td></td>
<td>BTNCh</td>
<td>38 (36–40)</td>
<td>13</td>
<td>0.8</td>
<td>45 (36–54)</td>
</tr>
<tr>
<td>AaAChE1</td>
<td>ATCh</td>
<td>528 (505–552)</td>
<td>124</td>
<td>19.6</td>
<td>27 (21–33)</td>
</tr>
<tr>
<td></td>
<td>PTCh</td>
<td>407 (393–421)</td>
<td>95</td>
<td>16.3</td>
<td>25 (20–29)</td>
</tr>
<tr>
<td></td>
<td>BTNCh</td>
<td>58 (55–61)</td>
<td>14</td>
<td>1.6</td>
<td>36 (27–45)</td>
</tr>
<tr>
<td>AgAChE1</td>
<td>ATCh</td>
<td>318 (310–326)</td>
<td>-</td>
<td>5.5</td>
<td>58 (52–65)</td>
</tr>
<tr>
<td></td>
<td>PTCh</td>
<td>105 (87–123)</td>
<td>-</td>
<td>0.27</td>
<td>303 (172–435)</td>
</tr>
<tr>
<td></td>
<td>BTNCh</td>
<td>34 (24–45)</td>
<td>-</td>
<td>0.08</td>
<td>431 (136–727)</td>
</tr>
<tr>
<td>AgAChE1-G122S</td>
<td>ATCh</td>
<td>821 (782–860)</td>
<td>-</td>
<td>5.6</td>
<td>146 (128–165)</td>
</tr>
<tr>
<td></td>
<td>PTCh</td>
<td>429 (415–442)</td>
<td>-</td>
<td>2.9</td>
<td>150 (137–162)</td>
</tr>
<tr>
<td></td>
<td>BTNCh</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>hAChE</td>
<td>ATCh</td>
<td>725 (694–756)</td>
<td>-</td>
<td>8.6</td>
<td>84 (72–95)</td>
</tr>
<tr>
<td></td>
<td>PTCh</td>
<td>367 (354–380)</td>
<td>-</td>
<td>6.2</td>
<td>59 (51–66)</td>
</tr>
<tr>
<td></td>
<td>BTNCh</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mAChE</td>
<td>ATCh</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PTCh</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BTNCh</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Parentheses indicate the 95% confidence interval (CI).

Post translational glycosylation was investigated for Ag- and AaAChE1 using LC-MS/MS (data presented in more detail in paper I). The mass spectral data indicated N-linked glycosylation at position Asn509 and three possible variants of a core glycopeptide were identified. In addition to the Asn509 site, Han et al. also identified a glycosylation site at Asn220 based on crystallographic data.\(^\text{73}\)

4.3. Using inhibitors to profile the molecular recognition of AChEs

To investigate the molecular recognition of mosquito AChE1s, chemically distinct inhibitors were used to profile the sensitivity of evolutionary distant AChEs. A panel of seven previously reported AChE inhibitors with different mechanisms of action was used (Table 4.2).

Analogous to the characterization of basal kinetic parameters, no significant difference between AgAChE1 and AaAChE1 was observed based on this panel of inhibitors. Surprisingly, the experiments revealed important differences between the mosquito- and vertebrate enzymes. For example, the covalent inhibitors propoxur (1) and eserine (2) displayed a tenfold stronger inhibition of AChE1 compared to the vertebrate AChEs. Furthermore, the non-covalent inhibitor ethopropazine (3) selectively inhibited AChE1 while C7653 (4) displayed an unselective profile, and donepezil (5), C568R (6) and
C568S (7) were more potent inhibitors of h- and mAChE. These differences encouraged the concept of designing inhibitors that target the enzymes selectively.

Table 4.2. Inhibition analysis of five AChE proteins.

<table>
<thead>
<tr>
<th>ID</th>
<th>Compound</th>
<th>( k_i ) values of covalent inhibitors (( \mu M \cdot min^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AaAChE1</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.1-1.4)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(18-32)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IC( _{50} ) values of non-covalent inhibitors (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
</tr>
<tr>
<td>----</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
</tbody>
</table>

Parentheses indicate the 95% confidence interval (CI). *not determined. \( b \)values in nM, \( *12, **13 \).

4.4. Inhibition of the G122S mutant

The G122S mutant showed similar sensitivity to the covalent inhibitor 2 as the vertebrate AChEs (Table 4.2). On the other hand, the G122S mutant was insensitive to the carbamate 1. Interestingly, when using non-covalent inhibitors, AgAChE1-G122S displayed a mixed profile, somewhere between the vertebrate and the mosquito enzymes. The IC\( _{50} \) values of AgAChE1-G122S for the compounds 5, 6, and 7 were very similar to the unmodified mosquito enzymes. However, in contrast to the selective inhibition of AChE1 by 3, no inhibitory effect on the AgAChE1-G122S mutant was observed up to the tested concentration of 1 mM. The G122S mutant displayed a slightly reduced affinity for the so far non-selective compound 4 compared to the other four enzymes, with an approximately threefold higher IC\( _{50} \) value. The mixed profile of the G122S mutant indicated that some chemotypes also
allow selective inhibition of the insensitive mutant over vertebrate AChEs. This is a promising and desirable feature from an insecticide development point of view.

The enzymatic characterization and the inhibition profiles of evolutionary distant AChEs indicated a possibility to design potent and selective compounds, and encouraged a HTS campaign to identify chemical starting points for this purpose (paper II).

4.5. Summary and conclusions

- Active AChE1 enzymes of the two most important disease-transmitting mosquitoes were successfully produced using a baculovirus expression system and insect cells.

- The mosquito and vertebrate enzymes are functionally similar; small but important differences in their basal kinetics and sensitivity for inhibitors indicates a possibility to target the enzymes selectively.

- Based on the characterization performed herein, no significant differences were observed between AgAChE1 and AaAChE1.

- The insecticide resistant G122S mutant commonly displayed a mixed profile between the AChE1 and vertebrate AChEs, both regarding kinetics and inhibition.
5. Identification of diverse and selective AChE1 inhibitors by differential HTS

HTS is a method that enables the testing of a large number of compounds in a short time period. Screening large compound libraries requires a stable and simple assay that preferably can be automated and scaled down to small assay volumes. The aim of our screening project (reported in paper II) was twofold: 1. to discover small molecules to be used as tools in our exploration of similarities and differences between mosquito and vertebrate AChE proteins, 2. to identify chemical starting points to be developed into potent and selective insecticides.

