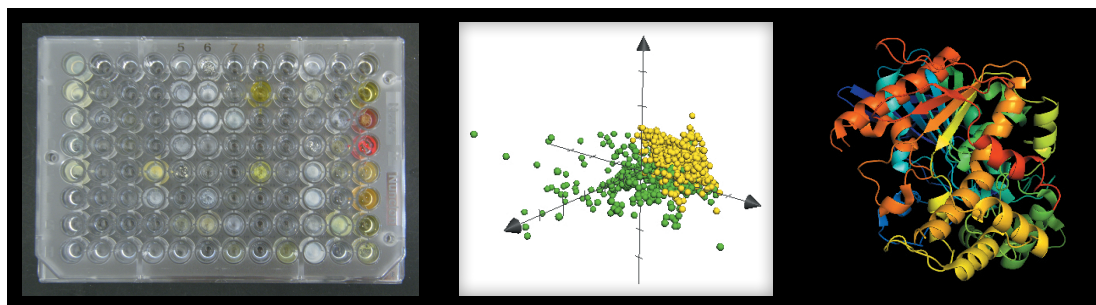


Daniela Karlsson

BIOMOLECULAR SCREENING FOR INHIBITORS OF BUTYRYLCHOLINESTERASE:

Identification and characterization using *in vitro*
and *in silico* tools



PHARMACEUTICAL SCIENCES
DEPARTMENT OF BIOSCIENCES
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**BIOMOLECULAR SCREENING FOR INHIBITORS OF
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CHARACTERIZATION USING *IN VITRO* AND *IN SILICO* TOOLS**

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Cover: Front, from left: A 96-well microplate containing compounds for screening, chemical space occupied by a synthetic (yellow) and natural (green) compound library and the structure of butyrylcholinesterase (PDB: 1POI) drawn with PyMOL. Back: Picture by Lindén-Montes Photography.

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ABSTRACT

Drug discovery is a continuous process where researchers are constantly trying to find new and better drugs for the treatment of various conditions. Alzheimer's disease, a neurodegenerative disease mostly affecting the elderly, has a complex etiology with several possible drug targets. Some of these targets have been known for years while other new targets and theories have emerged more recently. Cholinesterase inhibitors are the major class of drugs currently used for the symptomatic treatment of Alzheimer's disease. In the Alzheimer's disease brain there is a deficit of acetylcholine and an impairment in signal transmission. Acetylcholinesterase has therefore been the main target as this is the main enzyme hydrolysing acetylcholine and ending neurotransmission. It is believed that by inhibiting acetylcholinesterase the cholinergic signalling can be enhanced and the cognitive symptoms that arise in Alzheimer's disease can be improved.

Butyrylcholinesterase, the second enzyme of the cholinesterase family, has more recently attracted interest among researchers. Its function is still not fully known, but it is believed to play a role in several diseases, one of them being Alzheimer's disease. In this contribution the aim has primarily been to identify butyrylcholinesterase inhibitors to be used as drug molecules or molecular probes in the future. Both synthetic and natural compounds in diverse and targeted screening libraries have been used for this purpose. The active compounds have been further characterized regarding their potencies, cytotoxicity, and furthermore, in two of the publications, the inhibitors ability to also inhibit A β aggregation in an attempt to discover bifunctional compounds.

Further, *in silico* methods were used to evaluate the binding position of the active compounds with the enzyme targets. Mostly to differentiate between the selectivity towards acetylcholinesterase and butyrylcholinesterase, but also to assess the structural features required for enzyme inhibition. We also evaluated the compounds, active and non-active, in chemical space using the web-based tool ChemGPS-NP to try and determine the relevant chemical space occupied by cholinesterase inhibitors.

In this study, we have succeeded in finding potent butyrylcholinesterase inhibitors with a diverse set of structures, nine chemical classes in total. In addition, some of the compounds are bifunctional as they also inhibit A β aggregation. The data gathered from all publications regarding the chemical space occupied by butyrylcholinesterase inhibitors we believe will give an insight into the chemically active space occupied by this type of inhibitors and will hopefully facilitate future screening and result in an even deeper knowledge of butyrylcholinesterase inhibitors.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the thesis with their roman numerals:

- I. **Karlsson D**, Fallarero A, Brunhofer G, Guzik P, Prinz M, Holzgrabe U, Erker T, Vuorela P. Identification and characterization of diarylimidazoles as hybrid inhibitors of butyrylcholinesterase and amyloid beta fibril formation. *European Journal of Pharmaceutical Sciences* 45: 169-183, 2012.
- II. **Karlsson D**, Fallarero A, Brunhofer G, Mayer C, Prakash O, Mohan CG, Vuorela P, Erker T. The exploration of thienothiazines as selective butyrylcholinesterase inhibitors. *European Journal of Pharmaceutical Sciences* 47: 190-205, 2012.
- III. Brunhofer G, Fallarero A, **Karlsson D**, Batista-Gonzalez A, Shinde P, Mohan CG, Vuorela PM. Exploration of natural compounds as sources of new bifunctional scaffolds targeting cholinesterases and beta amyloid aggregation: the case of chelerythrine. *Bioorganic & Medicinal Chemistry* 22: 6669-6679, 2012.
- IV. **Karlsson D**, Fallarero A, Shinde P, Anju CP, Busygin I, Leino R, Mohan CG, Vuorela P. Chemical modifications of cinchona alkaloids lead to enhanced inhibition of human butyrylcholinesterase. Submitted, 2013.

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Prinz M, Parlar S, Bayraktar G, Alptüzün V, Erciyas E, Fallarero A, **Karlsson D**, Vuorela P, Burek M, Förster C, Turunc E, Armagan G, Yalcin A, Schiller C, Leuner K, Krug M, Sotriffer CA, Holzgrabe U. 1,4-Substituted 4-(1H)-pyridylene-hydrazone-type inhibitors of AChE, BuChE, and amyloid- β aggregation crossing the blood-brain barrier. *European Journal of Pharmaceutical Sciences* 49: 603-613, 2013.

CONTRIBUTIONS OF THE AUTHOR

This work was planned and performed by the author under the supervision of Docent Adyary Fallarero and Professor Pia Vuorela, the detailed contributions are listed below:

- I. The biological activity, chemical space analysis and all data processing was done by the author. The compounds were synthesized by the group of Professor Thomas Erker. The thioflavin T assays were performed by Dr Gerda Brunhofer and the group of Professor Ulrike Holzgrabe. Cytotoxicity studies were performed by MSc Ingeborg Smeds.

- II. The biological activity, chemical space analysis and all data processing was done by the author. The compounds were synthesized by the group of Professor Thomas Erker. Cytotoxicity studies were conducted by MSc Ana Elsa Batista and the docking studies were performed by the group of Associate Professor C. Gopi Mohan.

- III. The author took part in the cholinesterase assay and thioflavin T assay with Dr Gerda Brunhofer. The cytotoxicity studies were conducted by MSc Ana Elsa Batista and the group of Associate Professor C. Gopi Mohan performed the docking study.

- IV. The biological activity, chemical space analysis and all data processing was done by the author. The compounds were synthesized by the group of Professor Reko Leino. Cytotoxicity studies were conducted by MSc Ana Elsa Batista while the group of Associate Professor C Gopi Mohan performed the docking studies.

Publications I, II and IV were written by the author with contributions from the co-authors and under the supervision of Docent Adyary Fallarero and Professor Pia Vuorela.

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ABBREVIATIONS

A β	Amyloid beta
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADME	Absorption, distribution, metabolism and excretion
APP	Amyloid precursor protein
ATCI	Acetylthiocholine iodide
BChE	Butyrylcholinesterase
BTCCI	S- butyrylthiocholine chloride
BSA	Bovine serum albumin
ChemGPS	Chemical global positioning system
CNS	Central nervous system
CV _A	Coefficient of variation of the assay
DMSO	Dimethyl sulfoxide
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid) = Ellman's reagent
FDA	US Food and Drug Administration
GSK3 β	Glycogen synthase kinase 3 β
GT1-7	Mouse hypothalamic immortalized cells
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HL	Human lung epithelial cells
HTS	High-throughput screening
IC ₅₀	Mean inhibitory concentration
K _M	Michaelis-Menten constant; represents the substrate concentration at which the velocity of the enzyme is half of the maximal velocity
MCI	Mild cognitive impairment
NFTs	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
PC	Principal component
PCA	Principal component analysis
PDB	Protein Data Bank
S/B ratio	Signal-to-background ratio
S/N ratio	Signal-to-noise ratio
SAR	Structure-activity relationship
SMILES	Simplified molecular input line entry specification
ThT	Thioflavin T
V _{max}	Maximal velocity of the enzyme
Z' factor	Signal window coefficient

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1 REVIEW OF THE LITERATURE

1.1 Cholinesterases

Cholinesterases belong to the enzyme family of serine hydrolases, the name comes from their ability to hydrolyse substrates using an active site nucleophilic serine residue. The serine hydrolase superfamily is a large group of proteins involved in many important physiological processes e.g. digestion (Whitcomb and Lowe, 2007), blood coagulation (Flemmig and Melzig, 2012) and neurotransmission (Pohanka, 2011), and therefore many of these enzymes have also been linked to various diseases such as pancreatitis, thrombosis, and Alzheimer's disease (AD). Thus they serve as valid targets for drug discovery and in fact many inhibitors of these enzymes have been developed into drugs that are in clinical use today, for example Exelon[®] (Novartis) and Aricept[®] (Eisai) for AD, Pradaxa[®] (Boehringer Ingelheim) and Xarelto[®] (Bayer) for thrombosis and Januvia[®] (Merck) and Onglyza[®] (Bristol-Myers Squibb) for type 2 diabetes (Bachovchin and Cravatt, 2012). However, there are still many of the serine hydrolases that need to be characterized as their function and substrate specificity is still not known. The cholinesterases consist of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), both of these have known structures but only the first one has a well-established function. In the next sections the profile of these two enzymes will be discussed.

1.1.1 Acetylcholinesterase

Acetylcholinesterase (AChE, EC 3.1.1.7), also known as e.g. cholinesterase, acetylcholine acetylhydrolase, true cholinesterase and choline esterase I, is considered the main enzyme in the cholinesterase family. In humans, AChE is encoded by a single gene, *ACHE*, that is located on the long arm of chromosome 7 at position 7q22 (Getman et al., 1992). Different molecular forms of AChE are produced by alternative mRNA splicing and post-translational associations of catalytic and structural subunits, which gives AChE its structural diversity. The hydrophilic species can form disulfide-linked dimers and tetramers and they are the main form of AChE. Additionally, AChE can be attached to the cell membrane using glycopospholipid anchors or collagen links (Taylor and Radić, 1994; Massoulié et al., 1999). AChE is found in most tissues, but most notably in neuromuscular junctions (Guerra et al., 2005), brain cholinergic synapses (Adler et al., 2011), autonomic ganglia (Vernino et al., 2008) and red blood cell membranes (Delaunay, 1977).

Crystallization of AChE has identified the residues encompassing the substrate binding pocket as well as the catalytic triad responsible for the hydrolase activity. The structure of AChE has been extensively investigated and almost 50 % of the structures published in the Protein Data Bank (PDB) (<http://www.rcsb.org/pdb/home/home.do>) to date have been prepared using the electric ray (*Torpedo californica*) enzyme, 36 % using mouse enzyme and only 8 % using the human AChE. The first crystal structure of AChE was solved in 1991

using *Torpedo californica* (Harel et al., 1993) while the first structure of human AChE appeared in 2000 (Kryger et al., 2000) (**Figure 1**). In addition, the human AChE in complex with therapeutic drugs aiming to inhibit AChE has recently been solved (Cheung et al., 2012).

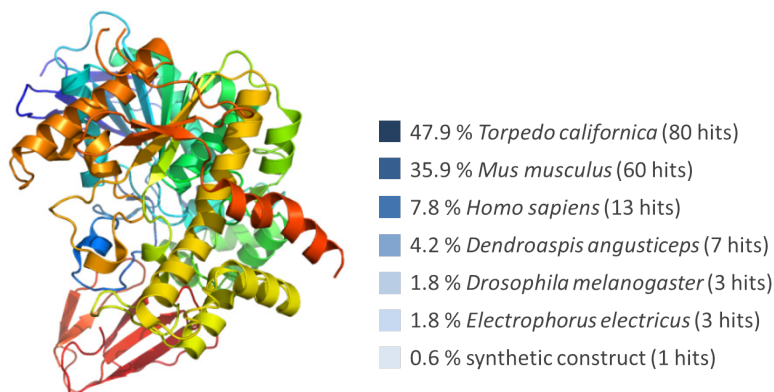


Figure 1. The first crystal structure of human AChE (PDB ID: 1B41 (Kryger et al., 2000) drawn with PyMOL (The PyMOL Molecular Graphics System, Schrödinger LCC, open source v.1.2x. <http://www.pymol.org>) and the origin of the available crystal structures of AChE in PDB (July 2013, keyword: 3.1.1.7).

AChE is best known for its function as a modulator of neurotransmission by hydrolysing acetylcholine (**Figure 2**). Acetylcholine is synthesised by choline acetyltransferase and concentrated into synaptic vesicles where it is stored until released into the synaptic cleft (Gauthier, 2002). In the synaptic cleft acetylcholine stimulates presynaptic nicotinic and muscarinic type 2 receptors and postsynaptic muscarinic type 1 receptors. AChE is also involved in many other functions such as apoptosis (Zhang et al., 2002; Zhang and Greenberg, 2012), cellular differentiation and tumorigenesis (Small et al., 1996). The kinetics of AChE is among the fastest in nature (Massoulié et al., 1993) and it allows the rapid termination of the neural signal. AChE is sensitive to organophosphates, as they irreversibly inhibit AChE by forming a covalent bond with the active serine residue, which has made organophosphates usable as pesticides, and sadly enough in chemical warfare (Pohanka, 2011). A continuous receptor stimulation by acetylcholine results in symptoms such as convulsion, vomiting, confusion and respiratory failure (Eddleston et al., 2008). On the other hand, a lack of acetylcholine reduces receptor stimulation which can be seen for example as the cognitive impairment occurring in AD (Garcia-Alloza et al., 2005). Thus, keeping a balance of acetylcholine activity is essential.

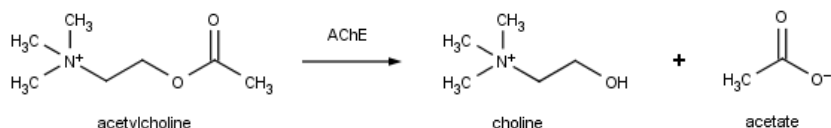


Figure 2. Reaction cascade of acetylcholinesterase hydrolysing acetylcholine.

