

Division of Pharmaceutical Biology
Faculty of Pharmacy
University of Helsinki
Finland

**Assessment of In-House Natural Product and Synthetic
Compound Libraries Based on *In vitro* Inhibition of
Cholinesterases**

Päivi Järvinen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 2 at Viikki Infocenter (Viikinkaari 11), on April 20th, 2011, at 12 noon.

Helsinki 2011

Supervisors

Professor Pia Vuorela
Pharmaceutical Sciences
Department of Biosciences
Åbo Akademi University
Turku, Finland

Professor Annele Hatakka
Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki
Helsinki, Finland

Professor Heikki Vuorela
Division of Pharmaceutical Biology
Faculty of Pharmacy
University of Helsinki
Helsinki, Finland

Reviewers

Professor Seppo Auriola
School of Pharmacy
Faculty of Health Sciences
University of Eastern Finland
Kuopio, Finland

Docent Merja Perälä
Medical Biotechnology
VTT Technical Research Centre of Finland
Turku, Finland

Opponent

Professor emeritus Kurt Hostettmann
University of Geneva
Geneva, Switzerland

© Päivi Järvinen 2011

ISBN 978-952-10-6826-3 (Paperback)

ISBN 978-952-10-6827-0 (PDF)

Unifragia Oy
Helsinki 2011
Finland

Abstract

The first line medication for mild to moderate Alzheimer's disease (AD) is based on cholinesterase inhibitors which prolong the effect of the neurotransmitter acetylcholine in cholinergic nerve synapses which relieves the symptoms of the disease. Implications of cholinesterases involvement in disease modifying processes, has increased interest in this research area.

The drug discovery and development process is a long and expensive process that takes on average 13.5 years and costs approximately 0.9 billion US dollars. Drug attritions in the clinical phases are common due to several reasons, e.g., poor bioavailability of compounds leading to low efficacy or toxic effects. Thus, improvements in the early drug discovery process are needed to create highly potent non-toxic compounds with predicted drug-like properties.

Nature has been a good source for the discovery of new medicines accounting for around half of the new drugs approved to market during the last three decades. These compounds are direct isolates from the nature, their synthetic derivatives or natural mimics. Synthetic chemistry is an alternative way to produce compounds for drug discovery purposes. Both sources have pros and cons. The screening of new bioactive compounds *in vitro* is based on assaying compound libraries against targets. Assay set-up has to be adapted and validated for each screen to produce high quality data. Depending on the size of the library, miniaturization and automation are often requirements to reduce solvent and compound amounts and fasten the process.

In this contribution, natural extract, natural pure compound and synthetic compound libraries were assessed as sources for new bioactive compounds. The libraries were screened primarily for acetylcholinesterase inhibitory effect and secondarily for butyrylcholinesterase inhibitory effect. To be able to screen the libraries, two assays were evaluated as screening tools and adapted to be compatible with special features of each library. The assays were validated to create high quality data.

Cholinesterase inhibitors with various potencies and selectivity were found in natural product and synthetic compound libraries which indicates that the two sources complement each other. It is acknowledged that natural compounds differ structurally from compounds in synthetic compound libraries which further support the view of complementation especially if a high diversity of structures is the criterion for selection of compounds in a library.

Acknowledgements

This work was carried out at the Division of Pharmaceutical Biology, Faculty of Pharmacy, University of Helsinki in close collaboration with the Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki.

I wish to thank my supervisors Prof. Pia Vuorela, Prof. Annele Hatakka and Prof. Heikki Vuorela for believing in my work, providing me with excellent supervision and support throughout the work. Especially I wish to acknowledge my gratitude to Prof. Pia Vuorela for advice in matters related to research and also to other issues in life. Her enthusiasm and dedication are overwhelming and very inspiring.

I appreciate Prof. Raimo Hiltunen, Head of Division of Pharmaceutical Biology, for his support and providing me with the facilities for my work.

I wish to acknowledge the providers of the libraries used in this contribution. The natural product libraries were provided by Prof. Annele Hatakka at the Department of Food and Environmental Sciences, University of Helsinki; Doc. Adyary Fallarero at the Department of Biosciences, Åbo Akademi University, and Dr. Bertalam Galambosi at the Agrifood Research Centre, Finland. M.Sci. Juhani Lahdenperä is acknowledged for assisting in the use of synthetic compound library at the High Throughput Centre, University of Helsinki.

Prof. Seppo Auriola and Doc. Merja Perälä are acknowledged for reviewing the thesis. Their comments and suggestions were valuable in improving and finalizing the work.

Dr. Damien Dorman is acknowledged for revising the language of the thesis.

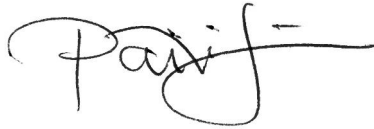
I sincerely wish to thank all the co-authors for their contribution to this work: Doc. Adyary Fallarero, M.Sci. Aila Mettälä, Doc. Jouni Jokela, M.Sci. Shikhar Gupta, Assistant Prof. Gopi Mohan, Doc. Jari Sinkkonen, Dr. Olli Martiskainen, Prof. Kalevi Pihlaja, Dr. Anna Galkin, and M.Sci. Pia Blom. Especially, I wish to thank Doc. Adyary Fallarero for her excellent ideas, conversations and work effort related to the contributions of this thesis and, in addition, for kicking me forward when I needed it. She has become a dear friend of mine which has made working together a pleasure.