5.1. Identification of AChE1-hits

An HTS campaign was conducted, separately targeting recombinant AChE1 from An. gambiae and Ae. aegypti with 17 500 small organic molecules. The Ellman assay was semi-automated, adapted to a 96 well plate format and used for the screening. In total, 338 unique hits were identified in the screen where the compounds’ ability to inhibit the enzymatic activity at 50 µM were tested (Figure 5.1). Compounds ability to reduce the enzymatic activity ≥ 3 x standard deviation of the mean for the 17 500 compounds were regarded as a hit. This corresponded to at least 33% and 31% inhibition of AgAChE1 and AaAChE1, respectively. The two mosquito enzymes behaved very similar in the HTS, consistent with our findings in paper I, only a small group of molecules displayed different inhibition capacity of the two enzymes at this one concentration.

The results from the reference plate, which was repeatedly tested throughout the screening, indicated no trends in the data over time, no effect on flanking wells and no effect on enzyme activity over time.

![Figure 5.1](image-url) The differential HTS generated a total of 338 unique hits for AgAChE1 and AaAChE1. 163 compounds were categorized as potentially selective for AChE1 over hAChE.
5.2. A large diversity within the AChE1 hits

A manual inspection of the hits revealed that the hit compounds comprise a chemically diverse set of molecules. All AChE1 hits were sorted into classes based on their core chemical structure, and eight compound classes were chosen for further investigation. These compounds varied in their calculated physicochemical properties; the molecular weights varied between 253 and 482 g/mole, the logarithm of the octanol/water partition coefficient (clogP) describing hydrophobicity spanned from -1.1 to 5.4, and the number of rotatable bonds relating to flexibility spanned from 3 to 8. To visualize the diversity within the group of hit compounds, principal component analysis (PCA)\textsuperscript{115,116} of all eight compound classes was performed based on 2D physicochemical descriptors. The result confirmed the manual inspection and illustrated the diversity among the hits’ properties. The largest variation was observed for molecular size and hydrophilicity (Figure 5.2).

Representative OPs and carbamates were predicted by the PCA-model and projected into the chemical space established by the eight compound classes. Figure 5.2 shows that the AChE1 hits spanned a different and larger property space than these insecticides.

Figure 5.2. The result of a PCA presented as a score plot (left) together with a loading plot (right. Values for total polar surface area (TPSA) and molecular weight (MW) are given for selected compounds. The result of the PCA based on 71 calculated 2D descriptors relating to the hits chemical structure and properties illustrates the large diversity within the eight compound classes of AChE1 hits. Five significant principal components were determined using cross validation\textsuperscript{117} and scree-plots, yielding a model describing 92% of the original variation in the chemical properties (R\textsuperscript{2}X=0.92), with a cross-validated Q\textsuperscript{2} of 0.84.
5.3. Potential selectivity for AChE1 over hAChE

The HTS data was further analyzed in relation to another screen targeting hAChE performed by us a few years earlier using the same assay format and conditions, and the same compound library.112 By comparing the HTS results, in what we call a differential HTS (dHTS) approach, enabled the detection of potential hAChE/AChE1 selectivity already at the screening level. In total, the three screens generated 425 unique hits. Despite the scarce structural and functional differences between the target proteins, surprisingly, only 10% of the AChE1 hits were also hAChE hits. AChE1 was selectively targeted by 163 compounds. These results truly strengthen our conclusion from the AChE1 characterization and limited inhibition profiling study in paper I; that the development of selective inhibitors of AChE1 is possible. A comparison between the hits for the three targets revealed similarities with respect to their overall molecular size, hydrophobicity, flexibility and compounds containing a tertiary amine were common.

5.4. Confirmation of inhibition

The single dose testing of the compounds during the screening required a more thorough investigation to confirm true hits. A representative set of 76 compounds was selected for follow-up experiments where dose-response curves and IC50 values were determined for all three targets. The selection of compounds included both AChE1 and hAChE hits that displayed both high and low inhibition capacity. The set also included compounds with physiochemical properties representing the eight compound classes. In addition, the group of molecules that in the HTS displayed different inhibition capacity of Ag- and AaAChE1 was included.

In general, the re-testing of this representative set of compounds confirmed the result from the screen both in terms of potency and selectivity, as exemplified in Figure 5.3. The majority of the tested AChE1-hits from the HTS displayed dose dependent inhibition of AChE1 with IC50 values ranging from 0.21 to 86 µM. The compounds displaying selectivity towards either of the two AChE1 in the HTS was discarded following the dose-response experiments; either no inhibition was observed, or both Ag- and AaAChE1 were inhibited to the same extent. Thus, no significant difference was observed between the two mosquito enzymes. Regarding selectivity, compounds that displayed more than fivefold stronger inhibition of AChE1 than of hAChE in the HTSs, displayed at least tenfold stronger inhibition of AChE1 than of hAChE, based on IC50 values.
<table>
<thead>
<tr>
<th>Compound</th>
<th>HTS (%)</th>
<th>IC₅₀ (µM)</th>
<th>HTS (%)</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgAChE1</td>
<td>93</td>
<td>0.26</td>
<td>92</td>
<td>0.21</td>
</tr>
<tr>
<td>AaAChE1</td>
<td>91</td>
<td>0.44</td>
<td>76</td>
<td>0.22</td>
</tr>
<tr>
<td>hAChE</td>
<td>99</td>
<td>0.030</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td>mAChE</td>
<td>-</td>
<td>0.026</td>
<td>-</td>
<td>18</td>
</tr>
</tbody>
</table>

**Figure 5.3.** The chemical structures, the % inhibition in the HTS and IC₅₀ values for the two hit compounds 4 and 8. The dose-response curves in A and B are for 4 and 8, respectively.