1.1.2 Butyrylcholinesterase

Butyrylcholinesterase (BChE, EC 3.1.1.8), also known as e.g. pseudocholinesterase, acylcholine acylhydrolase, non-specific cholinesterase and choline esterase II, is a glycoprotein of 340 kDa (Asojo et al., 2011) encoded by a gene, *BCHE*, that, in humans, is located on the long arm of chromosome 3 at position 3q26.1-q26.2 (Gaughan et al., 1991). As for AChE, BChE also exists in several molecular forms. BChE can form soluble monomers (G_1), dimers (G_2) and tetramers (G_4) and also exists in a G_4 membrane-bound form (Darvesh et al., 2003). In contrast to AChE, BChE is more active in the peripheral tissue than in the brain (Liston et al., 2004) and mostly found in serum and glial cells, but also present in neurons (Darvesh et al., 1998; Darvesh and Hopkins, 2003). The longer half-life of BChE has been proposed to be due to its high amount of glycosylation (Nachon et al., 2002).

In contrast to AChE, the crystal structures of BChE that are currently available in PDB use the human enzyme. The first crystal structure of a human BChE was solved in 2003 (Nicolet et al., 2003) (**Figure 3**). However, it was lacking some of the amino acids and four years later the structure of the full-length human BChE was published (Ngamelue et al., 2007). Several other structures of human BChE have since then been published (e.g. Asojo et al., 2011; Carletti et al., 2011; Carletti et al., 2013; Wandhammer et al., 2013).

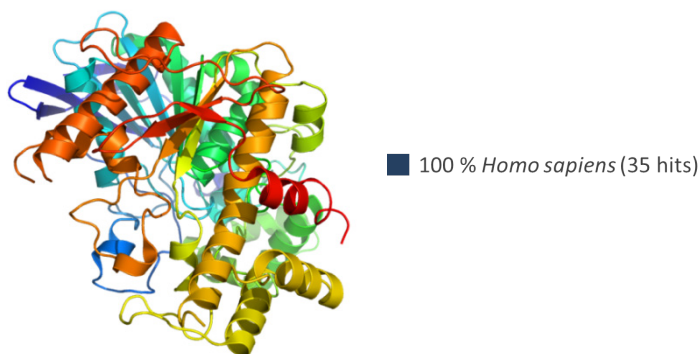


Figure 3. The first crystal structure of human BChE (PDB ID: 1POI (Nicolet et al., 2003) drawn with PyMOL (The PyMOL Molecular Graphics System, Schrödinger LCC, open source v.1.2x. <http://www.pymol.org>) and the origin of the available crystal structures of BChE in PDB (July 2013, keyword: 3.1.1.8).

BChE has been implicated in various physiological processes, the most prominent being the hydrolysis of several choline and non-choline esters, such as acetylcholine (Mesulam et al., 2002), succinylcholine (Kaufman et al., 2011), cocaine (Xue et al., 2011) and aspirin (Masson et al., 1998), thus, playing an important part in neurotransmission, anaesthesia and drug abuse. In contrast to AChE, which is sensitive to organophosphates, BChE is not affected by them and is in fact being studied for use as a detoxification enzyme, a bioscavenger, for organophosphates (Mumford and Troyer, 2011; Mumford et al., 2012;

Zhang et al., 2012a) so that they are destroyed (hydrolysed) before reaching their target in the central nervous system (CNS). However, the function of BChE is still being debated.

1.1.3 Structural differences in the active site

Although, AChE and BChE are encoded on different chromosomes they show 65 % amino acid sequence homology (Giacobini, 2004) and they both contain a catalytic triad that is comprised of the amino acids serine (Ser), histidine (His), and glutamic acid (Glu) which are located at the bottom of a 20 Å gorge (Nicolet et al., 2003; Dvir et al., 2010). The amino acids lining this gorge seem to determine the substrate selectivity as the entry to AChE is narrower than that of BChE, which is shown in **Figure 4A** and **Figure 4B**. This is mainly due to the aromatic residues Tyr-124 and Trp-286 which are located at the gorge entrance and which are occupied by Gln-119 and Ala-277 in BChE. Inside the gorge, there is a difference in the acyl binding site residues which in AChE consist of the aromatic residues Phe-295 and Phe-297, while BChE contains the smaller residues Leu-286 and Val-288 (Nicolet et al., 2003; Dvir et al., 2010) (**Figure 4C**). This allows BChE to bind bulkier substrates into the active site. Tyr-337 (Ala-328 in BChE) in AChE also hinders bulkier substrates from interacting with the catalytic triad.

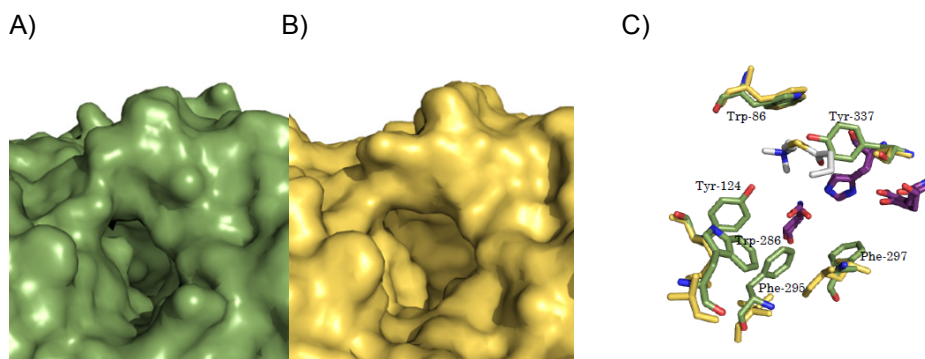


Figure 4. The active site entrance of **A)** human AChE [PDB code 3LII (Dvir et al., 2010)] and **B)** human BChE [PDB code 2PM8 (Ngamelue et al., 2007)]. **C)** A comparison between the residues in the active sites is also shown (the catalytic triad is visualized in violet). Drawn with PyMOL (The PyMOL Molecular Graphics System, Schrödinger LCC, open source v.1.2x. <http://www.pymol.org>).

1.2 Cholinesterases as targets in drug discovery

Due to the role of cholinesterases in several key functions in the human body, it has been postulated that they play a crucial role in several diseases and thus they have become central targets in drug discovery. Inhibitors of cholinesterase have been used in the therapy of AD (Birks, 2006) and myasthenia gravis (Mehndiratta et al., 2011), and have been suggested to be beneficial in the management of several other conditions e.g. chronic pain (Folkesson et al., 2010; Wehrfritz et al., 2010) and type 2 diabetes (Kamal et al., 2009). The use of cholinesterase inhibitors is not a cure but a temporary aid, usually at the earlier stages of the

disease (Birks, 2006). Cholinesterase inhibitors have received much criticism, and even though they have been the first-line therapy for AD patients for 20 years, their efficacy is still being questioned (see e.g. Lanctôt et al., 2009; Rountree et al., 2013). Although they do not benefit all AD patients they still remain an important part of disease therapy improving the daily life of many of those receiving this medical treatment. The most significant disease benefiting from cholinesterase inhibition, AD, will be discussed in the next section.

1.2.1 Dementia: Alzheimer's disease

Dementia is a condition that affects the function of the brain as nerve cells stop functioning properly and start deteriorating. Depending on the area of the brain that is affected, cells that manage functions such as memory, language, judgment and behaviour, fail (Aranda-Abreu et al., 2011), making it a difficult condition not only for the patients but also for the caregivers. The cause of dementia is usually an underlying neurological disorder, but it can also be caused by changes in the brain due to factors such as brain injuries or vitamin B12 deficiency (Moore et al., 2012; Sivanandam and Thakur, 2012). The types of dementia include vascular dementia, frontotemporal dementia, dementia with Lewy bodies and AD. Of these, AD is the most common form of dementia and estimated to affect over 35 million people worldwide (<http://www.alz.co.uk/research/files/WorldAlzheimerReport-ExecutiveSummary.pdf>). This number is growing rapidly as the population ages, and it has been predicted it will affect 1 in 85 people by the year 2050 (Brookmeyer et al., 2007). This high incidence, together with the severity of the disorder has made it a major research area; however, no cure is yet available and life expectancy after the disease is diagnosed is 3-10 years (Zanetti et al., 2009). In fact, AD is one of the leading causes of death in the high-income countries (WHO fact sheet Nr 310. The top 10 causes of death. World Health Organization, 2011).

The gradual deterioration of the AD brain is seen as a worsening of the patient's condition from not remembering names to not being able to dress themselves and ultimately needing full-time care. In a report published in 2012 by WHO and the Alzheimer's Disease International (ADI), it was emphasized that dementia needs to be considered a public health priority (Dementia: A public health priority. World Health Organization and Alzheimer's Disease International, 2012). The population is growing older and to be able to cope with this new burden awareness needs to be raised and treatment plans made. The slow progression of AD makes it a difficult condition to detect. The first signs include personality changes and problems with recent memory, but these, however are common signs that can appear as result of normal aging and do not necessarily mean a person has AD.

Elderly people with memory problems may have a condition called mild cognitive impairment (MIC), a sort of middle stage between normal aging and dementia (Gauthier et al., 2006), which can often develop into AD. Generally, AD is divided into three stages; mild, moderate, and severe AD. Mild AD is considered when memory loss begins occurring, and the patient starts forgetting names and where things are. In addition, personality changes can be observed. When a patient develops moderate AD, memory becomes even more

impaired and, for example, recognizing familiar faces becomes problematic. At this stage daily activities such as getting dressed are difficult and more personality changes can be observed. The patient might even experience hallucinations, paranoia and start to become violent. At the severe AD stage the disease is so advanced that the patient is unaware of their surrounding and unable to talk, eat, and take care of themselves. There are several different scales to assess the cognitive impairment of AD patients, among them are the most widely used Mini-Mental State Examination and the Alzheimer's Disease Assessment Scale – Cognitive subscale (Robert et al., 2010). These are continuously used in clinical trials to evaluate the effect of the treatment.

The disease is said to first have been described by Dr Alois Alzheimer in 1906 as he presented a peculiar disease of the brain which formed plaques and tangles (Holtzman et al., 2011). Considering that AD has been known for over 100 years, it is only in recent years that AD research has truly expanded with new drug targets emerging with new hope for AD pharmacotherapy. However, at the same time pharmaceutical companies that have invested in AD drug therapy and other CNS disorders are becoming more reluctant to continue to invest in these programs as they are considered to be risky with many drug candidates failing in clinical trials. The problem lies in the numerous physiological changes that occur in the AD brain that cannot be simplified into one single affected pathway. This complexity has become more apparent as the knowledge of this pathology progresses. There are many unanswered questions regarding the cause of AD and it is not an easy task to identify which is the original cause of the disease and which are consequences of the body's response to the original physiological change. Over the years several theories have emerged, but there is still no definite answer. Some of the hypotheses will be discussed briefly in the following section.

The cholinergic hypothesis and cholinesterases

The cholinergic hypothesis is the oldest of the hypotheses and arose from the fact that there is a significant loss of cholinergic neurons in the brain of AD patients as well as a reduced activity of choline acetyltransferase which catalyzes the production of acetylcholine, resulting in decreased neurotransmission and cognitive dysfunction (Whitehouse et al., 1982; Francis et al., 1999; Gauthier, 2002). Also a reduction in nicotinic and muscarinic receptors has been observed (Francis et al., 2010). By using cholinesterase inhibitors the levels of acetylcholine, which have a central role in cognitive functions (Liston et al., 2004), can be restored. In research, AChE has usually been the main drug target but lately the research has also been focused on the search for BChE inhibitors (e.g. Darvesh et al., 2007; Decker et al., 2008; Carolan et al., 2010; Nawaz et al., 2011). This is due partly to the fact that the activity of BChE seems to be unaffected by the changes occurring in the AD brain while the activity of AChE seems to decrease (Giacobini, 2004). Thus in the AD brain, BChE performs a more central role in cholinergic transmission and with already depleted acetylcholine levels (Greig et al., 2005), this is believed to cause further cognitive decline. Both enzymes are present in neuritic plaques and neurofibrillary tangles (Mesulam et al., 1987) and inhibition of AChE and BChE increase the amount of free acetylcholine that can interact with neuronal receptors

(Lane et al., 2006). The importance of selectively inhibiting BChE has further been shown using aged rats where BChE inhibition augmented acetylcholine levels, increased cognitive function and decreased amyloid deposits (Greig et al., 2005). Researchers have also found a link between a less active variant of BChE (Ala539Thr) and a lower tendency to develop AD (Podoly et al., 2009). This supports the value of inhibiting BChE and is also the basis for selecting BChE as a primary target for this thesis project. Current therapies (discussed in section 1.2.1.1) are mostly based on the cholinergic hypothesis.

The amyloid hypothesis

The next two major hypotheses involve the original hallmarks of the disease, amyloid plaques and neurofibrillary tangles, which have central roles in the pursuit of a next level strategy to combat AD (Karran et al., 2011; Duan et al., 2012). Amyloid plaques consist of amyloid beta ($A\beta$) peptides that cluster together and form extracellular aggregates impeding neuron function and causing neurotoxicity (Lorenzo and Yankner, 1994). The neurotoxic species of $A\beta$ have been identified as the $A\beta_{1-40/42}$ peptide species and are produced by proteolytic cleavage of the amyloid precursor protein (APP). APP is an integral membrane protein that is found throughout the body and is believed to regulate several processes such as synapse formation, neural plasticity (Turner et al., 2003), and dendritic spine formation and maintenance (Lee et al., 2010). APP can be processed in two ways, through the amyloidogenic pathway which produces $A\beta$ by being cleaved by β - and γ -secretase or the non-amyloidogenic pathway where APP is cleaved by α - and γ -secretase producing the non-toxic component α -sAPP. The increased level of $A\beta$ in the AD brain has led to the amyloid cascade hypothesis (Hardy and Higgins, 1992), where researchers believe that targeting $A\beta$ could reduce, or even stop, the progression of AD (Hardy and Selkoe, 2002; Karran et al., 2011). In 1998, a study was made where oligomers instead of amyloid fibrils were found to be the neurotoxic species, resulting in the oligomer hypothesis (Lambert et al., 1998).

Supporting the hypothesis are the recent findings that mutations in the APP gene have shown to be risk factors (e.g. at position 670, 671, 687, 692 and 693) (Cras et al., 1998; Kaden et al., 2012) or protective (position 673) (Jonsson et al., 2012) against developing AD in certain populations. It seems that humans carrying the Ala673Thr mutation form less amyloidogenic peptides, this is probable because it is adjacent to the aspartyl protease β site in APP and thus reduces β cleavage (the amyloidogenic pathway). However, there are many researchers that oppose this hypothesis as it seems to be difficult to obtain data showing any substantial relationship between amyloid burden and cognitive decline in AD patients (Bishop and Robinson, 2002; Zhang et al., 2012b). Nevertheless, $A\beta$ is still widely accepted as a potential target and therapy strategies include inhibition of $A\beta$ aggregation (e.g. Stevens et al., 2013; Zhang et al., 2013), β -secretase (BACE) (e.g. Roberds et al., 2001; Efremov et al., 2012) and γ -secretase (e.g. Meunier et al., 2013), and the stimulation of α -secretase (e.g. Marcade et al., 2008). It has also been shown that there is a connection between AChE and the formation of $A\beta$ fibrils (Inestrosa et al., 1996) and that stimulation of muscarinic acetylcholine

receptors promotes the non-amyloidogenic pathway (Davis et al., 2010), thus linking the cholinergic and amyloid hypotheses.