Dr. Anna Galkin is acknowledged scientifically but most of all for being the best roommate, a good friend in need and much more during the years. I wish to thank Dr. Laura Riihimäki-Lampén for her friendship and good companionship in exploring the world. Academic life in general would not have been the same without our triplet! All former and present colleagues at the Division of Pharmaceutical Biology, especially Dr. Nahed El-Najjar, M.Sci.(Pharm) Olli Salin and M.Sci. Tiina Lantto, have earned a special place in my heart. I also wish to thank Tarja Hiltunen for all the help during the years.

I wish to express my deepest gratitude to my parents and my brother for their endless support and encouragement. Jukka's family has become my second family with whom I have shared great moments during the years. I also wish to thank all my dear friends for filling my life with many hilarious moments and happiness. Special thanks are due to Minna and Naasse who have been my closest friends nearly 30 years now which itself tells it all. On top of all, I wish to thank Jukka for his love and for being my best friend but also for being the greatest believer in my thesis project.

I wish to thank the Academy of Finland: This work was partly funded by the grant to the Center of Excellence on Microbial Resources (project nr. 53305). I also wish to thank the Finnish Cultural Foundation for personal funding.

Helsinki, April 2011

A handwritten signature in black ink, appearing to read "Pasi". The signature is stylized with a large, looped 'P' and a long horizontal stroke extending to the right.

Contents

ABSTRACT	3
ACKNOWLEDGEMENTS	4
CONTENTS	6
LIST OF ORIGINAL PUBLICATIONS	9
ABBREVIATIONS	10
1 INTRODUCTION	12
2 REVIEW OF THE LITERATURE	14
2.1 DEMENTIA	14
2.2 ALZHEIMER’S DISEASE	14
2.2.1 Cholinergic Hypothesis of AD	15
2.2.2 Amyloid Hypothesis of AD	16
2.3 CHOLINESTERASES	17
2.3.1 Acetylcholinesterase.....	18
2.3.2 Butyrylcholinesterase	19
2.3.5 Cholinesterase Inhibitors	19
2.4. CHOLINESTERASE INHIBITORS FROM NATURAL AND SYNTHETIC ORIGINS ...	22
2.4.1 Drug Discovery Process	22
2.4.2 <i>In vitro</i> Screening of Cholinesterase Inhibitors.....	24
2.4.2.1 Natural Products.....	25
2.4.2.2 Natural Product Libraries for Screening.....	27
2.4.2.3 Synthetic Compound Libraries for Screening	28
2.4.3 <i>In vitro</i> Screening as a Basis for <i>In silico</i> Predictions.....	30
3 AIMS OF THE STUDY	34
4 EXPERIMENTAL	35
4.1 MATERIALS	35
4.1.1 Fungal Cultures and Extraction (I)	35
4.1.2 Plant Material and Extraction (II).....	35
4.1.3 Coumarin Library (III)	35
4.1.4 Synthetic Compound Library (IV).....	38
4.2 ENZYMATIC METHODS	38
4.2.1 Ellman’s Reagent Based Photometric Microplate Assay (II, III, IV, V)	38
4.2.2 Bioautographical Assay on TLC (I, II, III)	39
4.3 SEPARATION AND ANALYTICAL METHODS	40

4.3.1 HPLC Micro-Fractionation of the Extracts (II).....	40
4.3.2 TLC (I, II, III).....	40
4.3.3 LC-MS (I, II).....	40
4.3.4 NMR (I).....	41
5 RESULTS AND DISCUSSION.....	42
5.1 ELLMAN’S REAGENT BASED PHOTOMETRICAL MICROPLATE ASSAY.....	42
5.1.1 Adaptation of the Assay for Screening of AChE Inhibitors.....	42
5.1.2 Effect of Assay Variant on Potency of Inhibition.....	45
5.2 BIOAUTOGRAPHICAL ASSAY ON TLC.....	50
5.2.1 Evaluation of TLC Bioautography for the Screening of AChE and BChE Inhibitors.....	50
5.3 COUPLING CHOLINESTERASE INHIBITORY ASSAYS AND ANALYTICAL TECHNIQUES FOR THE SCREENING OF EXTRACTS.....	51
5.3.1 Tracking of Bioactive Compounds in Extracts.....	51
5.3.2 The Effect of Solvents on Fractionation and Enzyme Activity.....	53
5.4 CHOLINESTERASE INHIBITORS FROM IN-HOUSE LIBRARIES.....	54
5.4.1 Fungal and Plant Extract Libraries.....	54
5.4.1.1 2,5-Dihydroxy- α -oxo-benzeneacetic Acid Isolated from <i>Piptoporus betulinus</i> Extract.....	55
5.4.1.2 Linarin from <i>Mentha arvensis</i> Extract.....	56
5.4.2 Compound Libraries.....	58
5.4.2.1 Hits in the Coumarin Library.....	58
5.4.2.2 Hits in the Synthetic Compound Library.....	60
5.4.3 Chemical Evaluation of the Hits in the Libraries.....	63
5.5 PRODUCTION AND MANAGEMENT OF THE LIBRARIES FOR SCREENING.....	67
5.5.1 Extract Libraries.....	67
5.5.2 Compound Libraries.....	68
5.6 SELECTION OF COMPOUNDS IN A LIBRARY FOR SCREENING.....	70
6 SUMMARY AND CONCLUSIONS.....	72
APPENDIX 1.....	74
REFERENCES.....	77
Original Publications	