5.5. **Inhibition by compound 4 and 8**

Figure 5.3 shows two hits where the follow up dose-response experiments agreed well with the inhibition profiles from the HTS. In the screens, the hit compound 4 inhibited over 90% of the activity of all three targets while the compound 8 selectively inhibited the mosquito enzymes (Figure 5.3). The selective behaviour of 8 was confirmed in the follow-up experiments where a 100-fold stronger inhibition of AChE1 than of h- or mAChE was observed (Figure 5.3 B). Also, the non-selective appearance of 4 was confirmed and, in fact, h/mAChE was slightly more sensitive to 4 than AChE1 was, based on its IC₅₀ values (Figure 5.3 A).
Interestingly, the vertebrate enzymes, but not the mosquito enzymes, displayed very distinct sensitivity to 4 and 8, despite the similar chemical features of the two compounds. In paper II we explored the binding mode and analyzed the interaction patterns of these two compounds in mAChE and mosquito AChE1 to investigate the contributions to sensitivity. A hypothesis based on the structural study was that 8 adopts a slightly different binding pose in mAChE compared to AChE1, which enables more favorable interactions in the CAS of AChE1 and may thus explain its higher affinity for compound 8.

5.6. Evaluation of an orthogonal activity assay

We evaluated the chemiluminescence-based ChOx assay to independently confirm the Ellman-based results from the HTS and the follow-up experiments, and investigated its applicability as an orthogonal activity-based assay. A selection of compounds belonging to four of the eight compound classes identified in the HTS was used for dose-response experiments.

IC$_{50}$ values for one of the four classes correlated well with the values determined by the Ellman assay, demonstrating the possibility to use ChOx as an orthogonal assay. The IC$_{50}$ values of one of the compounds from this class (called EC1, structure not shown, Figure 5.4 A) were 27 and 12 µM for AgAChE1 and 51 and 40 µM for hAChE using the ChOx and the Ellman assay, respectively. However, the remaining three compound classes displayed discrepancies between the IC$_{50}$ values determined from the different assays. The IC$_{50}$ values of compound EC2 (structure not shown, Figure 5.4 C) were 2.8 and 7.8 µM for AgAChE1 and 4.8 and 90 µM for hAChE using the ChOx and the Ellman assay, respectively. These discrepant results could be explained by that enzymes downstream AChE in the ChOx assay reaction were targeted by the tested compounds, displaying dose-response inhibition even in the absence of AChE (Figure 5.4 D). Whether they target ChOx or HRP was not further investigated.

False results can be detected by using correct control experiments and thus, in principle, the ChOx assay could serve as an orthogonal method to the Ellman assay. It would especially be useful to approach troublesome compounds e.g. compounds that absorb light at 412 nm (which is the readout wavelength in Ellman) or compounds that have free thiols that possibly could react with Ellman’s reagent DTNB. However, it is doubtful that the assay is suitable or efficient for e.g. HTS of compound libraries, and certain chemotypes appear to interfere with this assay. For example, the tested compounds that interfered with the ChOx assay in our experiments all had tertiary amines.
Figure 5.4. A and C show the ChOx assay determined dose-response curves of compounds EC₁ and EC₂, respectively, targeting AgAChE₁ (dots) and hAChE (squares). B shows the control experiment for compound EC₁ where no inhibition was observed when the ChOx assay was run in absence of AChE. D shows the control experiment for EC₂ where a clear dose-response inhibition was observed even when the ChOx assay was run in absence of AChE, the IC₅₀ value was determined to 7.3 µM.

5.7. Summary and conclusions

- 338 compounds were identified by HTS as potential inhibitors of Ag- and/or AaAChE₁.

- No significant difference between the two mosquito AChE₁s molecular recognition was observed.

- The dHTS analysis indicated that the AChE₁ enzymes were selectively targeted by 163 hits. The hits can be used as chemical starting points for development of potent and selective insecticides.

- The follow-up experiments confirmed the HTS data and dose-dependent inhibition was displayed by the majority of the re-tested compounds.

- The ChOx assay is not optimal for HTS-purposes but could, with the correct controls, potentially be used as an orthogonal assay to Ellman.
6. Structure-based analysis of AChE-compound interactions

X-ray crystallography generates high-resolution information in the form of atomic coordinates and is today the dominating technique for three dimensional (3D) structure determination of proteins and other biological macromolecules. The technique has contributed significantly to physics, chemistry, biology and medicine, which is reflected in the 29 Nobel Prizes that are associated with crystallography.118

The aim of our structure determination effort was to: 1. investigate structural similarities and differences between mosquito and vertebrate AChEs, 2. explore AChE-inhibitor interactions, and 3. understand what structural features of AChE that contribute to potency and selectivity of inhibitors. Structure-based analyses are found in paper II and IV.

6.1. Generation of AChE crystals

A critical part of crystallography is to identify conditions where a highly purified and concentrated protein sample assembles into ordered, diffracting crystals. By using crystallization conditions that have been optimized previously,96 crystals of mAChE were obtained within days (Figure 6.1). Despite the similarity between AChE1 and mAChE, the established crystallization conditions for mAChE were not transferable to AChE1. This was not unexpected as the crystallization of a protein is often very sensitive and specific for a certain protein construct or purification method. Instead, a screening approach testing 192 crystallization conditions was applied to AChE1 enzymes and generated tiny, needle like crystals for both Ag- and AaAChE1 at similar conditions (Figure 6.1). Unfortunately, the crystals were fragile and diffraction tests were not possible. The crystallization condition where the needles were obtained was subsequently explored and refined in secondary screens where the effect of small adjustments to pH and the concentrations of polyethylene glycol (PEG) and ions were investigated. Additional parameters that were explored included the effect of adding different (stabilizing) inhibitors, altering the protein concentration, seeding from existing crystals and changing from sitting to hanging drops. The growth of crystals was reproducible but they were still too fragile to be handled.

The first protein crystal structure of mosquito AChE1 was published in March this year at a resolution of 3.4 Å (pdb code: 5X61).73
6.2. Comparison of an AgAChE1 model and crystal structure

As no crystal structure of a mosquito AChE was available when this work started, the 3D structures of Ag- and AaAChE1 were modeled from multiple sequence- and structure alignments of AChE crystal structures from other species. A known inhibitor of AChE1 spanning the length of the active site gorge was used when modelling AChE1. The homology models, created with DmAChE as template, were used in paper II. A superposition of our AgAChE1 model and the recently deposited structure of AgAChE1 (5X61) revealed a high structural similarity. The overall fold and the three cysteine bridges superposed well for the structures. Some minor differences were observed in loops distant from the active site gorge. A close inspection of the active site gorge revealed that a loop at the rim of the gorge (herein termed loop 1) containing the AChE1-unique cysteine and residues involved in the acyl pocket, shows slightly different conformations in the model compared to the crystal structure. However, the free cysteine is still facing the gorge in both structures. The aromatic residues lining the gorge, Tyr124 and Tyr337 making up the waist, Trp86, Trp286 and the catalytic triad all overlap. One exception was Tyr337 whose position was modelled differently in the model compared to in the crystal structure due to the presence of the inhibitor in the modelling procedure.