Tau hyperphosphorylation and emerging theories

Tau stabilizes microtubules in the neurons, which are important for the cell structure as well as cellular trafficking (Gendron and Petrucelli, 2009). When tau becomes phosphorylated the dynamics of the microtubule becomes affected and thus also the cell functions it regulates. Increased kinase activity leads to persistent tau phosphorylation which leads to neurite retraction and synaptic dysfunction (Sayas et al., 2002; Sun et al., 2011) and in the long run to the appearance of insoluble tau neurofibrillary tangles (NFTs). Glycogen synthase kinase 3 β (GSK3 β) is a serine/threonine protein kinase that is involved in regulating cellular functions such as cytoskeletal organization, cell cycle regulation, and apoptosis (Hardt and Sadoshima, 2002). It co-localizes with dystrophic neurites, pre-tangles and NFTs (Boutajangout et al., 2011), and seems to have an important role in neuronal development (Kim and Snider, 2011). GSK3 β inhibition decrease tau phosphorylation and thus microtubule stability in neurons remain intact (Hardt and Sadoshima, 2002). Inhibition of GSK3 β has recently been proven to reduce memory deficit using a mice model (Ly et al., 2013).

The cholinergic deficit, amyloid burden and hyperphosphorylated tau are the most accepted disease theories. However, there are several other mechanisms that are believed to result in AD. Some researchers believe that the failure in metal transportation contributes to AD pathology as A β PP, presenilins and tau have been shown to participate in this transportation and the fact that metal perturbances in absence of AD pathology can lead to neurodegeneration and loss of cognition (Bush, 2013). Inflammation is thought to play a part in AD pathology as inflammatory constituents such as cytokines and cyclooxygenase-2 (Rubio-Perez and Morillas-Ruiz, 2012) are present in the AD brain and the use of non-steroidal anti-inflammatory drugs has been shown to reduces the risk of AD (in 't Veld et al., 2001). However, it has not been proven to work as a treatment for patients already diagnosed with AD (Jaturapatporn et al., 2012). Mitochondrial dysfunction is also being investigated (Lunnon et al., 2012; Morán et al., 2012). Several recent reviews discuss these and other therapy approaches that are being considered for future successful AD treatment (see e.g. Hong-Qi et al., 2012; Huang and Mucke, 2012; Singh et al., 2012).

1.2.1.1 Current therapies

The treatment options for AD are cholinesterase inhibitors and an *N*-methyl-D-aspartate (NMDA) receptor antagonist (Farlow and Cummings, 2007; Farlow et al., 2008; van Marum, 2008) (**Figure 5**). As AChE is the main enzyme to hydrolyse acetylcholine and thereby ending neurotransmission, AChE inhibitors were developed as a treatment strategy for AD. Tacrine, a non-competitive synthetic AChE inhibitor, which also inhibits BChE (Ibach and

Haen, 2004) was the first drug to enter the market and was approved by the U.S. Food and Drug Administration (FDA) in 1993 (Summers, 2006). At that time there were already discussions about the toxic effect of tacrine (Watkins et al., 1994) and nowadays tacrine is not commonly used due to tolerability and hepatotoxicity issues (Bachovchin and Cravatt, 2012). However, the scaffold of tacrine has served as a starting point for medicinal chemists and many cholinesterase inhibitors taking advantage of the same ring structure as tacrine have been published (e.g. Chen et al., 2012; Hamulakova et al., 2012; Wang et al., 2012). The next generations of cholinesterase inhibitors included donepezil in 1996, rivastigmine in 2000 and galanthamine in 2001 (van Marum, 2008), all of which have a greater affinity for AChE, although rivastigmine is considered a dual inhibitor as the difference in potency between AChE and BChE is minor (Giacobini, 2004). All the drugs were also authorized for the European market and are still used today for the symptomatic treatment of AD. Memantine is a low-affinity, uncompetitive inhibitor of NMDA receptors, which allows it to normalize glutamergic neurotransmission without hindering transmission completely (Parsons et al., 2007). Memantine was approved in Europe in 2002 and in the US one year later for patients with moderate-to-severe AD (Möbius, 2003; Hussar, 2004). The combinational therapy of cholinesterase inhibitors and memantine has also been extensively studied and has shown a positive outcome (Atri et al., 2013).

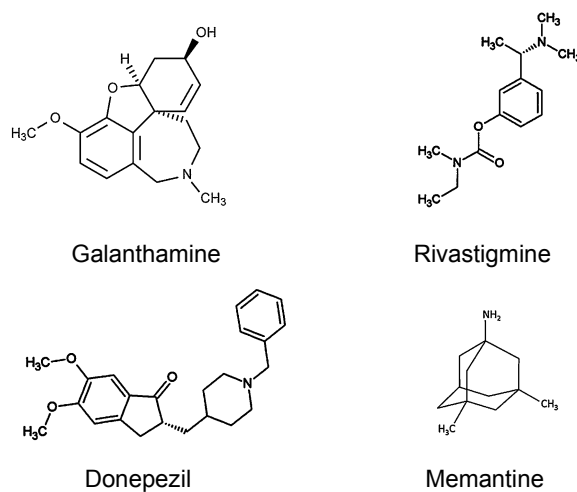


Figure 5. Currently approved drugs in the US and Europe for the symptomatic treatment of AD. Galanthamine, rivastigmine and donepezil are cholinesterase inhibitors and memantine is an NMDA receptor antagonist.

With regard to cholinesterase inhibitors, their benefits are often considered short-lived with no enduring effects. In several large controlled trials, cholinesterase inhibitors did not show significant effects in delaying or preventing conversion of patients from MCI to dementia (Petersen et al., 2005; Feldman et al., 2007; Winblad et al., 2008). The limitations of using cholinesterase inhibitors include several adverse effects (such as nausea, vomiting and diarrhoea) as well as the problem of accurately predicting which patients will respond to treatment. However, the failure of these studies could be explained by problems in trial

methods and analysis (Shanks et al., 2009) and the symptomatic effects of using cholinesterase inhibitors have been positively demonstrated in a number of other large, controlled trials. For example, a Cochrane review of 13 randomized, placebo-controlled, double blinded trials stated that galanthamine, donepezil and rivastigmine improve cognitive functions of patients with mild to moderate AD which could also be seen in measures of daily living and behaviour (Birks, 2006). The positive effects of cholinesterase inhibitors are regarded significant enough in order to use these drugs as a first-line therapy for mild to moderate AD, even though it is several decades since their discovery. However, considering the impact AD has on the patient as well as the caretakers and society, a better solution than symptomatic relief is sought. Ideally, the beneficial effect of an AD drug should be reflected in cognitive or behavioural improvements that would take place even after the treatment is stopped or interrupted. This would require a drug that modifies pathological steps leading to AD (disease-modifying drugs) and could be achieved by combining several targets, as has been done previously for example with the cholinesterases and A β aggregation. This is also something we have striven towards during this thesis work.

1.2.1.2 Importance of biomarkers for AD

As the therapeutic strategy for AD is likely to change in the near future, one important factor in upcoming drug development would be a more careful selection of patients during clinical trials. This could be achieved by using biomarkers that could aid in the process. The word biomarker, a biological marker, is used to define substances that can identify biological processes or disease states in an organism. Biomarkers are valuable for disease diagnosis and the requirement of a biomarker is that it should be reliable and stable enough to allow an early and accurate diagnosis. Biomarkers are also important for selecting drug candidates as they can aid in evaluating whether this candidate affects the underlying pathophysiology of the disease in question (Blennow, 2010). In order to combat AD in the most effective way, treatment needs to be started at an earlier stage before the disease has caused too much irreversible damage. The difficulty with AD is that the diagnostic criterion is too unspecific and drugs that are tested in clinical trials often seem to lack efficiency, which may be due to the fact that trial durations are too short and they are typically performed on advanced AD cases (Hempel, 2012). The search and validation of biomarkers that can help identify pre-dementia and even pre-clinical asymptomatic stages of AD, is a very current and important field. Cerebrospinal fluid biomarker candidates reflecting AD pathology are total tau, phosphorylated tau and the 42 amino acid form of A β (Blennow, 2010). However, patients can have different burdens of A β , phosphorylated tau, inflammation and other factors involved in AD. Reliable biomarkers for AD can be used for predicting the transition from MCI to dementia (Parnetti et al., 2012) as well as future cognitive decline in healthy individuals (Bendlin et al., 2012; Paternicò et al., 2012). Neuroimaging is being used as a non-invasive way of monitoring the physiological changes occurring in the diseased brain (Reiman and Jagust, 2012) and by using radiotracers it is possible to measure the A β load in the brain as well as observe the changes in A β over time (Villemagne and Rowe, 2013). This enables monitoring of patients selected for clinical trials, make deeper assessments of trial

outcome and, further on, it could be used to identify patients that will benefit from specific drug treatments. Several drugs in clinical trials are thought to have failed due to patient selection and the identification of valuable biomarkers to assess the effect of the drug candidates is crucial (Blennow, 2010).

1.3 The early drug discovery process

Discovering and developing a new drug is a very long and costly process that usually takes between 10-15 years and costs an average of 1 billion dollars (Khanna, 2012). The greatest problem in the pharmaceutical industry is the high attrition rate. A majority of drug candidates fail in clinical trials, or even before, either due to adverse effects or the lack of efficiency (Khanna, 2012; Mullard, 2012). An important part of the drug discovery process is a good understanding of the disease in question so that a good drug target can be chosen. A drug target can be considered a gene or a gene product which is involved in the manifestation of a disease in an organism. When a drug target has been identified researchers initiate the search for drug candidates. This is usually done by screening sets of compounds (discussed in the next section) which are then narrowed down (**Figure 6**) by criteria such as activity, cytotoxicity and absorption, distribution, metabolism and excretion (ADME) properties.

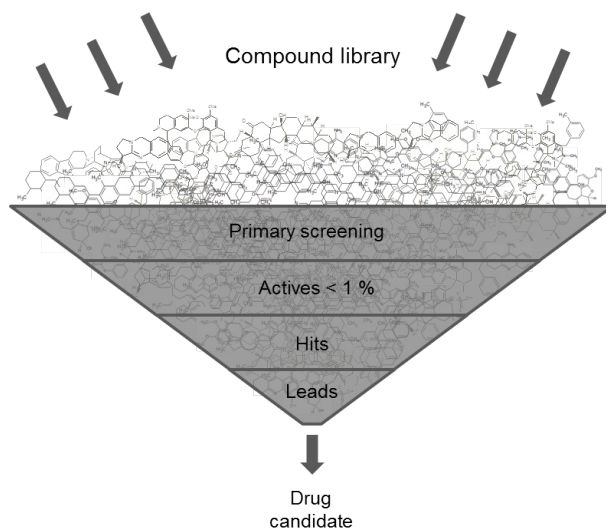


Figure 6. The compound attrition during the early drug discovery process. Of all the compounds tested during primary screening only a small number of compounds eventually continue along the drug discovery path and ultimately become drug candidates. Active compounds are generally considered those compounds that show desirable activity in the primary screening while hits are the active compounds from the primary screening where the activity has been confirmed and potencies determined. Further, leads are compounds that have been optimized from the hit compounds in order to improve the potency, selectivity, and *in vitro* ADME profile of the hit.

1.3.1 Screening approach

Screening, in the early drug discovery process, implies searching for a certain activity among a selected set of compounds. The goal of screening diverse compound libraries is to identify chemical scaffolds which can serve as interesting starting points for development into drug candidates. Before the first microplate (also termed microtitre or multiwell plates) arrived in the 1950s, compounds were tested in a cuvette or test-tube and the screening process was low-throughput, that is, few compounds could be tested at a time. The introduction of microplates allowed for faster screening of larger libraries with reduced costs as less time and reagents were needed. Nowadays, high-throughput screening (HTS) and Ultra HTS (UHTS) is applied in the pharmaceutical industry which allows for faster screening of a large amount of compounds where 96-, 384-, and 1536-well formats are the most commonly used (Mayr and Bojanic, 2009). In the pharmaceutical industry, workstations are often fully automated with robots handling the plates, from taking the plate containing the compounds out of the storage, picking the compounds to be tested, preparation of the screening plate and reading it. In an academic environment the workstations are seldom connected and the plate handling between workstations is manual. Automation is not only beneficial because it is faster and lightens the workload for the researchers, but it also reduces variations between plates and potential human errors. The most common way of screening compounds is in a one-well composition, that is one compound is tested in one well. It is also possible to pool compounds so as to test several compounds in the same well (5-20 compounds/well) in order to increase throughput and reduce costs. However, this may lead to problems such as higher false positive and negative hits, aggregation or interference (Kainkaryam and Woolf, 2009). As the assay selection has an impact on the screening results it is crucial to choose a good assay which is cost-effective, rapid, accurate, and precise. The assay performance should be monitored in order to validate the assay and ensure that high quality data can be obtained from the screening (Zhang et al., 1999).

There have been discussions regarding how screening libraries should be composed. Some believe that random libraries, libraries containing a diversity of structures, are more beneficial as this is more likely to yield novel structure classes for lead optimization. On the other hand, focused or targeted libraries, as the name implies, contains a smaller subset of compounds of a certain chemical class that is believed to have an effect on the desired target. Both approaches have their benefits and drawbacks, but are considered to complement each other, and so both are commonly used (Valler and Green, 2000). Lipinski's rule of five (Lipinski et al., 1997) is often used as a guideline when preparing compound libraries. Lipinski and co-workers stated that molecules that have a molecular weight over 500, more than 5 H-bond donors or 10 H-bond acceptors and a LogP over 5 are more likely to have a poor absorption and permeation, thus making poor drugs. However, there are several drugs that do not apply to these rules, for example many antibiotics belonging to the glycopeptide and aminoglycoside chemical classes have a higher molecular weight as well as higher lipophilicity (O'Shea and Moser, 2008). Natural products are also considered to be an exception to this rule, although they generally comply with having low LogP and H-bond donors (Ganesan, 2008). The storage condition of a compound library is important as well, as

this will ensure that the quality of the compounds remains intact. Dimethyl sulphoxide (DMSO) is the most common solvent to dissolve compounds aimed for storage in compound libraries, which are usually stored at temperatures between -20 and +4 °C (Di and Kerns, 2006).