List of Original Publications

This thesis of Päivi Järvinen (née Oinonen) is based on the following publications:

- I Päivi Järvinen, Aila Mettälä, Jari Sinkkonen, Olli Martiskainen, Kalevi Pihlaja, Heikki Vuorela, Annele Hatakka, Pia Vuorela: TLC bioautographical screening of dual cholinesterase inhibitors in extracts of wood rotting fungi and identification of 2,5-dihydroxy- α -oxo-benzeneacetic acid from *Piptoporus betulinus*. (manuscript)
- II Päivi Oinonen, Jouni Jokela, Annele Hatakka, Pia Vuorela: Linarin, a selective acetylcholinesterase inhibitor from *Mentha arvensis*. *Fitoterapia*, 77: 429-434, 2006
- III Adyary Fallarero, Päivi Oinonen, Shikhar Gupta, Pia Blom, Anna Galkin, Gopi Mohan, Pia Vuorela: Inhibition of acetylcholinesterase by coumarins: The case of coumarin 106. *Pharmacological Research*, 58 (3-4): 215-221, 2008
- IV Päivi Järvinen, Adyary Fallarero, Shikhar Gupta, Gopi Mohan, Annele Hatakka, Pia Vuorela: Miniaturization and validation of the Ellman's reaction based acetylcholinesterase inhibitory assay into 384-well plate format and screening of a chemical library. *Combinatorial Chemistry and High Throughput Screening*, 13 (3): 278-284, 2010
- V Päivi Järvinen, Pia Vuorela, Annele Hatakka, Adyary Fallarero: Potency determinations of acetylcholinesterase inhibitors using Ellman's reaction-based assay in screening: effect of assay variants. *Analytical Biochemistry*, 408: 166-168, 2011

The publications are referred to in the text by their roman numerals.
The articles are reprinted by kind permission from the publishers.

Abbreviations

1D, 2D, 3D	one-dimensional, two-dimensional, three-dimensional
A β	amyloid- β peptide
AChE	acetylcholinesterase
ACN	acetonitrile
AD	Alzheimer's disease
ADME	absorption, distribution, metabolism, excretion
APP	amyloid precursor protein
aq	aqueous
ATCI	acetylthiocholineiodide
BBB	blood-brain barrier
BChE	butyrylcholinesterase
ca.	circa
CHCl ₃	chloroform
CNS	central nervous system
CV	coefficient of variance
DAD	diode array detector
DMSO	dimethylsulphoxide
DTNB	dithionitrobenzoic acid, Ellman's reagent
ESI	electrospray ionization
e.g.	exempli gratia, for example
FDA	Food and Drug Administration in U.S.A.
HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrometry
HTS	high-throughput screening
IC ₅₀	concentration yielding 50% inhibition
i.e.	id est, that is
K _i	dissociation constant for an inhibitor for binding to the enzyme
LC	liquid chromatography
MeOH	methanol
MCI	mild cognitive impairment
MS	mass spectroscopy, mass spectrometry
NMDA	N-methyl-D-aspartic acid
NMR	nuclear magnetic resonance spectroscopy
qNMR	quantitative nuclear magnetic resonance spectroscopy
PAS	peripheral anionic site
PC	principal component
QSAR	quantitative structure-activity relationship
R _f	retardation factor or retention factor
RP	reversed-phase
SAR	structure-activity relationship
SD _b	standard deviation of the background
SD _s	standard deviation of the signal

S/B	signal-to-background
S/N	signal-to-noise
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
UV	ultra violet
Vis	visible
X_b	average of the background
X_s	average of the signal
Z'	screening window coefficient

1 Introduction

Drug discovery and development is a long and expensive process that takes ca. 13.5 years on average and costs approximately 0.9 billion US dollars per new molecular entity launch (Paul et al. 2010). Clinical trials (I-IV) are the most expensive phases in the whole process accounting for around 63% of the total costs. Withdrawals in these phases are still common since nine molecules are needed to enter the clinical phase I to yield one new molecular entity launch. More attention should be paid to the quality of research in the proceeding stages to create better lead molecules for clinical trials that are effective, non-toxic and drug-like and whose bioavailability is good. The improvement of the quality of data created and combination of *in vitro* and *in silico* approaches are means to make the process more efficient.

Several molecular sources for *in vitro* drug discovery are available from which natural products and synthetic compound libraries are the main ones. Nature has been a good source for new medicines accounting for nearly half of all the new chemical entities launched on market during 1981-2006 (Newman et al. 2003, Newman and Cragg 2007). These compounds are direct isolates from the nature, semi-synthetic or synthetic derivatives of natural molecules, proteins or natural mimics. In spite of this knowledge, there is reduced interest in natural product research among pharmaceutical companies because natural compounds tend to be problematic in lead optimization phase from medicinal chemistry perspective due to asymmetric carbon atoms and poor ADME properties (ADME, absorption, distribution, metabolism, excretion). With help of combinatorial chemistry it is possible to make large synthetic compound libraries of hundreds of thousands of compounds. Low volume assays and automatic liquid handling robotics allow screening for bioactive compounds from the large pure compound libraries. The realization that increases in throughput have not increased the number of approvals of synthetic entities has been a huge disappointment for the pharmaceutical industry (Li and Vederas 2009).