6.3. Comparison of evolutionary distant AChEs

A superposition of the crystal structures of AgAChE1 (5X61), mAChE (1J06) and hAChE (4EY4) confirmed that the overall fold and structure of the enzymes were conserved between the species (Figure 6.2). The enzymes were particularly similar in the CAS region and the main differences were seen at the rim of the active site gorge.

The two loops, located at the rim of the gorge, differed in length and sequence between AgAChE1 and vertebrate AChE and therefore adopt slightly different conformations, confirming previous models of AChE1.
Loop 1 (Leu289 to Phe297) is important for the structure of the acyl pocket (therefore commonly referred to as the acyl loop) and is three amino acids shorter in AgAChE than in vertebrate AChE. The second loop (herein termed loop 2), from Gly342 to Lys348, is one amino acid longer in AgAChE and extends from an alpha-helix lining the active site gorge that contains the aromatic residues, Tyr337, Phe338 and Tyr341. Although not obvious from the superposition of the structures, these differences of the loops could potentially affect the water distribution and the dynamics of the entire gorge.

Figure 6.2. Close-up on the active site gorge of the superposed crystal structure of AgAChE (cyan) and mAChE (white) illustrates the loop differences at the rim of the gorge, the AChE1-unique cysteine residue corresponding to Phe295 in mAChE, and the well superposed catalytic triad.

6.4. Crystal structures of mAChE-inhibitor complexes

Several crystal structures of mAChE in complex with AChE inhibitors were determined and have contributed to selectivity and potency discussions in this thesis (paper II and IV). Here, the 2.1 Å and 2.7 Å resolution structures of mAChE in complex with compound 9 and 10, respectively, are used to investigate mAChE’s different sensitivity for the two compounds. Compound 9 have a thiourea-based scaffold discussed in chapter 7 and paper III, and 10 is a phenoxyacetamide-based analog of 8 (paper II) discussed in chapter 8 and paper IV. Their potency and selectivity profiles make them interesting molecules for investigating potency- and selectivity-conferring properties and interactions (Figure 6.3). Refinement statistics in Appendix 1.
The electron density map for the mAChE•9 crystal complex clearly defined the compound and revealed a compact binding pose close to the base of the active site gorge (Figure 6.4 A). The compound formed an internal hydrogen bond between the sulfur and the nitrogen atom of the dimethylamine, with a heavy atom distance of 3.3 Å. The dimethylamine potentially also formed activated CH-arene interactions with Tyr341. The geometrical arrangement of the methoxy-substituted phenyl and the indole ring of Trp86 was suboptimal for a stacking interaction (Figure 6.4 B).

**Figure 6.3.** The chemical structures and IC₅₀ values of 9 and 10.

<table>
<thead>
<tr>
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<th>IC₅₀ (µM)</th>
<th></th>
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<td>29</td>
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<td>AgAChE1</td>
<td>7.1</td>
<td>(6.5-7.6)</td>
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**Figure 6.4.** Crystal structure of mAChE in complex with the thiourea-based inhibitor 9. The electron density map in A is the Fₒ-Fc simulated annealing omit map contoured at 3 sigma, showing the compact binding pose of 9 in the active site gorge. B shows the nearby amino acids in a more close up illustration.
In contrast to the compact binding pose of 9, the electron density map of the mAChE•10 crystal complex revealed an elongated binding pose spanning the entire active site gorge (Figure 6.5 A). The biphenyl of the inhibitor interacted with the rim of the gorge via a parallel stacking of the proximal phenyl and the indole ring of Trp286, enabling aromatic interactions. The distal phenyl ring of the biphenyl fragment was not explained by the electron density map (Figure 6.5 A). The electron density map clearly defined a water molecule between the amide carbonyl group of 10 and the backbone Phe295N suggesting a water mediated hydrogen bond. The ethylpiperazine fragment of 10 was extending towards the base of the gorge, but the geometrical arrangement of the ethylpiperazine and the indole of Trp86 was suboptimal for interactions, however, the distance suggest some contact to occur (Figure 6.5 B).

**Figure 6.5.** Crystal structure of mAChE in complex with the phenoxyacetamide-based inhibitor 10. The electron density maps in A are the Fo-Fc (blue) and the 2Fo-Fc (green) simulated annealing omit maps contoured at 3 and 1 sigma, respectively, showing the elongated binding pose of 10 in the active site gorge. B shows the nearby amino acids in a more close up illustration.

mAChE was sevenfold more sensitive to inhibition of 10 than of 9, based on their IC_{50} values. A contributing reason to this difference could be the larger number of contacts between 10 and the active site gorge, including key interactions with Trp286, which was not present for compound 9.
6.5. Structural differences could explain selectivity

Both 9 and 10 were better inhibitors of AChE1 than of mAChE, with IC₅₀ values of 6-7 µM and 0.23-0.40 µM for the AChE1 enzymes (Figure 6.3). Different explanations for their selective behavior are proposed for the two inhibitors. The CAS of AChE1 could accommodate a similar binding position for 9 as observed in the mAChE-9 crystal structure complex. However, the higher affinity for larger substrates indicated that AChE1 has a slightly larger CAS than vertebrate AChE (paper I). One can speculate that his may allow 9 to adopt a more relaxed and favorable binding pose in the CAS of AChE1 that could allow a more favorable geometry for interactions with Trp86.

It is likely that the AChE1 gorge would accommodate compound 10 in a similar elongated pose as was observed for 10 in mAChE. The higher potency for 10 in AChE1 compared to mAChE may be related to the residue and conformation differences in loop 1 and loop 2. These structural differences in the PAS region probably affect the interaction between the biphenyl fragment of 10 and the indole of Trp286, which may contribute to the increased potency in AChE1. Another contributing factor could be similar to what was seen for the similar compound 8 in paper II. The homology model of the AgAChE1-8 complex suggested a somewhat shifted binding position of 8 in AChE1 compared to mAChE. This allowed a more optimal position of the ethylpiperazine in CAS, resulting in potentially stronger interactions with Trp86. The mAChE-10 complex and the suggested interaction pattern have been studied in more detail using molecular dynamic (MD) simulations in paper IV and chapter 8.