1.3.2 The screening approach during this thesis

The primary target during this work has been BChE and the secondary target has been AChE. As mentioned earlier in section 1.2.1 Dementia: Alzheimer's disease (The cholinergic hypothesis), considering recent experimental data, it is believed that inhibiting BChE could be beneficial and in a way offers a new target to a known subject. Cholinesterase activity can be measured using Ellman's reagent [5,5'-dithiobis (2-nitrobenzoic acid), DTNB] (Ellman et al., 1961), Fast Blue B salt [3,3'-dimethoxy-4,4'-biphenylbis(diazonium) zinc chloride] (van Asperen, 1962) or assays using fluorescent labelling (Guilbault and Kramer, 1965; Parvari et al., 1983; Gainullina et al., 2006). Ellman's reagent, which has been used in this thesis, react with the product of cholinesterase hydrolysis and forms a coloured product (5-thio-2-nitrobenzoate) that can be spectrophotometrically measured at 412 nm (Ellman et al., 1961) (a detailed description of the assay can be found in section 3.2.1 Ellman's reaction). In **Figure 7** the procedure using the 96-well layout is visualized in a simplified way.

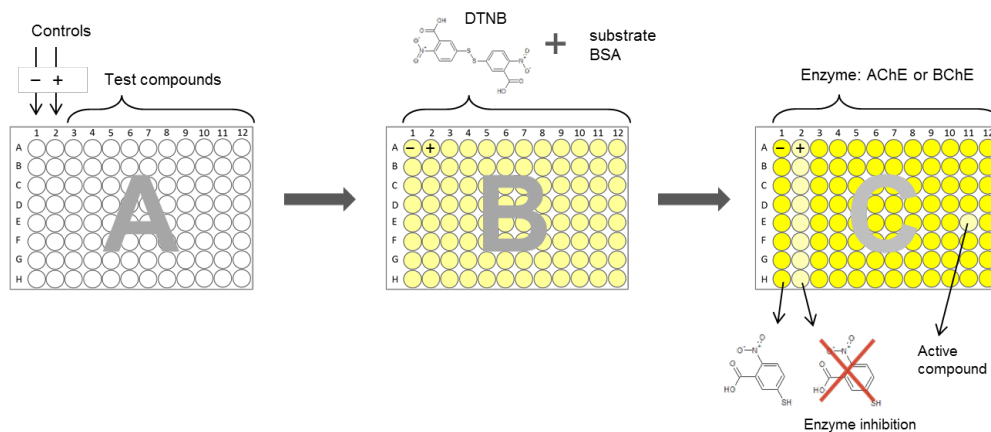


Figure 7. Screening procedure using Ellman's reagent (DTNB). **A)** Test compounds are transferred from the compound library to the screening plate and a positive and negative control is included in order to assess assay performance. **B)** Assay reagents are added into the screening plate [substrate, DTNB and a buffer containing bovine serum albumin (BSA)] and background signal measured. **C)** Enzyme addition starts the hydrolysis of the substrate and the formation of a coloured anion occurs due to the reaction of the hydrolysis product with the DTNB. Depending on the degree of enzyme inhibition, differences in the absorbance is observed. Maximal and minimal signal is obtained from the column containing the negative control (control not inhibiting the enzyme).

Considering that both the target and the assay had previously been validated, it allowed this project to start directly with the biomolecular screening for cholinesterase inhibitors, with a focus on BChE. The origin of the libraries was both natural and synthetic and they were tested on the enzyme target by the biochemical assay using Ellman's reagent (**Figure 7**). The screening was monitored using the statistical parameters: signal window coefficient (Z' factor), signal-to-background (S/B) ratio (Zhang et al., 1999), signal-to-noise (S/N) ratio (Bollini et al., 2002), and coefficient of variation of the assay (CV_A) (Iversen et al., 2006) (equations can be found in section 3.3.3 Statistical analysis). The Z' factor has been widely applied in both the industry and academia (Sui and Wu, 2007) since its introduction in 1999, as S/B and S/N in themselves are considered insufficient to evaluate the screening quality (Zhang et al., 1999). Further, the potency and kinetic mechanisms of the active compounds were determined in order to verify the hits. Additionally, all libraries and active compounds were analysed in chemical space using ChemGPS-NP (discussed in more detail on the next page; ChemGPS-NP as a tool in drug discovery), to assess the chemically relevant space for BChE inhibitors. With the help of medicinal chemists, in publication **I** and **II**, tasks such as hit refinement and structure-activity relationship was addressed and even a pharmacophore model was proposed. Additional assays were also used in order to shed light onto possible dual-activity and safety of the active compounds, such as the thioflavin T assay which detects $A\beta$ aggregation and cytotoxicity studies. All of these methods have made it possible for us to find the hit compounds that will be presented in this thesis. The screening approach during this thesis project is visualized in **Figure 8**.

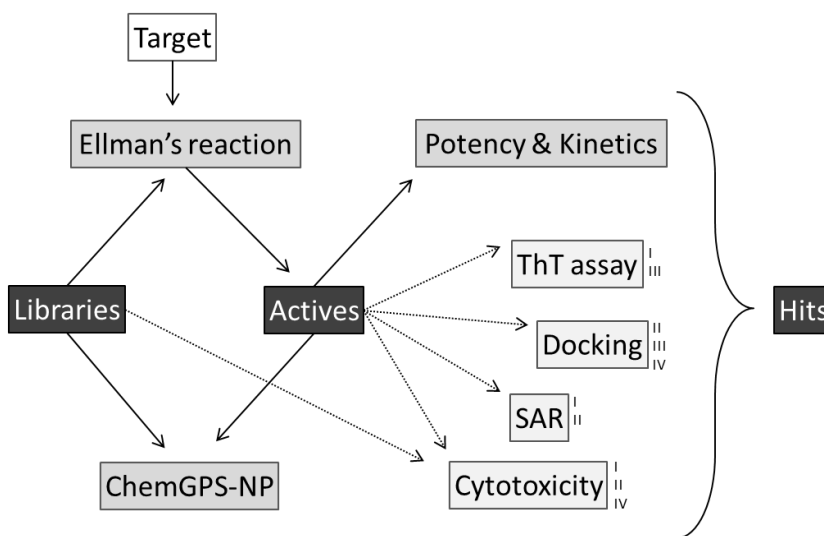


Figure 8. The screening approach during this thesis project. The full arrows indicate paths taken in all four publications while the dotted arrows represent approaches taken in different publications (indicated in the figure). In the case of publication **II** the entire library was tested for cytotoxicity.

It is a common practice nowadays in drug discovery to use a combination of *in vitro* and *in silico* methods to gather data on compounds. The information obtained from using ChemGPS-NP together with potency, molecular docking, and cytotoxicity studies gives a first profile of the active compounds. Thus, combining these methods, we have sought to identify new and potent cholinesterase inhibitors in this thesis project. Although cholinesterase inhibitors are symptomatic treatment of AD, new and improved inhibitors are needed and using a combination of *in vitro* and *in silico* tools to evaluate compound characteristics is a good way forward.

2 AIMS OF THE STUDY

The majority of the screening to find new cholinesterase inhibitors have been focused on AChE inhibitors. In this project, the focus has been on identifying new chemical classes of inhibitors that are either selective inhibitors of BChE or dual BChE/AChE inhibitors.

The specific aims were as follows

- I. To identify BChE inhibitors from a collection of diverse synthetic compounds based on naturally present scaffolds using an *in vitro* assay and to further test their ability to inhibit A β aggregation, as this has an implication in AD.
- II. Phenothiazines have been shown to inhibit BChE. In this study we wanted to verify if thienothiazines, which share structural features with the phenothiazines, could also inhibit BChE activity and if so, which are the structural requirements to do so.
- III. To identify compounds that could target two of the important features in AD: cholinergic depletion and A β aggregates. Thus, a diverse library of natural products was tested *in vitro* for an effect on cholinesterase activity and further tested for A β aggregation as well as disaggregation.
- IV. As we identified cinchona alkaloids as BChE inhibitors in publication **III** and there have previously been reports on cinchona alkaloids as cholinesterase inhibitors, we wanted to investigate whether other modifications to the cinchona scaffold could also yield BChE inhibitors.

3 MATERIALS AND METHODS

3.1 Compound libraries

3.1.1 Synthetic compound libraries (I and II)

The two synthetic compound libraries that have been screened contain a wide variety of compounds synthesized by the group of Professor Thomas Erker at the University of Vienna, Austria. The larger library consisted of 697 compounds partly containing naturally-present scaffolds such as alkaloids, benzanilides, coumarins, flavonoids, imidazoles, and thiophenes (**I**), while the smaller library of 45 compounds focused on thienothiazine derivatives (**II**). The compounds were prepared in cryogenic vials (external threaded polypropylene vials with plug seal caps) to a concentration of 20 mM and stored at -20 °C. The compounds were diluted in Tris-HCl 50 mM pH 8 before screening at a final concentration of 10 µM. The larger library (**I**) was transferred to 96-well microplates using a Biomek 3000 liquid handling station (Beckman Coulter, USA).

3.1.2 Enzo library (III)

The Enzo[®] Screen-Well[®] Natural Product Library (Enzo Life Sciences, Inc. USA) is a commercially available compound library that contains 502 natural and naturally derived compounds. The concentration of the compounds is 2 mg/ml in DMSO. Compounds are maintained at -70 °C and are thawed in a water bath at +37 °C before usage. The library was transferred to 96-well microplates using a Biomek 3000 liquid handling station (Beckman Coulter, USA) and diluted in Tris-HCl 50 mM pH 8 to concentrations ranging from 0.117 to 1.624 mM, which means a final concentration between 2.35 and 32.65 µM in the primary screening. The sealed plates were stored at +4 °C and screened within one week of preparation.

3.1.3 Cinchona alkaloids (IV)

The small subset of 23 cinchona alkaloids contained commercially available cinchonine and cinchonidine and their derivatives synthesized by the group of Professor Reko Leino at the Laboratory of Organic Chemistry at Åbo Akademi University, Finland. All compounds were prepared as described in section 3.1.1 Synthetic compound libraries.

3.2 Biochemical assays (*in vitro*)

3.2.1 Ellman's reaction (I-IV)

The Ellman's reaction (Ellman et al., 1961) was used in all publications to detect the anticholinesterase activity of the compound libraries as well as determining the mean inhibitory concentrations (IC_{50}) and kinetic mechanisms of the hits. When BChE cleaves butyrylthiocholine or when AChE cleaves acetylthiocholine they produce thiocholine and butyrate or acetate, respectively. The hydrolytic activity of BChE and AChE can be spectrophotometrically detected due to the formation of a coloured anion (5-thio-2-nitrobenzoate) after the Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoid acid) (DTNB), reacts with the thiocholine. The spontaneous and enzymatic hydrolysis is measured 10 times during 10 min with Victor2 1420 multilabel counter (PerkinElmer, Finland) at $\lambda=405$ nm (**I** and **IV**) or Varioskan Flash multimode plate reader (Thermo Fisher Scientific, Finland) at $\lambda=412$ nm (**II** and **III**). This is done in a 96-well microplate where the final concentrations of the reaction components in the primary screening are: between 2.35 and 32.65 μ M of compound in Tris-HCl 50 mM pH 8 depending on the compound library screened (see previous section 3.1 Compound libraries), 1.5 mM substrate in purified water (*S*-butyrylthiocholine chloride (BTCCI) for BChE and acetylthiocholine iodide (ATCI) for AChE), 1.5 mM DTNB in Tris-HCl 50 mM pH 8 containing 0.1 M NaCl and 0.02 M $MgCl \times 6 H_2O$ and finally 0.1 % (w/v) bovine serum albumin (BSA) in Tris-HCl 50 mM pH 8. The reaction is started by the addition of the enzyme at concentrations of 0.350 U/ml for equine BChE (BChE; EC 3.1.1.8, from equine serum, Sigma-Aldrich, USA); 0.192 U/ml for recombinant human BChE (huBChE; EC 3.1.1.8, expressed in transgenic goat, Sigma-Aldrich, USA); 0.224 U/ml for electric eel AChE (AChE; EC 3.1.1.7, from Electric eel, Sigma-Aldrich, USA) and 0.198 U/ml for human AChE (huAChE; EC 3.1.1.7, from human erythrocytes, Sunnyslab, UK). Physostigmine was used as a positive control and DMSO:buffer Tris-HCl 50 mM pH 8 was used as a solvent control. Galanthamine was also used as a positive control compound. The final DMSO concentration during primary screening was 0.05 % in **I**, **II** and **IV** and less than 0.16 % in **III**.

3.2.1.1 Potency determination (I-IV)

To determine the potency of the active compounds the IC_{50} values were calculated, that is the concentration needed to inhibit half of the enzyme activity. This was done by testing the inhibitory activity, as described in section 3.2.1 Ellman's reaction, against BChE and/or AChE at several concentrations so as to obtain a concentration-response curve. For this purpose a minimum of seven concentrations in at least three replicates were used. In the case of selective BChE inhibitors, AChE was also tested up to concentrations of 250 (**I** and **IV**) or 500 (**II**) μ M to determine the selectivity of the active compounds.

3.2.1.2 Kinetic measurement (I-IV)

The mode of action of the hits on BChE or AChE was determined using a minimum of five substrate concentrations in the range of 0.065 – 1.5 mM for BTCCl (**I**, **II** and **IV**) and 0.2 – 2 mM for ATCI (**III**). Results were fitted into Lineweaver-Burk double-reciprocal ($1/V$ vs $1/[S]$) and Dixon ($1/V$ vs $[I]$) kinetic plots and apparent K_i values were calculated as follows:

Uncompetitive (K_{iuc}):

$$K_{iuc} = \frac{V_{\max \text{ app}}[I]}{V_{\max} - V_{\max \text{ app}}}$$

Competitive (K_{ic}):

$$K_{ic} = \frac{K_M V_{\max \text{ app}}[I]}{K_{M \text{ app}} V_{\max} - K_M V_{\max \text{ app}}}$$

The kinetic constants determined in absence of inhibitors (I) are marked as K_M ; V_{\max} and in the presence as $K_{M \text{ app}}$; $V_{\max \text{ app}}$. For mixed inhibition a combination of both inhibition types occurs.