Natural product scaffolds are more complex in structure than synthetic drugs or compounds in synthetic compound libraries (Lee and Schneider 2001, Feher and Schmidt 2003, Ortholand and Ganesan 2004, Newman 2008, Rosen et al. 2009, Sukuru et al. 2009). This has been postulated to be the reason why synthetic compounds have failed to supplant natural products. The handling of extracts as well as the isolation and identification of active compounds from extracts have traditionally been pitfalls in natural product research (Hamburger 2003, Potterat and Hamburger 2006). Recent improvement in analytical technologies, especially chromatographic and spectroscopic methods, have shortened the times of analyses and decreased the amount of compounds needed for identification of molecular structures (Eldridge et al. 2002, Dalisay and Molinski 2010). This development will draw attention back to natural products. Natural product pure compound libraries and well designed pre-fractionated extract libraries are compatible with HTS platforms, and therefore are available for large scale screening (Sukuru et al. 2009, Wagenaar 2008). In addition, coupling of analytical methods to bioactivity assays is

a means to facilitate the drug discovery of natural products because simultaneously created information on bioactivity and structural data eases the identification of bioactive compounds (Potterat and Hamburger 2006).

Approximately 13,000 new dementia cases are diagnosed every year in Finland (Suhonen et al. 2010). It was estimated in 2005 that 24 million people worldwide suffered from dementia and the amount of incidents doubles every 20 years because of the trend in the aging of the population which makes dementia a huge burden for health care systems (Ferri et al. 2005). Alzheimer's disease is the most common form of dementia. Alzheimer's disease is a neurodegenerative disorder that slowly and steadily deteriorates memory and other cognitive functions (McKhann et al. 1984, Dubois et al. 2007). There is no cure for the disease or means to stop its progression. The first line medication is based on cholinesterase inhibitors which are compounds that prolong the effect of neurotransmitter acetylcholine in cholinergic synapses by inhibiting the degrading enzyme acetylcholinesterase (Birks 2006). The medication relieves the symptoms of the disease. Indications that cholinesterase inhibitors could also modify the disease have increased research efforts on this target.

2 Review of the Literature

2.1 Dementia

Dementia has been defined as the deterioration of more than one of the cognitive functions to the extent that it impairs a person's ability to perform daily functions independently (McKhann et al. 1984, Sulkava 2010). In dementia, the cognitive impairment is due to an organic cause. People with mild cognitive impairment (MCI) have memory impairments but do not meet the clinical criteria for dementia. MCI is often considered a transition stage between normal aging and dementia (Dubois et al. 2007, Petersen et al. 1999).

Age and genetic susceptibility are the only well established risk factor for dementia (Qiu et al. 2007). Incidences of dementia increase exponentially with age (Fratiglioni et al. 2000, Berr et al. 2005). Consequently, the trend in the aging of population will increase the number of dementia incidents. The number of incidents is estimated to be near 50 million worldwide in the year 2025 (Ferri et al. 2005). There is no cure for dementia so far which makes it a huge burden for health care systems worldwide. Costs of care increase with the progression of dementia (Jönsson and Berr 2005, Jönsson and Wimo 2009). Etiological studies have implicated that control of risk factors for vascular disorders including hypertension, type 2 diabetes, hyperlipidaemia and smoking decrease the risk of dementia and may delay the onset of disease (Ferri et al. 2005, Qiu et al. 2007).

Alzheimer's disease (AD) is the most common form of dementia in people older than the age of 65. AD represents 60 to 70% of demented cases whereas the second most common dementia, vascular dementia, accounts for about 15 to 20% of the cases (Fratiglioni et al. 2000). Other forms of dementia are e.g. Lewy body dementia, Parkinson's disease with dementia, Frontotemporal dementia, Huntington's disease and Creutzfeldt-Jakob disease. This literature review focuses further on AD.

2.2 Alzheimer's Disease

AD is a progressive neurodegenerative disease that slowly and steadily deteriorates memory and other cognitive functions (McKhann et al. 1984, Dubois et al. 2007). Other symptoms are alteration of mood and delusions but also hallucinations are possible. Symptoms of AD usually begin with difficulties in the acquisition of new skills and finding words because of memory disturbances. Duration of the disease from first symptoms to death is approximately 12 years (range 2–20 years) (Sulkava 2010). Men diagnosed with AD die earlier after the onset of the disease than females with AD (Williams et al. 2006).

Hallmark findings in postmortem AD brains are extracellular amyloid plaques and intracellular neurofibrillary tangles (McKhann et al. 1984, Glenner and Wong 1984, Braak et al. 1996, Schliebs and Arendt 2006, Kern and Behl 2009). The spatial and temporal connections between these two pathological hallmarks are not completely understood and are, thus, under constant debate. In addition, cholinergic neuronal depletion is established in AD which influences the cognitive deficits (Mesulam 2004). AD proceeds in stages. Destructive process begins in areas of the cerebral cortex and specific sub-cortical nuclei in a consistent and predictable sequence (Braak et al. 1999). Eventually AD damages the primary motor area and primary sensory fields.

The correlation is strong between accumulation of amyloid plaques and neurofibrillary tangles in the hippocampal region and memory decline over time (Näslund et al. 2000, Reitz et al. 2009). The pathological features of AD are strongly associated within the age group of 75 year olds (Savva et al. 2009). At the same time the association is not that evident in older people. People who died at the age of 95 and did not have dementia still had the pathological findings within their brains to those of AD patients which again underlines the many unresolved questions about this disease. Recent research has shown that healthy children of late onset AD patients have increased fibrillar amyloid burden in AD-vulnerable brain regions compared to controls but the meaning of this finding as a predictive risk factor for developing of the symptoms later on needs further clarification (Mosconi et al. 2010).