6.6. AChE mutants designed to explore interactions

In an ongoing project, we have introduced specific mutations in AChE to investigate interactions between AChE and inhibitors. By this molecular genetic approach we generated chimeric proteins of AgAChE1 and mAChE to explore if, and how, the structural differences between AChE1 and vertebrate AChE relate to potency and selectivity of compounds. For example, mAChE was made more “mosquito-like” by introducing loop 1 or loop 2 from AgAChE1, and corresponding loops from mAChE were introduced in AgAChE1. Expression and characterization of these constructs are ongoing, but preliminary results indicate that the Michaelis constants were affected by the mutations. Next, the chimeric proteins will be probed with a selection of inhibitors to study the chimeric inhibition profiles in relation to the unmodified AgAChE1 and mAChE enzymes.
6.7. Summary and conclusions

- Protein crystals of both AgAChE1 and AaAChE1 were obtained. Further refinement of the quality and size of the crystals might enable structure determination.

- The homology model of AgAChE1 agreed well with the newly published crystal structure and was useful to rationalize SAR and SSR of inhibitors.

- Mosquito AChE1 and vertebrate AChE are structurally very similar; the main differences in two loops at the rim of the active site gorge potentially affect the structure and dynamics of the entire gorge.

- The different binding poses of 9 and 10 in mAChE allows more contacts with AChE for 10, including key interactions with Trp286, which probably contributes to its higher potency.

- Structural differences at the rim of the active site gorge could contribute to selectivity between AChE1 and mAChE.
7. Exploring the insecticidal potential of thiourea-based compounds

In the dHTS presented in paper II and in chapter 5, a large number of non-covalent hit compounds showing selectivity for the mosquito enzymes over human AChE were identified. Here, the insecticide-potential of one compound class is explored and evaluated in vitro, ex vivo and in vivo. The results and highlights of paper III are presented in this chapter.

7.1. Design of analogues of three hit compounds

Hit compounds 11, 12, and the already introduced compound 9, were identified in the HTS targeting Ag- and AaAChE1 (paper II), and their potencies were confirmed by determination of IC₅₀ values (Figure 7.1 A and Table 7.1). With the aim to investigate the SAR and SSR between this compound class and AChE, the core structure containing the thiourea-scaffold became the basis for a design of three sets of analogous compounds (Figure 7.1 B). In the design, the N-(2-aminoethyl)-N'-phenylthiourea moiety was kept constant and the substituents and their position on the aromatic ring were varied along with variations of tertiary amines on the other end of the molecule. In total, 21 thiourea-based compounds belonging to set A-C were synthesized and biochemically evaluated (see paper III for complete list of compounds).

Figure 7.1. A shows the hit compounds 11, 12 and 9 identified in the HTS. B shows the common scaffold and the variation sites for the analogues.

7.2. Inhibition of AChE1

Four different enzymes were probed with the 21 synthesized compounds; Ag-, AaAChE1, hAChE and the naturally occurring resistant mutant AgAChE1-G122S (Table 7.1). The complete set of analogous compounds displayed a wide range in potency with IC₅₀ values ranging from 90 nM to
inactive (IC\textsubscript{50}>1 mM). In total, 13 compounds displayed dose-dependent inhibition, and again, no significant difference regarding inhibition was observed between Ag- and AaAChE1.

Interestingly, the thiourea-based analogues showed that the level of AChE-inhibition was strongly affected by subtle changes in the chemical structure of the inhibitor. For example, a drastic effect was seen when removing one of the two substituents on the phenyl ring (cf. compound 11 and 13 or 14) as the inhibitory capacity was lost (IC\textsubscript{50} values >200 µM). Another example was the tenfold gain in potency that was observed when the methyl-piperidine of 11 was exchanged for a morpholine, 15. Comparing the inhibitory capacity of 9 and 16 gives one additional example; by changing the position of the methoxy substituent on the phenyl ring from ortho to para position caused the IC\textsubscript{50} values to increase from 6-7 µM to >200 µM. This resulted in a complex SAR that suggested different interaction patterns and binding modes for each set of compounds, as is discussed in more detail in paper III. A divergent SAR has been observed for mAChE previously, when probed with chemically similar non-covalent inhibitors.\textsuperscript{119}

Table 7.1. Chemical structures and IC\textsubscript{50} values for a selection of the analogues.

<table>
<thead>
<tr>
<th>ID</th>
<th>Structure</th>
<th>IC\textsubscript{50}\textsuperscript{a} (µM)</th>
<th>S.R.\textsuperscript{b}</th>
<th>IC\textsubscript{50}\textsuperscript{a} (µM)</th>
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<td>hAChE</td>
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</table>

\textsuperscript{a}IC\textsubscript{50} values were determined using 2-3 replicates, the 95% confidence interval is given in parentheses. \textsuperscript{b}Selectivity ratios computed by taking the compound’s hAChE IC\textsubscript{50} value divided by the higher IC\textsubscript{50} value for AgAChE1 or AaAChE1. \textsuperscript{c}na = not applicable.
7.3. Inhibition of hAChE and AgAChE1-G122S

Six compounds displayed more than a tenfold stronger inhibition of AChE compared to hAChE. The most potent inhibitor of AChE, compound 15, was also the most selective compound showing a hAChE/AChE selectivity ratio of 117. Analysis of the inhibition evaluation of the complete set of analogues showed that the variations of the tertiary amine appeared to contribute the most to selectivity (paper III). Similar to what was seen for AChE, hAChE inhibition was also largely affected by relatively small modifications of the inhibitors.

Generally, the G122S mutation reduced AgAChE1’s sensitivity for the thioureas; eight of the 21 analogues inhibited the resistant G122S mutant, displaying IC_{50} values of 17 to 94 µM (Table 7.1). Thus, the inhibition profile of AgAChE1-G122S when probed with this compound class was more similar than that of hAChE. Only five compounds displayed lower IC_{50} values for the G122S mutant than for hAChE. AgAChE1-G122S was much less sensitive to the earlier highlighted compound 15, displaying an IC_{50} value of 84 µM.