3.2.2 Thioflavin T (I and III)

The fluorometric assay using Thioflavin T (ThT) (LeVine, 1993) was used to detect aggregation of two peptides as a shift in the emission and excitation spectra ($\lambda_{\text{excitation}} = 385$ nm and $\lambda_{\text{emission}} = 445$ nm to $\lambda_{\text{excitation}} = 450$ nm and $\lambda_{\text{emission}} = 482$ nm) of ThT as it interacts with the aggregates. The first peptide was an 11-amino acid fragment containing the sequence KLVFF (**I**) and experimental procedure was conducted as described by Alptüzün et al. (2010) at inhibitor concentrations of 2.5, 25 and 250 μM . The second peptide that was used was the $A\beta_{1-40}$ peptide (**I** and **III**) and the experiment was carried out as described by Liu et al. (2004) with several modifications as described in publication **I**. Nordihydroguaiaretic acid (Ono et al., 2004) and 4-aminophenol (De Felice et al., 2004), which are known to inhibit $A\beta$ aggregation, were used as control compounds. Generation of fluorescent ThT-positive $A\beta_{1-40}$ aggregates was accelerated using 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Catto et al., 2010). In publication **I**, at higher concentrations, the most active compound (6c) was incubated in the buffer/ThT mixture at 37 °C and 1000 rpm for 90 minutes before protein was added. The IC_{50} was determined using six concentrations between 5 and 50 μM in three replicates.

3.2.2.1 Disaggregation of A β (III)

In order to measure the effect of the compound on preformed aggregates, the A β_{1-40} peptide was allowed to incubate with ThT for 45 min at 1000 rpm in 37 °C prior to compound addition. Before adding the compounds (concentrations between 0.5 and 250 μ M) the fluorescence was measured to ensure that aggregates had been formed. Resveratrol (Feng et al., 2009) was used as a positive control.

3.2.2.2 AChE induced aggregation (III)

Induction of A β_{1-40} aggregation using the electric eel AChE was performed in analogy to the method described by Inestrosa et al. (1996). A β_{1-40} peptide and compound was dissolved in 0.1 M Tris-HCl, pH 7.4 to a concentration of 230 μ M and 1, 5, 10 and 100 μ M, respectively. Sodium phosphate buffer (0.1 M, pH 8) was used to prepare the electric eel AChE to a final concentration of 2.3 μ M. The final ThT concentration in the assay was 1.5 μ M and fluorescence was measured at $\lambda_{\text{excitation}} = 440$ nm and $\lambda_{\text{emission}} = 485$ nm after 24 h incubation at 25 °C with Varioskan Flash multimode plate reader (Thermo Fisher Scientific, Finland).

3.2.3 In vitro cytotoxicity assay (I, II and IV)

Cell lines of different origins were utilized to determine the cytotoxic effect of the active hits: mouse hypothalamic immortalized (GT1-7) (Mellon et al., 1990) (**I**, **II** and **IV**), human lung (HL) (**I**, **II** and **IV**), human liver (HepG2) (**II** and **IV**) and human epithelial (Caco-2) (**II**, **IV**) cells. Plates containing cell suspensions of 4×10^5 cells/ml (200 μ l) for GT1-7, HL and HepG2 and 4.5×10^5 cells/ml (100 μ l) for Caco-2 cells were incubated at 37 °C for 24 h. After which, 10 (for Caco-2 cells) or 20 μ l of culture media was replaced with compound solutions at different concentrations (10-100 μ M) and incubated for an additional 24 h (**I**, **II** and **IV**) or up to 72 h (**II**) at 37 °C. The culture media was replaced with 20 μ M resazurin (In vitro Toxicology Assay Kit, resazurin-based, Sigma-Aldrich, US) in PBS for HL, HepG2 and Caco-2 cells and 25 μ M for GT1-7 cells and maintained for 2 h at 37 °C in 5 % CO₂ in an air-ventilated humidified incubator. Inside viable cells, resazurin is reduced to fluorescent resorufin (O'Brien et al., 2000) which was measured using a Varioskan Flash multimode plate reader ($\lambda_{\text{excitation}} = 570$ nm and $\lambda_{\text{emission}} = 590$ nm). Results are expressed as relative fluorescence units (RFU) and a reduced resazurin signal indicates that cells are viable. Cells not treated with the active hits were used as positive control, only media was used as a negative control and 0.5 % DMSO as a solvent control. Cell viability was calculated as a percentage of the treated cells in relation to the untreated cells.

Cell cultures used in cytotoxicity assay

Cell lines were cultured using media and supplements as listed in **Table 1** in 75 cm² cell culture flasks at 37 °C in 5 % CO₂ in an air-ventilated humidified incubator to around 90 % confluence. Cells were harvested by adding 0.05 % (v/v) trypsin and 0.02 % (w/v) EDTA in PBS for GT1-7, HepG2 and Caco-2 cells and 0.25 % trypsin in PBS for HL cells.

Table 1. Cell culturing media and supplements according to cell line.

Cell line	Media	Supplements	Publications
GT1-7	DMEM ^{a)}	10 % inactivated FBS 50 IU/ml penicillin 50 µg/ml streptomycin	I, II and IV
HL	RPMI 1640 ^{b)}	7 % inactivated FBS 2 mM L-glutamine 20 µg/ml gentamicin	I, II and IV
HepG2	DMEM ^{a)}	10 % inactivated FBS 100 IU/ml penicillin 100 µg/ml streptomycin	II and IV
Caco-2	DMEM ^{a)}	10 % inactivated FBS 1 % nonessential amino acids 1 % (2mM) L-glutamine 100 IU/ml penicillin 100 µg/ml streptomycin	II and IV

^{a)} Dulbecco's Modified Eagle Medium (DMEM), Invitrogen, Gibco, Paisley, UK

^{b)} Biowhittaker, Lonza, Walkersville, USA

3.3 Computational methods (*in silico*)

3.3.1 Molecular docking (II, III and IV)

The crystal structures of AChE that were used were: *Torpedo californica* AChE PDB code 1FSS (resolution 3 Å) (Harel et al., 1995) (**III**) and huAChE PDB codes 1B41 (resolution 2.76 Å) (Kryger et al., 2000) (**II**) and 4EY7 (resolution 2.35 Å) (Cheung et al., 2012) (**IV**). The crystal structures of BChE that were used were: huBChE PDB codes 2XQF (resolution 2.1 Å) (Wandhammer et al., 2011) (**II**) and 1P0I (resolution 2 Å) (Nicolet et al., 2003) (**IV**). In publication **II**, ligand building was done using the ChemsSketch software (Advanced Chemistry Development, Inc.; ACD/Labs) and energy minimized to obtain the geometry-optimized structure. The Autodock4.2 (Morris et al., 2009) program was used to perform molecular docking of the ligand into the active site of huBChE and huAChE. In **III** and **IV** the proteins were prepared using the Protein Preparation Wizard in Schrödinger (Schrödinger

Suite 2011 Protein Preparation Wizard; Epik version 2.2, Ligprep 2.5, Glide 5.7, New York). The ligands were drawn in Maestro and prepared using the Ligprep module with an MMFF force field. For our docking studies the option to dock flexibly with standard precision mode (III) and extra precision (XP) mode (IV) was selected.

3.3.2 ChemGPS-NP (I-IV)

The compound libraries were analysed using the chemical global positioning system (ChemGPS) modified to also take into account biologically relevant natural products (NP), ChemGPS-NP (Larsson et al., 2007; Rosén et al., 2009). PC scores were obtained using SMILES for the compound libraries that had been acquired using web based programs ChemSpider (www.chemspider.com) or Molinspiration Chemoinformatics v2009.01. (www.molinspiration.com). Salts, hydration information, counter-ions and tautomeric structures were excluded. In our analysis only the first four of the eight dimensions were used, namely PC1 to PC4, mainly describing size, aromaticity, lipophilicity and flexibility and visualized using Grapher 2.1 software (MacOS X, US).

3.3.3 Statistical analysis (I-IV)

The concentration of the compounds in the primary screening was 10 μ M (I, II and IV) and 2.35 - 32.65 μ M (III). Compounds were tested in a single-well composition in the primary screening and at least two replicates of each concentration were used for the potency determination and kinetic measurement and four replicates in the cytotoxicity assays. The potency determination experiment was repeated at least twice and IC₅₀ values were calculated using non-linear regression analysis (sigmoidal fitting with variable slope) in GraphPad Prism v.4.0 (I and III) and v. 5.0c (II and IV) for Mac (GraphPad software Inc., US).

The assay performance was monitored using typical statistical parameters (Z' factor, S/B ratio, S/N ratio and CV_A) as described by Zhang et al. (1999), Bollini et al. (2002) and Iversen et al. (2006):

Z' factor:

$$Z' = 1 - \frac{3SD_S + 3SD_B}{|X_S - X_B|}$$

S/B ratio:

$$S/B = \frac{X_S}{X_B}$$

Materials and methods

S/N ratio:

$$S/N = \frac{X_S - X_B}{\sqrt{SD_S^2 - SD_B^2}}$$

CV_A

$$CV_A = \frac{SD_S}{X_S - X_B}$$

The equations above describe the relationship between the mean values and standard deviations of the minimal (X_B and SD_B) and maximal (X_S and SD_S) signals as determined by measuring the spectrophotometric absorbance in the solvent (negative) control wells at one (minimal) and ten (maximal) minutes after enzyme addition.

4 RESULTS

4.1 Assay performance (I-IV)

The assay performance was monitored using the statistical tools S/N ratio (Bollini et al., 2002), S/B ratio, Z' factor (Zhang et al., 1999) and CV_A (Iversen et al., 2006) and the theoretical hit limit was set at three times the standard deviation of the maximal signal (negative controls). In publication **I**, **II** and **IV** acceptance parameters were set as $Z' > 0.5$, $S/N > 7$ and $S/B > 4$ and the empirical hit limit was set at 50 % inhibition in order to identify compounds with $IC_{50} \leq 10 \mu\text{M}$. In publication **III** acceptance parameters were set as $Z' > 0.5$, $S/N > 15$ and $S/B > 15$ and the theoretical hit limit was set at 40 % of inhibition. However, since the screening was planned at an average compound concentration in the micromolar range (17.5 μM), the threshold was empirically set higher, at 50 % inhibition, to identify more potent inhibitors. Using the Ellman's kinetic reaction good statistical parameters are obtained with a good signal window. The absorbance was measured using Victor2 1420 multitable counter (**I** and **IV**), which required manual enzyme addition, and Varioskan Flash multimode plate reader (**II** and **III**), with automatic enzyme dispensing. Differences in the minimal and maximal signal can be observed between the two systems due to the time delay that occurs using Victor2 (**Figure 10**), however, this did not affect the quality of the assay as the separation band was still large and Z' values were ≥ 0.5 . On average, throughout the primary screening of all compound libraries, the Z' was 0.69 ± 0.14 .

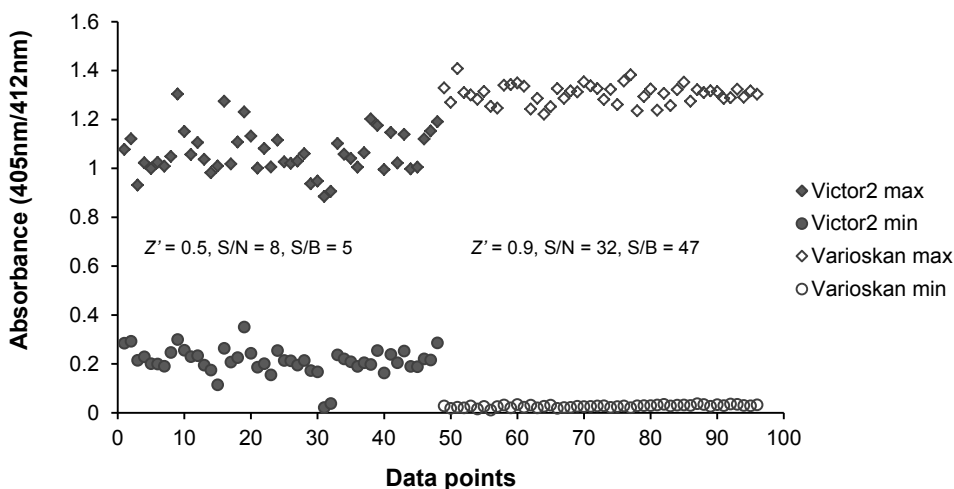
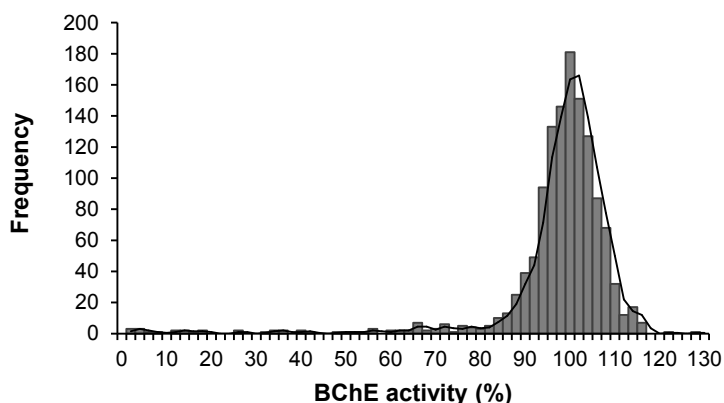


Figure 10. Variance between wells in maximal and minimal signal using Victor2 1420 multitable counter (405 nm) and Varioskan Flash multimode plate reader (412 nm). Manual enzyme addition using Victor2 causes more variance between the wells in addition to a delayed measurement of the initial kinetics which was eliminated using Varioskan with automatic enzyme dispensing. However, a good signal window using the Ellman's reaction was obtained using both plate readers.

4.2 Primary cholinesterase screening results (I-IV)

In publications **I-IV** a total of 1267 compounds was screened for inhibitory activity on equine BChE and electric eel AChE. The libraries contained natural molecules as well as compounds synthesized based on naturally-present scaffolds. The libraries used in this work can be divided into two categories; the larger and more diverse compound libraries (**I** and **III**) and the smaller, more focused libraries (**II** and **IV**). The frequency distribution of the BChE and AChE activity can be seen in **Figure 11**. Most of the compounds had no activity on the enzymes thus clustering around 100 % enzyme activity. A total of 27 BChE inhibitors, seven AChE inhibitors and three dual inhibitors were found, as summarized in **Table 2**. Additionally, the libraries contained compounds that showed some inhibitory activity but which however were under the applied hit limit and were therefore excluded from further investigations.

A)



B)

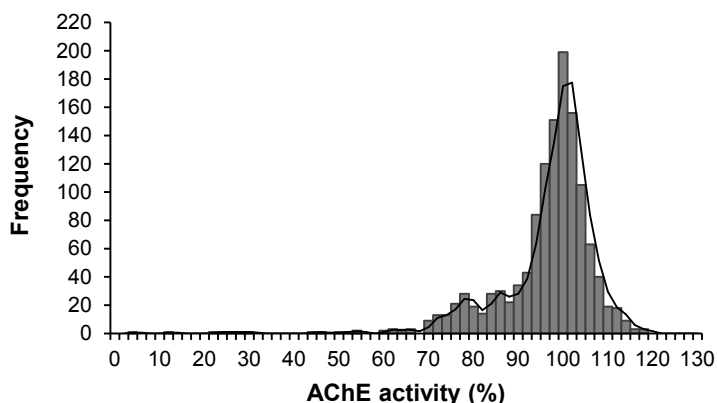


Figure 11. Summary of the frequency distribution of **A)** BChE and **B)** AChE activity during primary screening of all 1267 compounds (**I-IV**). The average activity for BChE was 96 ± 14 % and for AChE 95 ± 11 %.