2.2.1 Cholinergic Hypothesis of AD

The cholinergic hypothesis states that the loss of cholinergic function in the central nervous system is associated with the cognitive symptoms in age-related memory dysfunction as in AD (Bartus et al. 1982). Accordingly, enhancement of signal transmission in nerve synapses can be used as a treatment strategy to improve impaired memory and other cognitive symptoms. This has been achieved by inhibiting the catalytic site of cholinesterases which in turn prolongs the effect of the neurotransmitter acetylcholine in synapses.

To date, cholinesterase inhibitors are regarded as the first line in pharmacotherapy for the symptomatic relief of mild to moderate AD, accounting for 3 out of the 4 drugs currently approved for the treatment of the disease (Lleo et al. 2006, Farlow and Cummings 2007, van Marum 2008, Pepeu and Giovannini 2009). The effect of the cholinesterase inhibitors is moderate but they have been shown to improve cognitive functions of patients with mild to moderate AD which could also be seen in measures of daily living and behaviour (Birks 2006). Most of the studies on cholinesterase inhibitors have been conducted over a time period of one year or less which is too short of a time to evaluate true long term effects of these drugs. Rountree et al. (2009) evaluated the persistent treatment with dementia medication which consisted of either cholinesterase inhibitor alone or with memantine or memantine alone. Memantine is a NMDA receptor

(N-methyl-D-aspartic acid receptor) antagonist, the only non-cholinergic drug approved for clinical use of AD. They were able to show significantly slower rates of decline on measures of cognition and activities of daily living. The results were cumulative over time but more long term research needs to be done before cholinesterase inhibitors may be declared disease slowing compounds. So far other disease modifying therapy approaches have failed to show true efficacy in clinical trials which indicates that cholinesterase inhibitors will be the most important form of therapy in the near future (Golde et al. 2010).

Recent findings have shown that the gene coding acetylcholinesterase (AChE) can form alternative variants of the protein, and interestingly the N-terminally extended synaptic form of AChE (N-AChE-S form) induces programmed cell death (Toiber et al. 2008, Greenberg et al. 2010). This variant is over-expressed in AD which could explain the degeneration of cholinergic neurons in AD. Cholinesterase inhibitors were able to prevent this apoptotic effect of the N-AChE-S variant in preliminary studies which implies in a possibility of using cholinesterase inhibitors as preventive medication against neurodegeneration in AD. The catalytic properties of all studied AChE variants were similar. It is too early to conclude the real meaning of these findings but the preliminary results are intriguing.

2.2.2 Amyloid Hypothesis of AD

According to the amyloid hypothesis of Alzheimer's disease amyloid- β peptide ($A\beta$) initiates the pathological cascade of the disease (Hardy and Higgins 1992, Hardy and Selkoe 2002). Amyloid- β peptides are 39 to 43 amino acids in length and cleave from the much larger transmembrane amyloid precursor protein (APP) (Selkoe 1999, Vassar et al. 1999, Costantini et al. 2005). Amyloid- β peptides aggregate and form amyloid plaques which are extracellular abnormal protein deposits. The attempt to create disease modifying drugs has partly concentrated on boosting the clearance of amyloid- β peptides or preventing their aggregation.

Recent findings have shown that in addition to its catalytic function, AChE can mediate other processes related to its peripheral anionic site (PAS), one being enhancement of amyloid aggregation and fibril formation (Alvarez et al. 1998, Giacobini 2003b, Giacobini 2004, Inestrosa et al. 2008). AChE has been identified within amyloid- β deposits of both pre-amyloid diffuse deposits and mature senile plaques (Moran et al. 1993, Geula et al. 1994). AChE forms stable complexes with amyloid plaques (Alvarez et al. 1998, Inestrosa et al. 1996). Stable amyloid- β -AChE complexes are more toxic than those formed without the effect of the enzyme (Alvarez et al. 1998, Munoz and Inestrosa 1999, Reyes et al. 2004). It has been shown in APP-transgenic mice that release of AChE from β -amyloid plaques improves memory impairment (Dinamarca et al. 2008). This translates to a strategy to create new kinds of acetylcholinesterase inhibitors inhibiting both catalytic and peripheral sites of the enzyme, which could relieve the symptoms of AD as well as modify the disease. Dual binding site inhibitors have been shown to inhibit the

AChE-induced β -amyloid aggregation (Bolognesi et al. 2010, Camps et al. 2010). In addition, a monoclonal antibody that interacts with PAS of AChE has been shown to inhibit the promotion of amyloid fibril formation induced by AChE (Reyes et al. 1997).

As long as there is no disease modifying treatment for AD, there will be debate over different hypotheses because complex neurodegenerative diseases like AD are affected by numerous factors. It has been widely established that amyloid- β is involved in AD pathogenesis but is it the sole cause of the disease or is it one of the causes or even a downstream consequence for something else, is still under debate (Pimplikar 2009, Young and Bennett 2010).

2.3 Cholinesterases

Cholinesterases are serine hydrolyzing enzymes that catalyze the hydrolysis reaction of choline esters (Fig. 1) (Soreq and Seidman 2001, Darvesh et al. 2003a). Cholinesterases in human central nervous system (CNS) are divided into two classes: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) (Fig. 2). The former is often referred as “true cholinesterase” whereas the latter “pseudo-cholinesterase”.