7.4. Inhibitory effect on mosquito extract

With the ambition to design insecticides, it was critical to test the thiourea-based inhibitors ability to inhibit the enzymatic activity of AChE also in more complex systems than on recombinant enzymes in vitro. Three thiourea-based compounds, 9, 15 and 16, that displayed different potencies on recombinant AaAChE1 were therefore tested for their inhibitory effect on tissue extract of female Aa. aegypti adults. The compounds’ potencies in the ex vivo experiments corresponded well with the IC_{50} values determined in vitro on recombinant AaAChE1, regardless if the extracted enzyme originated from the head or the body of the mosquitoes (Figure 7.2). Interestingly, the compounds inhibitory capacity was unaffected by the more complex environment.

Figure 7.2. Left: dose-response curves showing the inhibition of esterase activity from Aa. aegypti adult mosquitoes head (filled symbols) and body (empty symbols) for compound 9 (circles), 15 (squares), and 16 (triangles). Right: the corresponding IC_{50} values (µM) determined for recombinant enzyme (rAaAChE1) and extract.
7.5. Cell toxicity of thiourea-based compounds

Prior to the initial in vivo phase 1 trial (laboratory testing)\(^{102}\) for insecticide development, the thiourea-based compound class was evaluated with regards to potential cell toxicity. Two potent and selective compounds (11 and 15) were selected as representatives for the compound class, and viability of Sf9 cells was recorded after 24 h exposure to these compounds.

We found that neither of these compounds caused any decrease in Sf9 cell viability after 24 h exposure. Concentrations up to 500 µM were tested, which corresponds to the highest concentration tested in the larvae in vivo experiments (discussed in 7.6). Cell viability was 114% and 105% for compound 11 and 15, respectively, when compared to DMSO controls, indicating that any insecticidal effect caused by this compound class is most likely not a result of general cell toxicity.

7.6. Insecticidal effect of thioureas in vivo

The promising in vitro and ex vivo potency of the thiourea-based compounds prompted us to investigate their insecticidal effect on living mosquitoes. The same three compounds that were investigated ex vivo were also explored for insecticidal capacity of five days old females and 3rd instar larvae of An. gambiae and Ae. aegypti.

The most promising compound 15 displayed a clear larvicidal effect on both An. gambiae and Ae. aegypti larvae, causing close to 100% mortality at 300 and 500 µM exposure concentrations, respectively (Figure 7.3). Furthermore, 15 killed approximately 70% of the An. gambiae adults at 1 nmol dose while Ae. aegypti mosquitoes were unaffected by the same dose. This could be evaluated in comparison to the commercial insecticide propoxur having a topical application LD\(_{50}\) of 0.015 nmol/mosquito\(^{120}\), indicating the rather poor insecticidal efficacy of 15. Compound 9 and 16 displayed lower insecticidal effect compared to 15, as is described in more detail in paper III.
45

Figure 7.3. The insecticidal effect of compound 15 recorded 24 hours after application. A) Mortality rates of larvae exposed to treated water. B) Mortality rates of adult mosquitoes exposed to compound 15 by topical application.

Interestingly, a significant difference in sensitivity was observed between the two mosquito species, whereas the in vitro studies gave no such indications. *Aedes* mosquitoes appeared more unaffected to the compounds in comparison to the more sensitive *Anopheles* mosquitoes. This indicates that the pharmacokinetic properties of the two mosquito species differ. It was also difficult to correlate the in vitro IC₅₀ values to the insecticidal capacity in vivo, highlighting the complexity of living organisms compared to the study of isolated reactions in a test tube. Properties such as intrinsic metabolism and inhibitor uptake (penetration through the insect exoskeleton) play a critical role that must be assessed by in vivo studies.

7.7. Summary and conclusions

- 21 thiourea-based analogues were designed, synthetized and evaluated for their inhibition of recombinant AChE1, hAChE and AgAChE1-G122S.

- *Ag*- and *Aa*AChE1 displayed very similar inhibition profiles, with IC₅₀ values as low as 0.1 μM, and several potent compounds showed selectivity for AChE1 over hAChE.

- The relatively large differences in inhibition effect caused by small changes to the molecules resulted in a complex SAR for the thiourea-based inhibitors.

- The inhibitory capacity of 9, 15 and 16 was unaffected by the more complex environment of *Ae. aegypti* tissue extract.

- *Ae. aegypti* and *An. gambiae* were killed by thiourea compounds, although high concentrations of the inhibitors were needed.
8. Structural differences govern potency and selectivity of phenoxyacetamide-based inhibitors

The non-covalent inhibitor 8, identified in the HTS (paper II), was recognized as a promising chemical starting point towards the development of new insecticides as it displayed sub micromolar potency for AChE1. In addition, 8 was a 100-fold stronger inhibitor of AChE1 and a 20-fold stronger inhibitor of AgAChE1-G122S than of hAChE. In paper IV and in this chapter we explored a set of analogues of 8 to better understand the structural basis for the compounds selectivity and potency.

8.1. Design of analogues of compound 8

To investigate the SAR and SSR for this compound class, a set of analogues was designed and evaluated. The phenoxyacetamide core structure of 8 was maintained in all analogues. The compounds were designed so that the CAS-binding part of the molecule was varied in size and electronic properties. The waist-binding part of the molecule was varied in flexibility by exchanging the rigid piperidine-linker to a more flexible propyl-linker. The PAS-binding part of the molecule was also varied; the distal phenyl ring was either replaced by an iodide or substituents were added to the distal phenyl. From the crystal structures of mAChE in complex with 8 (paper II) and its derivative 10 (chapter 6), it was observed that these two structurally similar inhibitors both bind in an elongated pose forming interactions with Trp286 in the PAS, and potentially with Trp86 in CAS.

8.2. Inhibition by phenoxyacetamide analogues

The set of 21 analogues were synthetized and their IC₅₀ values were determined for Ag-, AaAChE1, hAChE and the G122S mutant. A comprehensive SAR study of the complete set of compounds is presented in paper IV. Here, a selection of the analogues are presented and used to illustrate the interaction patterns described from the mAChE•8 and mAChE•10 crystal structures (Table 8.1).
Table 8.1. Chemical structures and IC₅₀ values for a selection of the analogues.

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aIC₅₀ values were determined using 2-3 replicates, the 95% confidence interval is given in parentheses. bSelectivity ratios were computed by taking the compound’s hAChE IC₅₀ value divided by the higher IC₅₀ value for AgAChE₁ or AaAChE₁. cna = not applicable.
Changes of the CAS binding part of the compounds influenced their potency on both hAChE and AChE1. When the size of the CAS binding fragment was reduced they became slightly more potent inhibitors of hAChE while the inhibition of AChE1 was unaffected (cf. 8 and 17). Affecting the electronic properties of the compound by changing the piperazine to a piperadine (18) reduced the IC₅₀ for AChE1 tenfold. Interestingly, the potency for both hAChE and AChE1 was abolished when the CAS-interacting part of the compound was removed; compound 19 had no inhibitory effect. Thus, CAS-interactions were important for both potency and selectivity.