Table 2. Size of screening libraries, number of inhibitors found and hit rate.

Publication	Compounds	Concentration (μM)	Inhibitors of			Active compounds	Hit rate ^{a)}
			BChE	AChE	Dual		
I	697	10	5	0	0	5	0.007
II	45	10	6	0	0	6	0.13
III	502	2.35-32.65	14	6	3	23	0.05
IV	23	10	2	1	0	3	0.13
Total	1267		27	7	3	37	0.03

^{a)} Calculated as total amount of inhibitors divided by total amount of compounds

4.3 Characterization of active compounds (I-IV)

4.3.1 Potency determination and structural optimization

Potency determination was performed on the compounds that inhibited 50 % of the enzyme target at the screening concentration (**I-IV**), with some exceptions as follows: in publication **III** already well-known cholinesterase inhibitors (galanthamine, ebelactone B, huperzine A and physostigmine) and compounds that were unstable in the assay condition (solasodine, tetrahydroalstonine and harmine) were excluded. A summary of the determined potencies of compounds in publication **I-IV** can be found in **Table 3**. In publication **I**, structural optimization was carried out on the most active chemical class, the diarylimidazoles (1a-c) and a total of 12 new compounds (6a-c, 9a-i) were tested for their BChE inhibitory activity. These results have also been included in **Table 3**. In total, 12 compounds have been found with inhibitory potencies under 1 μM against BChE. Some of the most active compounds were also tested against the huBChE and huAChE.

Table 3. Potencies of the original hits presented in publications **I-IV**.

Publication	Compound	IC ₅₀ (μM)			
		BChE	huBChE	AChE	huAChE
I	1a	3.6 \pm 0.7	n.t.	>250	n.t.
	1b	1.5 \pm 0.4	n.t.	>250	n.t.
	1c	0.20 \pm 0.03	n.t.	>250	n.t.
	6a	>10	n.t.	>250	n.t.
	6b	>10	n.t.	>250	n.t.
	6c	0.10 \pm 0.01	1.1	>250	n.t.
	9a	12 \pm 2	n.t.	>250	n.t.
	9b	1.8 \pm 0.1	n.t.	>250	n.t.
	9c	0.68 \pm 0.05	n.t.	>250	n.t.
	9d	0.86 \pm 0.04	n.t.	>250	n.t.
	9e	0.98 \pm 0.04	n.t.	>250	n.t.

Results

	9f	3.4 ± 0.1	n.t.	>250	n.t.
	9g	0.18 ± 0.06	n.t.	>250	n.t.
	9h	3.2 ± 0.5	n.t.	>250	n.t.
	9i	0.16 ± 0.07	n.t.	>250	n.t.
II	3a	1.4 ± 0.4	17 ± 3	>500	49 ± 8
	3b	0.17 ± 0.03	20 ± 4	110 ± 14	88 ± 1
	3c	3.8 ± 0.6	7.2 ± 3.2	210 ± 30	300 ± 59
	3d	0.49 ± 0.02	17 ± 4	140 ± 3	240 ± 6
	3e	0.34 ± 0.03	2.2 ± 0.4	220 ± 11	120 ± 9
	3f	0.037 ± 0.07	0.51 ± 0.07	140 ± 3	53 ± 1
III	Peganole	11 ± 0	> 20	n.a.	n.t.
	Vasicine	2.5 ± 0.4	3.1 ± 0.8	n.a.	n.t.
	Desoxypeganone	12 ± 0	n.t.	12 ± 0	n.t.
	Nitrarine	11 ± 0	9.0 ± 0.2	n.a.	n.t.
	Hirsutine	5.0 ± 0.3	2.0 ± 0.0	n.a.	n.t.
	Rauwolscine	8.4 ± 0.5	14 ± 1	n.a.	n.t.
	Catharanthine	5.2 ± 0.2	3.2 ± 0.1	n.a.	n.t.
	Sevedindione	3.7 ± 0.3	0.3 ± 0.0	n.a.	n.t.
	Veratramine	11 ± 1	19 ± 1	n.a.	n.t.
	E6 Berbamine	4.2 ± 0.2	2.1 ± 0.1	n.a.	n.t.
	Oxyacanthine sulfate	4.1 ± 0.2	1.9 ± 0.1	n.a.	n.t.
	Quinidine	7.4 ± 0.0	1.2 ± 0.1	n.a.	n.t.
	Himbacine	n.a.	n.t.	23 ± 1	n.t.
	Palmatine	n.a.	n.t.	4 ± 0.1	1.7 ± 0.1
Berberine	n.a.	n.t.	2.7 ± 0.2	0.6 ± 0.0	
Chelerythrine	6.3 ± 0.9	10 ± 0	3.8 ± 0.2	1.5 ± 0.1	
IV	11	9.8 ± 0.3	34 ± 5	21 ± 2	120 ± 29
	15	0.56 ± 0.14	0.24 ± 0.04	>250	>250
Controls	Galanthamine	17 ± 1	30 ± 0	1.6 ± 0.0	n.t.
	Physostigmine	2.1 ± 0.5	4.6 ± 0.0	1.8 ± 0.2 ^{a)}	n.t.

n.a. - not active

n.t. - not tested

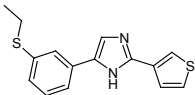
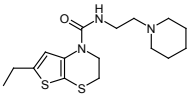
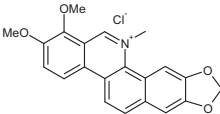
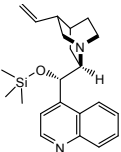
^{a)} previously reported by our group (Järvinen et al., 2011)

4.3.2 Kinetic mechanisms

The kinetic mechanism of the most active compounds against BChE or AChE was determined and K_i values calculated using Lineweaver-Burk double reciprocal plots. In

Table 4, the most active hit from each publication is presented, one uncompetitive and three mixed inhibitors were found, all with K_i values in the lower micromolar range.

Table 4. Structure and kinetic mechanism of the most potent compounds according to publication.

Publication	Compound	Structure	Enzyme/ Kinetic mechanism K_i values
I	6c		equine BChE/ uncompetitive $K_i = 0.073 \pm 0.011 \mu\text{M}$
II	3f		huBChE/ mixed $K_{ic} = 0.62 \pm 0.04 \mu\text{M}$ $K_{iuc} = 0.064 \pm 0.002 \mu\text{M}$
III	Chelerythrine		huAChE/ mixed ^{a)} $K_{ic} = 0.32 \pm 0.08 \mu\text{M}$ $K_{iuc} = 1.1 \pm 0.1 \mu\text{M}$
IV	15		equine BChE/ mixed $K_{ic} = 0.85 \pm 0.26 \mu\text{M}$ $K_{iuc} = 1.7 \pm 0.5 \mu\text{M}$

^{a)} Chelerythrine is a dual inhibitor with higher affinity for AChE than BChE

4.3.3 Cytotoxicity

Potential cytotoxic effect of the active compounds was evaluated using cell lines with different origins. In publication **I** the most active compound (6c) was tested in GT1-7 and HL cells and was not seen to affect cell viability at concentrations up to 50 μM . In publication **II** the cytotoxicity of the entire library was tested on GT1-7 immortalized neurons. Four compounds (3i, 3j, 6b and 6d) reduced cell viability under 80 % but none less than 75 % at 10 μM . The most active compounds (3a-f) were further tested at 10 and 50 μM for 48 and 72 hours in the same cell line to exclude delayed cytotoxic effect. Only one compound (3d) showed time-dependent cytotoxicity as it significantly reduced cell viability at 50 μM after 72 hours. The most active compound (3f) was also tested in HL, Caco-2 and HepG2 cells up to 100 μM with no cytotoxic effect observed. In publication **IV** the cytotoxicity of 11 and 15 was tested in GT1-7, HL, Caco-2 and HepG2 cell lines. Cell viability was reduced by 11 at the higher concentrations (80 and 100 μM), while 15 did not significantly reduce cell viability.

4.4 Structure-activity relationship and pharmacophore model (I and II)

A total of 15 compounds were selected for structure-activity relationship (SAR) studies in publication **I**. This included the original three active hits 1a-c and the derivatives 6a-c and 9a-i (activities shown previously in **Table 3**). Modifications included alkoxy and thioether groups and phenyl versus thienyl systems. Substitutions on both phenyl rings were not beneficial for BChE inhibition as this resulted in the lowest inhibitor activity. Substitution in *meta* position was favoured over *ortho* position and the results showed that the alkoxy residues in the *meta* position on the phenyl ring were favoured in the following order: ethoxy < methoxy < isobutoxy < butoxy < propoxy.

In publication **II** the compounds were clustered into eight groups, A-H, depending on their structures (Table 1 in publication **II**) and the active compounds all belonged to the same group, group C. A pharmacophore model based on the results from the thienothiazine BChE screening as well as the previous results by Darvesh et al. (2010) was proposed (Figure 3 in publication **II**). We concluded that two hydrophobic areas, a hydrogen bond acceptor and donor, an alkanediyl linker with a minimum of two carbon atoms and distal basic nitrogen are required for selective BChE inhibition using the thienothiazine scaffold.

4.5 Prevention and destruction of A β aggregates (I and III)

In publication **I** and **III** the ability of the active compounds to inhibit A β aggregation was tested. In publication **I** the active compounds (1a-c) were first tested on the short peptide HHQKLVFFAED and showed a concentration-dependent inhibition of A β aggregation. At 250 μ M inhibitory values of 45, 58 and 65 % inhibition, respectively, were obtained. They were further tested on the A β ₁₋₄₀ peptide and displayed similar values at 250 μ M; 57, 38 and 62 %, respectively. Lead optimization of the original hits resulted in an even more potent inhibitor of A β ₁₋₄₀ aggregation (6c) with an IC₅₀ value of 5.8 μ M. The most active cholinesterase inhibitor in publication **III**, chelerythrine, was proven to inhibit the aggregation of the A β ₁₋₄₀ peptide with an IC₅₀ value of 4.2 \pm 0.4 μ M. Two reference compounds were used; nordihydroguaiaretic acid showed a potency value of 18 μ M and 4-aminophenol a potency value of 83 μ M on inhibiting A β ₁₋₄₀ aggregation.

Chelerythrine (**III**) was further tested if it could inhibit electric eel AChE-induced aggregation of A β . The concentration tested was 5, 10 and 100 μ M and produced an inhibition of 49, 65 and 88 % of A β aggregation. Propidium iodide was used as a reference compound and showed 93.1 \pm 2.2 % inhibition at 100 μ M. We also tested if chelerythrine could destruct preformed A β aggregates as this would be a relevant feature in AD pharmacotherapy. In this case A β was allowed to aggregate 45 minutes before addition of chelerythrine and after 45 min incubation with the compound an IC₅₀ of 13.0 \pm 2.9 μ M was obtained. Resveratrol was used as a positive control and determined to have an IC₅₀ of 81.8 \pm 3.7 μ M.

4.6 Molecular docking studies (II, III and IV)

Molecular docking studies were performed in order to get insight into the structural features required for cholinesterase inhibition. The results from the docking studies of the most active compounds in publication **II** and **IV** can be found in **Figure 12** and in publication **III** in **Figure 13**. In publication **II**, 3f was docked into 2XQF (huBChE) (Wandhammer et al., 2011) and 1B41 (huAChE) (Kryger et al., 2000) using the Autodock4.2 program to specify interactions with huBChE but also to get some insight into the selectivity of the compound. In the huBChE crystal structure, the important and structurally conserved water molecules at the active site are W2139, W2138, W2129, W2067, W2170, W2066, W2082, W2128 and W2085, and are in **Figure 12** denoted as W1, W2, W3, W4, W5, W6, W7, W8 and W9, respectively. In the huAChE crystal structure the comparable water molecules are W737, W659, W664, W670 and W663 and are denoted as W1, W2, W4, W5 and W8, respectively. In huBChE, two strong hydrogen bonding interactions are formed between the oxygen atom of the carbonyl group of 3f with the catalytic site residues Ser-198 and His-438, while in huAChE, hydrogen bonding forms between two different functional groups of compound 3f (an amide group (NH) and a protonated tertiary amine group) and Tyr-337 (**Figure 12**).

In publication **IV**, the two most active compounds were docked into huAChE and huBChE in order to identify structural differences in enzyme specificity and inhibitory activity. In this case the crystal structure with PDB ID 1POI was used for huBChE (Nicolet et al., 2003) and 4EY7 for huAChE (Cheung et al., 2012) in order to dock the active compound 15. The active site of 1POI consists of residues Asp-70, Trp-82, Gly-116, Gly-117, Glu-197, Ser-198, Ala-199, Trp-231, Leu-286, Ser-287, Val-288, Tyr-332 and His-438 and conserved water molecules influencing the ligand binding W736, W786, W811, W825, W839, W875, W910, W919, W1042, W1076, W1177 are shown as W1, W2, W3, W4, W5, W6, W7, W8, W9, W10 and W11 respectively. The active site of 4EY7 consists primarily of residues Tyr-72, Trp-86, Tyr-124, Glu-202, Ser-203, Ala-226, Phe-295, Tyr-337, Tyr-341 and His-447 and conserved water molecules W728, W737, W856, W931, W952, W953, and W954 are depicted as W1, W2, W3, W4, W5, W6, and W7 respectively. It is plausible that 15 forms π - π stacking interactions with Trp-82 in huBChE while it is not able to enter the active site gorge of huAChE (**Figure 12**). The docking scores for 11 and 15 within the active sites of huBChE and huAChE were -7.549 and -6.515 and -8.813 and -4.345 , respectively.