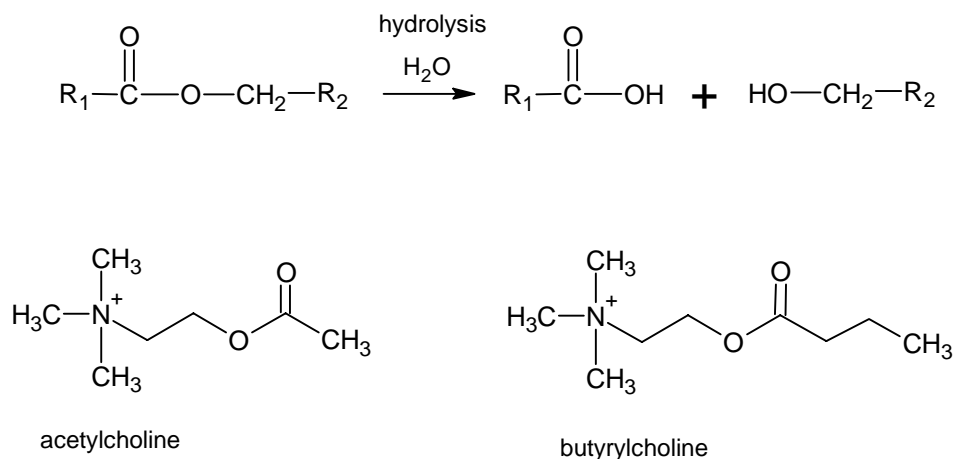


Figure 1. Cholinesterases catalyze hydrolysis reactions of choline esters like neurotransmitter acetylcholine. Butyrylcholinesterase is less substrate specific than acetylcholinesterase thus it also catalyze hydrolysis of more bulky substrates such as butyrylcholine.

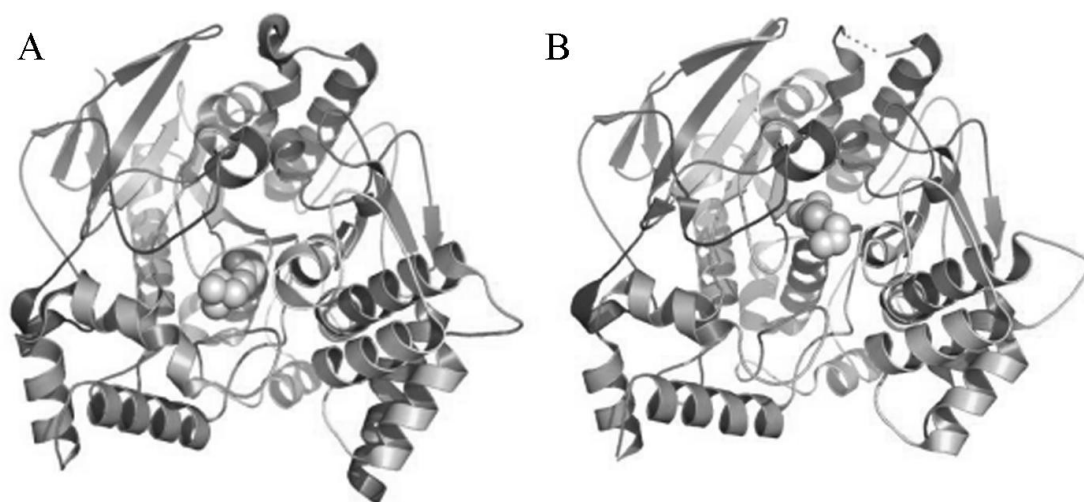


Figure 2. The figures of A) *Torpedo californica* AChE (with acetylthiocholine in the figure, protein data bank (PDB) code 2C58) and B) human BChE (with butyrylthiocholine in the figure, PDB code 1P0P).

2.3.1 Acetylcholinesterase

Acetylcholinesterase is an enzyme which plays a central role in neuronal signal transmission by regulating the synaptic concentration of the neurotransmitter acetylcholine (Fig. 2A) (Silman and Sussman 2005). Cholinergic neurons are rich in acetylcholinesterase and are present in all regions of the brain (Mesulam et al. 2002b). Acetylcholinesterase is a substrate specific enzyme that hydrolyses the natural substrate acetylcholine at one of the highest known enzymatic rates (Quinn 1987, Rosenberry 2010). Acetylcholinesterase is evolutionally well preserved in different species, especially the active site of the enzyme and its function (Wiesner et al. 2007).

The catalytic (also called acylation) site of AChE lies on the bottom of a deep gorge and is composed of three main amino acid residues: Ser-200 (serine), Glu-327 (glutamic acid) and His-440 (histidine) (*Torpedo* numbering) or Ser-203, Glu-334 and His-447 (mammalian numbering) (Shafferman et al. 1992, Saxena et al. 1997, Johnson and Moore 2006, Dvir et al. 2010). Anionic subsite consisting of Trp 84 (tryptophan), Tyr 130 (tyrosine), Tyr 330 and Phe 331 (phenylalanine) (*Torpedo* numbering) or Trp 86, Tyr 133, Tyr 337 and Phe 338 (mammalian numbering), interacts with the quaternary trimethylammonium of acetylcholine, and positions the ester moiety at the acylation site.