The potency of the compounds was reduced when the flexibility of the linker was increased by exchanging the piperidine to a propyl-linker (20-22).

Changes to the PAS binding part of the compounds were more inconclusive. Their potency for AChE1 was reduced with a factor of ten if the distal phenyl ring was changed to an iodide (cf. 23 and 8), while their effect on hAChE activity was basically unaffected by this change. Also, the activity of AChE1 and hAChE were either not affected by the addition of substituents to the distal phenyl (cf. 10 or 24 and 8) or it caused a drop in selectivity due to a slightly stronger inhibition of hAChE (25 and 26).

Interestingly, the G122S mutant followed the same SAR as the AChE1 enzymes, showing that the mutation did not disturb the proteins affinity for these compounds. The only notable difference in potency was observed for the more flexible compounds that were slightly less potent on AgAChE-G122S than on AgAChE1.

8.3. Molecular dynamics of AChE•10 complexes

MD simulations were used to investigate structural and dynamic contributions to potency and selectivity of the phenoxyacetamide analogues. 3D structures of four enzymes in complex with compound 10 were used as starting structures in the MD simulations. The mAChE•10 complex was introduced in chapter 6, and three additional complexes of 10 bound to AaAChE1, AgAChE1, and AgAChE1-G122S were created based on the crystal structure of AgAChE1 (5X61) and the mAChE•10 structure. For more details on how the structure complexes were prepared and set up for MD simulations, see paper IV.

The conformational dynamics of the simulated trajectories were studied by analysing root-mean-square deviations (RMSD) of the enzymes backbone as a function of time. Multiple parallel 200 ns MD simulations of the complexes showed that all four systems were stable over time, with RMSD values of the backbone around 1.5 Å. To monitor the dynamics of compound 10, its RMSD was calculated after superposing the protein backbone on its
The RMSD values showed that 10 was more dynamic when bound to mAChE compared to the mosquito enzymes (Figure 8.1). It was mainly the PAS-binding part of 10 that contributed to the dynamics as it was deviating more than the CAS-binding part. In the mosquito enzymes, compound 10 adopts highly similar binding poses throughout the trajectories in the three parallel simulations, while in the case of mAChE the separate simulation trajectories identified three distinct binding conformations for the PAS binding biphenyl. This conformational mobility may contribute to the weak and disordered electron density map of the distal phenyl ring in the crystal structure of mAChE•10.

Figure 8.1. Three parallel trajectories (colored blue, red and green) of 200 ns MD simulations of compound 10 in complex with a) mAChE, b) AgAChE1, c) AaAChE1 and d) AgAChE1-G122S.

8.4. Interaction pattern analysis of AChE with compound 10

A more detailed analysis of the active site gorge of AChEs during the MD simulations was performed. Here, the contact frequencies between inhibitor 10 and amino acids in AChE within 4 Å of the inhibitor were investigated throughout the simulations. The most dominating contacts for all four
enzymes were with Trp86 and Tyr341, based on contact frequencies and calculated interaction energies (i.e. electrostatic and van der Waals contribution). Despite the suboptimal geometry for 10 and Trp86 observed in the crystal structure, the MD simulations clearly indicate interactions. The interaction strength between Trp286 and 10 was lower in the mouse complex compared to the mosquitoes, which relates to the larger mobility of 10 in mACHe and thus more sporadic interactions with Trp286.

In addition to these, 10 in AChE1 showed important interactions with Tyr337 and Phe338 that were much less frequent in the simulation of mACHe-10. These differences in the interaction patterns between compound 10 and AChE1 and mACHe, respectively, were linked to the differences observed in loop 2 (introduced in chapter 6). Residues Tyr337 and Phe338 are part of an alpha helix that lines the active site gorge and extends into loop 2 at the rim of the gorge. From the MD simulations it was observed that this alpha helix behaved differently in AChE1 and mACHe, which enabled more frequent and strong contacts between compound 10 and residues Tyr337 and Phe338 in AChE1, while the corresponding interactions in mACHe were much weaker and less frequent. Both the crystal structure and the MD simulations showed that compound 10 does not occupy the space in CAS where the G122S mutation is located, which explains why the mutation does not affect the AChE1s sensitivity to the phenoxyacetamide compounds.

8.5. Insecticidal efficacy of analogues

A selection of three of the most potent analogues (24-26) was chosen for investigation of their insecticidal efficacy on adult mosquitoes (Figure 8.2). Topical application of these compounds on 5 days old Ae. aegypti mosquitoes revealed that the ortho-fluoro-substituted biphenyl compound 24 had the greatest adulticidal efficacy; a dose of 10 nmol killed 75% of adult Ae. aegypti females within 24 hours. Interestingly, 24 also displayed high selectivity for AChE1 over hAChE in vitro. Mortality rates for 25 and 26 were 53% and 24%, respectively, using a dose of 10 nmol.

Figure 8.2. Mortality rates 24 hours after topical application of compound 24, 26 and 25 on Ae. aegypti.
8.6. Summary and conclusions

- Several compounds in this class displayed the desired combination of AChE1 over hAChE selectivity and sub-micromolar IC$_{50}$ values for the G122S mutant.

- The G122S point mutation in AgAChE1 only marginally affect the proteins sensitivity for the phenoxyacetamide-based analogues.

- Compound 10 was more dynamic in complex with mAChE than AChE1.

- The MD simulations indicate that the selectivity of 10 could be due to the difference in loop 2, which affects the dynamics of Tyr337 and Phe338 and enables stronger and more frequent interactions between 10 and these amino acids in AChE1, compared to mAChE.

- Compound 24 displayed high selectivity in vitro and killed adult Ae. aegypti female mosquitoes.
9. Summary and discussion

Vector control by insecticide-based interventions is the primary method to avoid transmission of several mosquito-borne diseases, but new insecticides for this purpose are urgently needed. We have explored the protein AChE1 from the two disease-transmitting mosquitoes *Anopheles gambiae* and *Aedes aegypti* for the possibility to design and develop selective inhibitors, with a long term goal towards new insecticides for use in vector control.