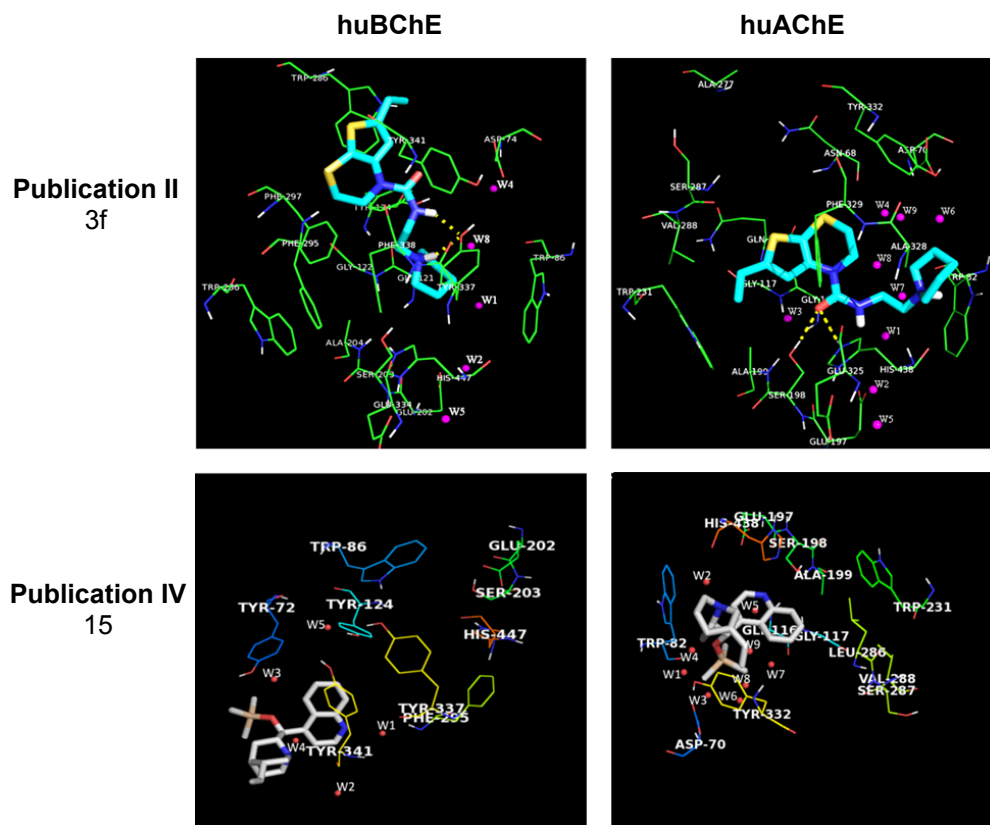


Figure 12. Docking of the most active compound in publication **II** and **IV** in huBChE and huAChE. In publication **II** the crystal structures 2XQF (huBChE) (Wandhammer et al., 2011) and 1B41 (huAChE) (Kryger et al., 2000) were used while in publication **IV** the crystal structures 1POI (huBChE) (Nicolet et al., 2003) and 4EY7 (huAChE) (Cheung et al., 2012) were used.

In publication **III**, chelerythrine was docked using the crystal structure of 1FSS (*Torpedo californica* AChE) (Harel et al., 1995) for which the active site consists of residues Tyr-70, Trp-84, Gly-118, Gly-119, Glu-200, Ser-201, Ala-226, Trp-279, Tyr-327, Phe-330 and His-440. Chelerythrine shows hydrogen bonding interaction with Tyr-130 and π -stacking interactions with Tyr-121 and Tyr-334 PAS residues. Conserved water molecules 710, 718, 722, 728 are denoted as W1, W2, W3 and W4 in **Figure 13** and are found to play an important role for binding inhibitors within the active site.

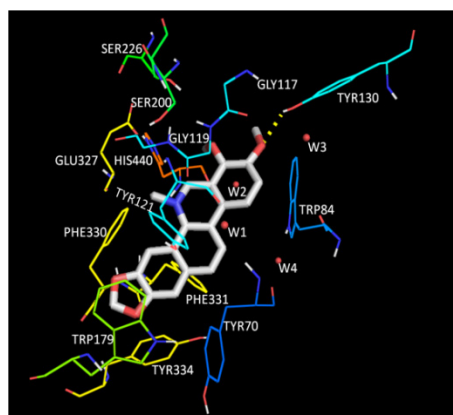


Figure 13. Molecular docking of the most active compound, chelerythrine, in publication **III** using *Torpedo californica* AChE (1FSS) (Harel et al., 1995).

4.7 ChemGPS-NP and chemical space (I-IV)

The compound libraries were all mapped into 3D graphs using PC scores obtained by ChemGPS-NP in order to visualize and analyse the chemical space occupied by our active and non-active compounds. In **Figure 14** the compound libraries (I-IV) have been clustered according to their size in order to facilitate the analysis. As can be seen the screening libraries occupy a wide space with different molecular sizes and different degrees of lipophilicity, aromaticity and flexibility.

The synthetic library including the diarylimidazoles (**I**) (yellow dots) contains compounds mostly on the negative side of PC1, positive side of PC2, both sides of PC3 and positive side of PC4. The Enzo[®] Screen-Well[®] Natural Product Library (green dots) (**III**), on the other hand includes compounds with properties that expands to all sides of the four dimensions included here (PC1-4) (**Figure 14A-B**). However, they tend to cluster more on the positive side of PC1 (higher molecular size) and negative side of PC4 (more rigid).

In **Figure 14C-D**, the purple dots represent the synthetic library containing derivatives of the thienothiazine type (**II**) while the blue dots represent the cinchona alkaloid library (**IV**). Consisting of a small group of derivatives, as already mentioned, they occupy a quite narrow chemical space. Both of these libraries contain compounds that span throughout the positive and negative side of PC1 (varying molecular size) while mostly being on the positive side of PC2 and PC3 (more aromatic and lipophilic). Some of the thienothiazine groups (A and F, Figure 1 in publication **II**) contained small compounds that were on the polar side (negative PC3). Both libraries have a trend of increasing lipophilicity with size. When including flexibility (PC4) the compounds within the libraries tend to cluster together, showing similarities in the flexibility. However, there is a difference between the cinchona alkaloid derivatives and the thienothiazine derivatives, with the former occupying the negative side of

PC4 (blue dots, more rigid) and the latter the positive side (purple dots, more flexible). In **Figure 15**, the active compounds have been indicated in red and are compared to the chemical space occupied by all compounds included in the primary screening. The active compounds mostly span the negative side of PC1 and PC4 and the positive side of PC2 and PC3.

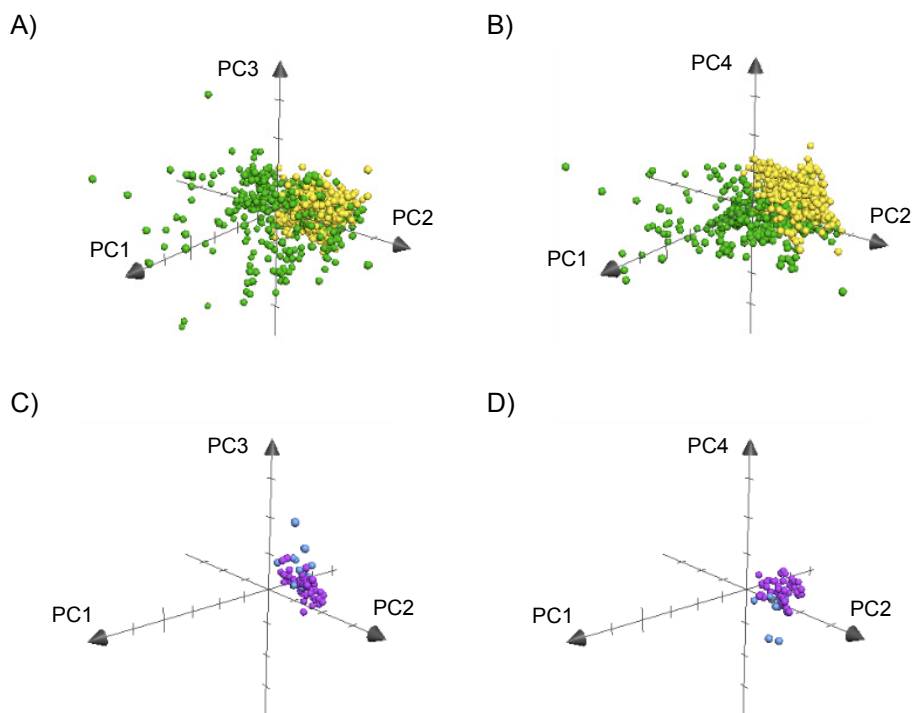


Figure 14. Three dimensional visualization of the chemical space occupied by the compounds included in the different screening libraries using ChemGPS-NP. The larger synthetic library containing the diarylimidazoles (**I**) is visualized using yellow dots, the small synthetic thienothiazine library (**II**) using purple dots, the Enzo[®] Screen-Well[®] Natural Product Library (**III**) using green dots and the cinchona alkaloid synthetic library (**IV**) using blue dots. In **A**) the first three dimensions; PC1 (size), PC2 (aromaticity) and PC3 (lipophilicity) of the larger compound libraries (**I** and **III**) are shown and in **B**) the fourth dimension (PC4) is taken into account. In **C**) and **D**) the two smaller libraries (**II** and **IV**) are similarly shown.

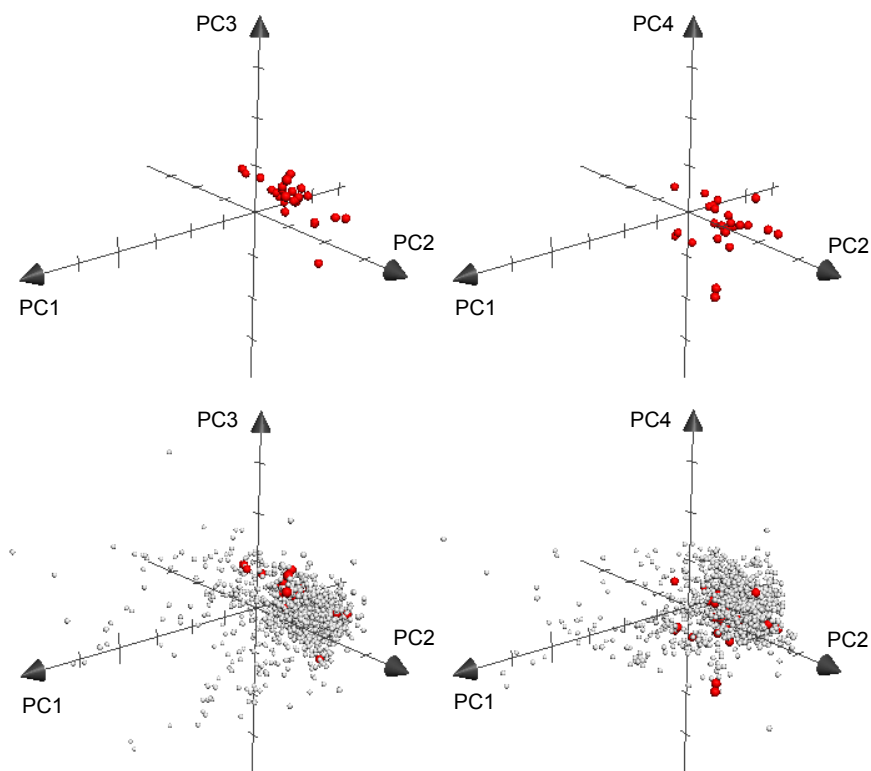


Figure 15. Identification of the chemical space occupied by the active cholinesterase inhibitors using ChemGPS-NP. The compound libraries are indicated with white dots and the active compounds are visualized with red dots.

5 DISCUSSION

It is estimated that in the world there is one new case of AD every seven seconds (Massoud and Gauthier, 2010), devastating the life of a whole family. Current treatments offer little relief and thus novel mechanisms and new approaches are required; however, CNS projects in general are prone to be less successful, more expensive, take a longer time as well as being scientifically more demanding, and so it seems also when it comes to the development of new AD pharmacotherapy. So far the patients of AD have two classes of drugs to choose from, the cholinesterase inhibitors and an NMDA receptor antagonist, both providing symptomatic relief but with several adverse effects. Considering that cholinesterase inhibitors are the first-line therapy for AD, and will probably continue to be so for many years, developing next generation cholinesterase inhibitors is important. For a long time, BChE was a neglected target in AD drug discovery as it has a lower activity than AChE and exist in the “wrong” areas in the healthy human brain (Greig et al., 2005). However, changes that occur in the AD brain call for a reassessment of the role of BChE and it has been demonstrated that BChE inhibitors may offer a new approach to managing AD (Giacobini, 2004; Greig et al., 2005; Lane et al., 2006). Considering the potential therapeutic benefit that BChE inhibitors may offer in the future, the focus of this work has been on finding novel BChE inhibitors.

Evolution has provided nature with a diversity of natural products that has not only been beneficial for the plants producing them but also for humans as plant extracts were the first medicines available to treat ailments (Ganesan, 2008). Natural products still play an important role in drug discovery as half of the current drugs have their roots in nature (Newman and Cragg, 2012) and in fact two out of four AD drugs have a natural origin (galanthamine, from *Galanthus woronowii* Losinsk., and rivastigmine, based on the structure of physostigmine from *Physostigma venenosum* Balf.). Although natural products are considered to have an advantage as they have been shaped by evolution to possess biological activity (Koch and Waldmann, 2005), natural products do have some limitations as well, such as: crude natural product extracts are not suitable for HTS, there are difficulties in collecting and isolating the natural compounds and, after identifying an active compound, obtaining a supply of the bioactive compound in larger amounts (Kingston, 2011). It is no surprise that natural products therefore often have to be synthesised, such as, for example, galanthamine (Marco-Contelles et al., 2006), as this is also generally a more efficient and economical way. During this study we therefore screened for cholinesterase inhibitors among compound libraries containing compounds with natural origin or synthetic compounds partly based on natural scaffolds, as we believe these types of compounds gives an advantage in finding new bioactive compounds with the possibility to conduct further structural optimization.

The solvent used for preparing the compound libraries was DMSO, as this is the most common solvent used for storing compound libraries (Di and Kerns, 2006). It is previously known that DMSO has an effect on cholinesterase activity (Jagota, 1992; Di Giovanni et al.,

2008); however, as the DMSO concentration was kept at a low final concentration (0.05 % and 0.16 %) during the primary screening this did not affect the screening outcome. The theoretical hit limit was set as three times the standard deviation of the maximal signal (negative controls, samples containing the uninhibited enzyme) as this ensures a clear separation between the variation of the maximal signal and the actual active compounds (Zhang et al., 1999). However, the limit was in some cases (**I-III**) further increased so as to ensure a high activity of the identified compounds as well as facilitate the management of the compounds during their characterization. The hit rate during primary screening is usually < 1 % (Coma et al., 2009; Posner et al., 2009; Ilouga and Hestekamp, 2012) and in our case it was 0.7 (**I**), 13 (**II** and **IV**) and 5 % (**III**). The higher hit rates in publication **II** and **IV** are due to the fact that the screened libraries are smaller and more focused. More specifically, this means they contained compounds with specific structures that were expected to have activity. In publication **III**, where the natural products library was used, the hit rate was also high. It is known that the hit rate in natural products libraries are usually higher as natural products, as noted earlier, have been “designed” by nature to possess bioactivity (Koch and Waldmann, 2005). Moreover, this library contained a set of already well-known cholinesterase inhibitors.