The peripheral anionic site (PAS) of AChE is composed mainly of five amino acid residues: Tyr-70, Asp-72 (aspartic acid), Tyr-121, Trp-279 and Tyr 334 (*Torpedo* numbering) or Tyr-72, Asp-74, Tyr-124, Trp-286, Tyr-341 (mammalian numbering) (Johnson and Moore 2006). It is practically located on the surface of the enzyme at the

entrance of the gorge to the catalytic triad. Ligand binding to the PAS, change allosterically the conformation of the active center (Shafferman et al. 1992). Substrates entering the gorge are transiently in contact with PAS which traps the substrate and enhances the efficiency of the catalysis. Non-classical functions of AChE are claimed to mediate through PAS of AChE (Johnson and Moore 2006).

2.3.2 Butyrylcholinesterase

Butyrylcholinesterase is a non-specific enzyme that hydrolyzes acetylcholine and other esters (Fig. 2B) (Masson et al. 2007, Masson et al. 2009). BChE is widely distributed in brain areas but it is also present in other tissues such as the liver (Li et al. 2000, Geula and Nagykerly 2007). The physiological role of BChE is not known. Because of its non-specific nature, BChE is involved in detoxification of harmful compounds in the human body (Masson et al. 2009). BChE expression varies during different developmental stages which implicates its role in neuronal development (Geula and Nagykerly 2007). Similarities in the molecular characteristics of BChE in the AD brain and in the developmental stages of the brain suggest stimulation of a neuronal repairing mechanism involving BChE in degenerative disorders. Selective inhibition of BChE has been shown to increase cortical extracellular level of acetylcholine (Giacobini 2004, Greig et al. 2005). Conversely, BChE in the normal brain can hydrolyze acetylcholine if AChE is completely inhibited (Mesulam et al. 2002a). AChE knockout mice have dysfunctions in the central and peripheral cholinergic pathways but are able to survive to adulthood which indicates that BChE contributes to normal cholinergic transmission and serves as a back-up for AChE (Mesulam et al. 2002b). It is noteworthy that the level of BChE activity increases in AD whereas the level of AChE activity decreases (Giacobini 2003a).

The active site of BChE is located in a gorge that is wider than the active site gorge of AChE (Saxena et al. 1997, Masson et al. 2009). The catalytic triad consists mainly of three amino acid residues: Ser-198, His-438, Glu-325 (Ser-226, His-466, Glu-353 human numbering) (Darvesh et al. 2003a). Two residues (Asp-70 and Tyr-332 mammalian numbering) where positively charged substrates bind before entering the gorge form the PAS of BChE. BChE lacks the PAS residues of AChE (Tyr-70, Tyr-121, Trp-279 *Torpedo* numbering; or Tyr-72, Tyr-124, Trp-286 mammalian numbering) which has been postulated to be the reason why BChE does not affect amyloid aggregation in the same way that AChE does (Inestrosa et al. 2008, Masson et al. 2009). On the contrary, the C terminal peptide of BChE has been shown to interact with soluble forms of β -amyloid and prolonging the kinetics of amyloid fibril formation (Diamant et al. 2006).

2.3.5 Cholinesterase Inhibitors

Currently approved cholinesterase inhibitors for the treatment of mild to moderate AD in Finland are galantamine, donepezil and rivastigmine (Fig. 3) (Fimea 2010). Tacrine was

the first FDA approved cholinesterase inhibitor for treatment of AD but poor bioavailability and adverse effects have limited its clinical usage (van Marum 2008). Cochrane review of 13 randomized, placebo-controlled, double blinded trials states that galantamine, 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 reviewer could not distinguish differences within clinical potency differences between these three compounds even though the modes of action are quite different.

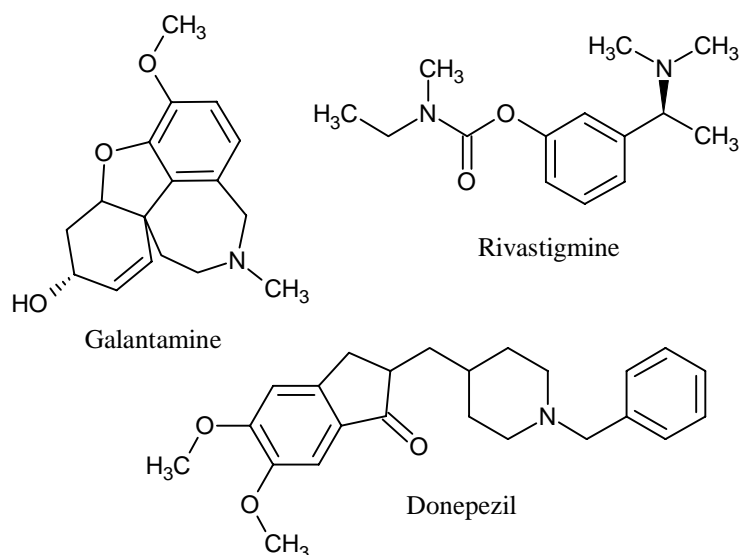


Figure 3. The structures of the cholinesterase inhibitors on the market for AD in Finland.