**AChE1 from mosquitoes as an insecticide target**

The biological role of AChE in the nervous system is essential for life and conserved over evolutionary distant organisms. Our studies have provided a complex structural and functional dataset that give an opportunity to discuss how these enzymes compares to each other. None of our studies indicated any significant differences between *Ag*- and *Aa*AChE1, which is in agreement with another study where inhibition profiles of AChEs from mosquito species of *Anopheles*, *Aedes* and *Culex* genus were compared.

We found that AChEs from vertebrates and mosquitoes share many kinetic properties, for example, the preference for small substrates before larger, and the substrate inhibition at elevated substrate concentrations (paper I). Throughout the work, we have also found that despite the overall structural and functional similarity, important differences between the vertebrate and the mosquito enzymes are evident. For example, the molecular recognition of mosquito AChE1 and vertebrate AChEs differ (paper I-IV), only 10% of the identified inhibitors in a HTS were common for mosquito AChE1 and hAChE (paper II). Interestingly, based on a small set of carbamate-like inhibitors, the inhibition profiles appeared to differ also between mosquitoes and agriculturally relevant insect species.

The majority of the investigated inhibitors herein show selectivity for AChE1 over hAChE (paper II-IV). Selectivity factors over 100 were achieved for thiourea- and phenoxyacetamide-based inhibitors, with AChE1-potencies of 100-200 nM. Selective compounds with different mechanisms of action than presented herein have been reported before; the design of carbamate-modified covalent compounds has proven valid for this purpose as a selectivity ratio above 500 was reported for the most selective 2-substituted aryl methylcarbamate, based on \( k_i \) values. Even better potency was achieved by covalently target the AChE1-unique cysteine residue, however, these compounds are somewhat compromised by unwanted reversible inhibition of hAChE.
Resistance-breaking inhibition of mosquito AChE1

New inhibitors targeting AChE1 should preferably also address the growing concern of insecticide resistance. The G122S mutation gives the AgAChE1 protein slightly different kinetic properties, which was observed by a higher \( K_M \) value and decreased \( V_{max} \) ([paper I](#)), the mutation is also a fitness cost for mosquitoes.\(^7\) Generally this mutation reduces AChE's affinity for inhibitors, as is seen in [paper I-IV](#), and by others.\(^7\) To the best of our knowledge, no compounds have previously been shown to combine selectivity for AChE1 over hAChE with potency also for the G122S mutant. However, in [paper IV](#) we show that selectivity, and a sub-micromolar affinity for the G122S mutant can be combined. For the AChE1-selective phenoxyacetamide-based analogues, the G122S mutant displayed similar SAR and IC\(_{50}\) values as Ag- and AaAChE1. The crystal structure of mAChE•10 and the MD simulations based on it ([paper IV](#)) showed that compound 10 did not occupy the space close to the mutation in the active site gorge, explaining why the mutation only marginally affect the proteins affinity for the compounds in this class.

The use of OPs and carbamates as insecticides is a proven strategy, however, the potency of such compounds are dependent on their ability to form a covalent bond with Ser203 at the CAS. This makes covalent inhibitors highly sensitive to active site mutations. A modification at the CAS may enable this bond-formation and completely abolish the insecticidal effect, as is seen for the G122S mutation. In contrast, non-covalent inhibitors gain their potency by a number of interactions and are, in theory, free from the drawbacks associated with a reactive bond formation at the CAS. We speculate that a non-covalent approach may reduce the selection pressure of resistance-conferring mutations. Similar reasoning in the development of proteasome inhibitors for cancer treatment, where existing covalent drugs are hampered by resistance, has recently been reported.\(^1\) Non-covalent inhibitors can bind in different parts of the active site gorge, as was shown for compound 9 and 10 in chapter 6. Thus, by a non-covalent strategy, other regions of the gorge than where existing insecticides bind, and more importantly, where the existing insecticide resistant conferring mutation is located, can be investigated.
**Reversible, non-covalent inhibitors are effective also in vivo**

We found that both thiourea- and phenoxyacetamide-based non-covalent inhibitors killed mosquitoes, although it was not straightforward to correlate in vitro potency with in vivo efficacy, as has been reported before.\textsuperscript{78,80,82,83,86} For example, the chemically similar inhibitors 24 and 25 with similar potency in vitro experienced very different response in *Ae. aegypti* (**paper IV**).

The difficulties to correlate in vitro potency and in vivo efficacy of inhibitors are likely linked to pharmacokinetics of the mosquitoes, such as uptake and metabolism. To investigate if low in vivo efficacy of potent compounds was related to problems of penetrating the mosquitoes’ exoskeleton, different approaches to avoid the exoskeleton barrier have been undertaken; *e.g.* direct injection of compounds into the mosquito thorax\textsuperscript{78,86} or a fumigation assay used on volatile compounds.\textsuperscript{86,122,123} To evaluate the effect of intrinsic metabolic events in the mosquito, such as detoxification, properties of the injected inhibitors were varied.\textsuperscript{83}

Pharmacokinetic aspects should be included as early as possible in insecticide development, preferably already in the design of compounds’ properties. In addition, ex vivo experiments using mosquito tissue extracts are beneficial to evaluate inhibition capacity of compounds in a more complex environment when transferring lead compounds from in vitro to in vivo evaluation.

**Concluding remarks and future aspects**

Apart from the challenges discussed in this thesis, the design and development of new insecticides is severely hampered by the limited number of insecticide targets. Future insecticide research should aim at identify and evaluate new (protein) targets. This would increase the number of possible compound classes that can be explored for insecticidal effect and thus lessen the heavy use of a few number of insecticide classes. However, new insecticides for vector control will not alone decrease the burden of vector-borne diseases. Instead, a combination of efforts is needed: vaccine, drug, and diagnostic-development, surveillance of vector and pathogen spread in combination with weather and climate-reports, proper information and education, and more insecticide-independent vector control interventions.
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Carlier, P. R. et al. Towards a species-selective acetylcholinesterase inhibitor to control the mosquito vector of malaria, Anopheles


screening platform of the Laboratories for Chemical Biology (LCBU), Umeå University. [http://www.chemistry.umu.se/english/research/infrastructure/lcbu/](http://www.chemistry.umu.se/english/research/infrastructure/lcbu/).


## Appendix

**Table A1.** Data collection and refinement statistics.

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