The assay using the Ellman’s reagent is considered the most appropriate assay for testing cholinesterase activity (Di Giovanni et al., 2008), it has been extensively applied with different modifications (e.g. Carolan et al., 2008; Rizzo et al., 2008; Järvinen et al., 2010) and is considered to be reliable and robust. Nevertheless, to ensure that the assay performed with high quality during the screening process, statistical parameters indicating the performance of the assay [S/N ratio (Bollini et al., 2002), S/B ratio, Z factor (Zhang et al., 1999) and CV_A (Iversen et al., 2006)] were applied. According to Zhang et al. (1999), in a biochemical assay, a Z' value ≥ 0.5 is considered a good-to-excellent assay where $Z' = 1$ is the highest value (the ideal assay). Thus during our screening we set the criteria for Z' at 0.5, however most of the time the Z' was well above this value. The S/B ratio indicates the ratio between the average maximal and minimal signal but does not take into account the variance of the two signals. The S/N ratio (Bollini et al., 2002), on the other hand, also reflects the differences between the standard deviations of the maximal and minimal signal, thus giving a more accurate view of the dispersion of the signal. A higher value indicates a larger separation between the maximal and minimal signal as well as less dispersion of the values.

During this thesis project we have found a total of nine chemical groups that inhibit BChE: diarylimidazoles, benzanilides (**I**), thienothiazines (**II**), quinazolines, indoles, steroid alkaloids, compounds with a polycyclic ring system, an isoquinoline (**III**) and cinchona alkaloids (**III** and **IV**). In the past, several chemical classes have been identified as BChE inhibitors (e.g. Greig et al., 2005; Elsinghorst et al., 2006; Decker et al., 2008; Carolan et al., 2010; Darvesh et al., 2010; Nawaz et al., 2011). Some of the different chemical classes found during this thesis project have previously been associated with either cholinesterase inhibition or other biological activities. The imidazole core structure, for example, is commonly present in drug molecules (Bemis and Murcko, 1996) and has also been present in previously reported cholinesterase inhibitors (Andreani et al., 2008; Kovárová et al., 2010).

Other of these chemical classes that have been shown to possess activity against cholinesterases are: quinazoline (e.g. Decker et al., 2008; Chen et al., 2011), indole (e.g. Ismail et al., 2012; Khorana et al., 2012; Passos et al., 2013), steroid alkaloids (e.g. Devkota et al., 2008; Yang et al., 2012), isoquinoline (e.g. Shan et al., 2011; Huang et al., 2012; Peng et al., 2012) and cinchona alkaloids (Kambam et al., 1987; Chemnitius et al., 1997; Nawaz et al., 2011). This, however, does not imply that the compounds found during this project would not be of interest, quite the contrary. The substitutions of the different chemical classes found in this project vary considerably from the previously published compounds and combining this previous data of cholinesterase inhibitors with our results may aid in developing even more potent inhibitors; as was the aim in publication **II** with the thienothiazines and their structural relationship to the phenothiazines, (Darvesh et al., 2005; Darvesh et al., 2007; Darvesh et al., 2010). No reports on benzanilides (**I**) or thienothiazines (**II**) as cholinesterase inhibitors were found while searching the literature, thus suggesting that this is the first time compounds of these chemical structures have shown activity against cholinesterase. In fact, it was among the thienothiazines (**II**) that we found the most potent inhibitor during this study, with IC_{50} values of 37 nM against BChE and 510 nM against huBChE. This was compared to the controls physostigmine and galanthamine which had IC_{50} values of 2.1 and 17 μ M, respectively, against BChE (**Table 3**). As a comparison, in **Table 5**, a collection of previously published BChE inhibitors have been listed along with their potencies and the control used in that study. In this work the potencies ranged from 0.037-2.5 μ M among the most potent BChE inhibitors in publications **I-IV** (**Table 3**).

Table 5. Published cholinesterase inhibitors with selectivity towards BChE according to their chemical class; selected examples.

Chemical class	Reference	ID	IC_{50} (μ M)	K_i (μ M)	Control IC_{50} (μ M) ^{b)}
Benzofuran	(Rizzo et al., 2008)	1	0.28 ^{a)}		RIV: 0.30 ^{a)}
Cinchona alkaloid	(Nawaz et al., 2011)	5	0.34	0.4	PHY: 1.85
Cymserine	(Greig et al., 2005)	BNC	0.001 ^{a)}		n.d.
Isosorbide	(Carolan et al., 2010)	5	0.00015 ^{a)}		PHY: 0.019 ^{a)}
Phenothiazine	(Darvesh et al., 2007)	23		0.0035 ^{a)}	GAL: K_i 2.09 ^{a)}
Quinazolinimine	(Chen et al., 2011)	33	0.003		GAL: 8.4
Steroid alkaloid	(Devkota et al., 2008)	4	0.3		GAL: 8.2

n.d. - not determined

^{a)} Enzyme origin: human

^{b)} RIV: Rivastigmine, PHY: Physostigmine and GAL: Galanthamine

The isosorbide compound (Carolan et al., 2010) is the most potent BChE inhibitor to date. However, these types of inhibitor seem to be limited as they are rapidly degraded by carboxylesterases in mouse plasma, restricting their use in mice AD models (Dillon et al., 2010). The cinchona alkaloid (**IV**) we found was in the same range as the most active by Nawaz et al. (2011), only our compounds required less extensive substitutions to achieve the same goal, and by additional hit refinement this potency could most probably be further improved. The most active diarylimidazole (**I**) had an IC_{50} value of 0.1 μ M (**Table 3**) and in

the Enzo[®] Screen-Well[®] Natural Product Library (**III**) (Enzo Life Sciences, Inc. USA) the most active BChE inhibitor was vasicine with an IC₅₀ value of 2.5 μM. However, although vasicine was the most potent compound against BChE (**III**), further investigation into the possible effect of the compounds on Aβ aggregates, made us turn our attention towards chelerythrine, an isoquinoline with a BChE IC₅₀ value of 6.3 μM. As AD is a multifactorial disorder and thus does not seem to fit into the category “one target-one disease”, finding bifunctional drugs, that is, one drug that can modify several targets of the disease at the same time, can prove an advantage. Therefore we also paid attention to the amyloid hypothesis, and the beneficial effect that inhibiting cholinesterase and Aβ aggregation may convey.

BChE and AChE are known to exist in Aβ plaques and neurofibrillary tangles (Mesulam et al., 1987) but while AChE induces Aβ fibril formation, BChE has not shown this inducing mechanism (Inestrosa et al., 1996) and in fact the imperfect amphipathic characteristics of the C-terminal of the human BChE has actually shown to slow down the formation of Aβ fibrils in vitro (Diamant et al., 2006). It has also been shown that Aβ plaques can occur in the brain of older adults, but is only associated with BChE in the AD brain (Darvesh, 2013) and observations in a transgenic mouse model of AD point to an involvement of BChE in the maturation of Aβ plaques (Darvesh et al., 2012). Nevertheless, the link between BChE inhibition and Aβ fibril formation is still unclear (Diamant et al., 2006; Podoly et al., 2010). Compounds inhibiting Aβ fibril formation/aggregation that have previously been found are e.g. 4-aminophenol (De Felice et al., 2004), nordihydroguaiaretic acid (Ono et al., 2004), propidium iodide (Bartolini et al., 2003), 4-(2-(2,6-dichlorobenzylidene) hydrazinyl)-1-(3-phenylpropyl) pyridin-1-ium bromide (Alptüzün et al., 2010) and 4-hydroxyindole (Cohen et al., 2006). By using bifunctional compounds the amount of drugs a patient may need to take during the day is reduced as well as the risk of drug-drug interactions, and the pharmaceutical companies can focus on developing one drug instead of two. However, it may become challenging to manage the dose needed to achieve the different activities simultaneously (Morphy and Rankovic, 2005).

During this thesis project, two compound classes were found to inhibit Aβ aggregation *in vitro*, the diarylimidazoles (**I**) and the isoquinolines (**III**), making them bifunctional compounds and thus more attractive from a drug discovery perspective. In addition, both compounds were found to be even more potent (IC₅₀ values 4-6 μM) than the control compounds, nordihydroguaiaretic acid (IC₅₀ 18 μM) and 4-aminophenol (64 % inhibition at 250 μM). Although the ThT assay is known to produce variations between runs and Aβ protein batches the ThT assay has been extensively used for quantification of Aβ aggregation (Reinke and Gestwicki, 2011; Sabate and Saupe, 2007). In publication **I**, the short peptide HHQKLVFFAED (Alptüzün et al., 2010) was first used before testing with the full peptide Aβ₍₁₋₄₀₎ peptide. The sequence KLVFF, which is present in this short peptide, has been defined as the fibril forming sequence (Tjernberg et al., 1996; Gazit, 2005), thus, using the shorter peptide, a first view of the compounds anti-amyloid properties can be assessed before continuing with the full peptide. Compounds exhibiting both cholinesterase and amyloid-β inhibitory activities have previously been published, i.e. benzofuran-based inhibitors with an IC₅₀ of 7.0 μM (Rizzo et al., 2008), phenylthiazole-tacrine hybrid inhibitors with activity of

72 % inhibition at 20 μM (Wang et al., 2012) and tacrine-coumarine hybrids 68 % inhibition at 20 μM (Xie et al., 2013). All of these A β aggregation studies have focused on the inhibition of the formation of the A β aggregates; however, considering that the A β aggregates have already formed in the AD brain, the destruction of the aggregates could be an additional and even more relevant feature. Chelerythrine (**III**) was found to destroy preformed aggregates (disaggregation) with an IC_{50} of 13 μM . Thus, chelerythrine is a potent inhibitor of AChE and BChE, it inhibits A β aggregation and AChE-induced aggregation (48.5 % at 5 μM) and it also has the ability to disaggregate preformed A β aggregates. Chelerythrine is therefore an attractive compound for future drug discovery and development.

During the drug discovery process it is very important to continue with the right compounds so as to minimize failure at later stages. Thus series of tests are made to eliminate hits with unbeneficial properties. One problem that reoccurs is safety issues regarding drug candidates, and testing for cytotoxicity at an early stage is therefore important. During this thesis project the active compounds have been tested against different cell lines to assess their cytotoxic effect. As the compounds are aimed at targeting the brain the cytotoxic effect on immortalized neurons was tested (GT1-7 cells) (**I**, **II** and **IV**). In addition, human lung (HL), liver (HepG2) and epithelial (Caco2) cells were exposed to the active compounds. The most active compounds in publication **I**, **II** and **IV** did not show any cytotoxic effect in the cell lines tested, thus showing that these compounds are not only potent selective BChE inhibitors but also that they do not affect the cell viability up to concentrations of 50 μM (**I**) or 100 μM (**II** and **IV**), which is well above the potency values. No cytotoxicity was tested in publication **III** as the most active compound, chelerythrine, had already been shown to be well-tolerated (Chmura et al., 2000; Niu et al., 2011). These compounds may still have unwanted effects, for example chelerythrine is a known protein kinase inhibitor and inhibits e.g. myotonic dystrophy kinase-related Cdc42-binding kinase (Tan et al., 2011) and protein kinase C (Chmura et al., 2000). Also as BChE is abundant in peripheral tissue (Liston et al., 2004) it is likely that these compounds could result in adverse effects in the periphery. However, this is difficult to say at this point as this project is a part of the early drug discovery process.

To give a better understanding of the interaction between the most active compounds with the cholinesterases and their selectivity towards BChE, molecular docking studies were performed (**II** and **IV**). In publication **II** the selectivity of the most active compound (3f) towards huBChE was shown to be due to differences in the binding mode of the compound to the enzymes. While in huBChE the compound interacted with the residues of the catalytic triad (Ser-198 and His-438), it did not do so in huAChE. In publication **IV**, on the other hand, the most active compound (15) was not able to enter the active site gorge of huAChE thus explaining its high selectivity towards huBChE. Although the structures of BChE and AChE are very similar and dual inhibitors are most frequently found (Musiał et al., 2007), the structural differences between the active site gorges (Ngamelue et al., 2007; Dvir et al., 2010) still offer an opportunity to develop selective BChE inhibitors.

It is thought that molecules with similar structural properties should possess similar biological activities. Indeed, there is a higher probability of finding biologically active compounds among a compound collection with similar structures to already known bioactive compounds, but this is not a rule (Martin et al., 2002). Another approach is to not focus solely on the structure but to focus more on the physico-chemical properties required of a compound that possesses a certain kind of biological activity. This way the probability of finding novel compounds with a desired activity is higher. In chemical space, the amount of possible molecules is vast and there is therefore a requirement to try and reduce this to an area which is considered to be the biologically relevant chemical space (Larsson et al., 2007). During this thesis project, we have tried to identify an area that would encompass compounds with BChE inhibitory activity. The chemical space that the libraries occupy is interesting as one can clearly see the features that have been described for natural and synthetic libraries in the past (**Figure 14**). Natural products are known to be more rigid due to their lower amount of rotatable bonds compared to synthetic compounds as well as having less aromatic rings (Feher and Schmidt, 2002; Ertl and Schuffenhauer, 2008). ChemGPS-NP can be used to identify an area of chemical space where most of the active compounds reside (Rosén et al., 2009). In our case, the active compounds span a narrower space, defining a chemical space relevant for the types of cholinesterase inhibitors found in this project (**Figure 15**). Of course, as with the Lipinski's rule of five (Lipinski et al., 1997), there are exceptions to this and there are several compounds that do not reside in the area of chemical space where our active compounds reside, as we noticed in publication **II**. However, in general, this type of analysis would facilitate future screening as a large part of the library can, similarly to using the Lipinski's rule of five, be left unscreened based on their physico-chemical properties, as their profile does not fit with the requirements of this type of inhibition.

6 CONCLUSIONS

Due to the complexity of AD it is challenging to find one drug to cure this disease, at least not until the disease mechanism is better understood. Until that day it is likely that a combination of therapies, symptomatic and disease-modifying, is the way forward. Cholinesterase inhibitors are thought to be symptomatic treatment, but evidence is pointing to the fact that they may have an impact on the progression of the disease as well. The existence of BChE has been known for a long time, but its function is still somewhat of a mystery. It could be that the task of BChE is to function as a bioscavenger, but BChE could also prove to play a more vital role in human health and disease.

During this thesis project, a total of nine chemical classes have been found to inhibit BChE (diarylimidazole, benzanilide, thienothiazine, quinazoline, indole, steroid alkaloid, isoquinoline, compounds with polycyclic ring system, cinchona alkaloid) and some also inhibit AChE (dual inhibitors: quinazoline and isoquinoline) and/or A β aggregation (bifunctional: diarylimidazole and isoquinoline). They offer structures that can be further optimized and importantly, these compounds do not show signs of cytotoxicity up to 50 μ M in the cell lines tested, suggesting a level of safety when using these scaffolds. The compounds found in this study may serve as scaffolds for future development of drug candidates in AD or be used as molecular probes in the search for the function of BChE.

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