Traditional cholinesterase inhibitors including FDA approved (FDA, Food and Drug Administration in U.S.A.) donepezil, rivastigmine and galantamine inhibit primarily the catalytic site of AChE, and some of them also inhibit the catalytic site of BChE. Galantamine is a reversible competitive inhibitor of AChE (Camps and Munoz-Torrero 2002, Darvesh et al. 2003b). It also inhibits BChE but its inhibitory potency toward this enzyme is four fold lower than for AChE. Donepezil is a reversible mixed non-competitive AChE inhibitor with 100 fold AChE selectivity over BChE. Rivastigmine inhibits AChE and BChE in pseudo-irreversible fashion i.e. it initially forms a covalent carbamyl complex with the enzymes that leads to a slow reactivation of the enzymes. The mechanism is common for cholinesterase inhibitors with a carbamate structure, including physostigmine. Rivastigmine and physostigmine inhibit AChE and BChE non-selectively if potencies are calculated using IC_{50} values (IC_{50} , concentration yielding 50% inhibition) (Giacobini 2004). Darvesh et al. (2003b) calculated a second order rate constant which takes into account the pseudo-irreversible mode of action of these compounds and came to the conclusion that selectivity of rivastigmine is 100 fold higher towards BChE compared

to AChE, whereas selectivity of physostigmine is 3 fold higher towards BChE compared to AChE.

Propidium which is an inhibitor that binds to the peripheral anionic site of AChE is able to inhibit AChE induced β -amyloid aggregation (Bartolini et al. 2003). Donepezil and physostigmine show slight inhibitory potential for AChE induced β -amyloid aggregation whereas tacrine is inactive. Series of inhibitors designed to bind to both sites of AChE have been shown to act as dual inhibitors that inhibit AChE-induced β -amyloid aggregation as well as catalytic action (Bolognesi et al. 2010, Camps et al. 2010). BChE has not been associated with β -amyloid aggregation in the same way as AChE is. The C-terminus of BChE has been attenuated to prolong the amyloid fibril formation therefore butyrylcholinesterase inhibitors should not interact with it (Diamant et al. 2006).

In addition to classical inhibitory effect, cholinesterase inhibitors may have other biological activities, e.g., galantamine modulates nicotinic acetylcholine receptors in a non-competitive fashion which potentiates the channel activation by the natural transmitter and leads to increased pre-synaptic release of acetylcholine (Storch et al. 1995). Physostigmine has similar nicotinic acetylcholine receptor modulating properties as galantamine. Donepezil has been shown to inhibit β -secretase *in vitro* (Mancini et al. 2007). Huperzine is a potent cholinesterase inhibitor which also has neuroprotective potential through inhibition of apoptosis, beneficial actions on A β processing and it reduces oxidative stress (Zhang and Tang 2006).

In general, multifunctional compounds that could modify the progression of AD in addition to the classical mode of cholinesterase inhibition have become a hot area of interest. Most often new compounds aim to also target the peripheral site of AChE to decrease amyloid aggregation (e.g., Bolognesi et al. 2005, Dickerson et al. 2005, Munoz-Ruiz et al. 2005a, He et al. 2007, Kwon et al. 2007, Camps et al. 2008, Leon et al. 2008, Marco-Contelles et al. 2009, Rizzo et al. 2010). Other approaches aim to find compounds that inhibit β -secretase (Zhu et al. 2009), scavenge free radicals (Decker et al. 2008, Spuch et al. 2010) or activate muscarinic M₁ receptors (Fang et al. 2010). One of the groups has taken a further step by creating a true multi-potent compound, memoquin, which inhibits AChE, AChE-mediated amyloid aggregation, β -secretase, tau-phosphorylation and free radical formation, also *in vivo* (Bolognesi et al. 2009). It will be interesting to see if such a compound could reach clinical trials or is the effect non-selective also for unwanted targets.

2.4. Cholinesterase Inhibitors from Natural and Synthetic Origins

2.4.1 Drug Discovery Process

The drug discovery and development process is a long and costly road from target identification to the market. It begins with basic research and identification of a target relevant to a disease and ends up with a new molecular entity that has been through preclinical and clinical phases and has been formulated into a product that is marketed to patients. The whole process takes ca. 13.5 years on average and costs 0.9 billion dollars per new molecular entity launch (Paul et al. 2010). Costs have been increasing exponentially since the 1950s at the rate of 13% a year (Munos 2009). Withdrawal rates are high in phases II and III, therefore nine compounds are need to enter clinical phase I to yield one new molecular entity launch. Clinical trials are the most expensive phases in the whole process accounting for around 63% of the costs (Paul et al. 2010). Therefore, improving the quality of research in the preclinical phase saves money and years of work if non-drug-like compounds are cut out of the pipeline as early as possible. The number of drug approvals has been a decreasing trend which raises concerns for public health care systems. The number of FDA drug approvals in 2009 was 26 (Hughes 2010) whereas the number of drug approvals in Europe was 29 (Eichler et al. 2010).

The drug discovery process can be roughly divided into four phases: i) target selection and validation, ii) assay selection, development and adaptation, iii) primary and secondary screening *in vitro* and iii) lead discovery and optimization (Fig. 4) (Johnston and Johnston 2002, Mayr and Bojanic 2009). When designing a screening campaign on a selected target the first decisions to be made are the selection of assay, the format of the assay and the detection method. High-throughput screening (HTS) is defined by the number of data points created per day; typically 10,000–100,000 compounds are assayed per day. Assay applicability for large scale screening limits the materials to be screened and vice versa. If tens of thousands of compounds or extracts are to be screened, the format of the assay should be in the low microlitre scale and automatable. A screening campaign for more targeted libraries leaves more options for assay formats. When screening extracts, it is wise to couple liquid chromatography separation technique to the bioactivity assay of choice (Wagenaar 2008). This enables the tracking of activity to a certain compound or fraction containing a low number of compounds which helps the following purification and structure elucidation steps. Complexity of the sample decreases with the fractionation which is advantageous because some compounds can mask detection in subsequent bioactivity measurements.

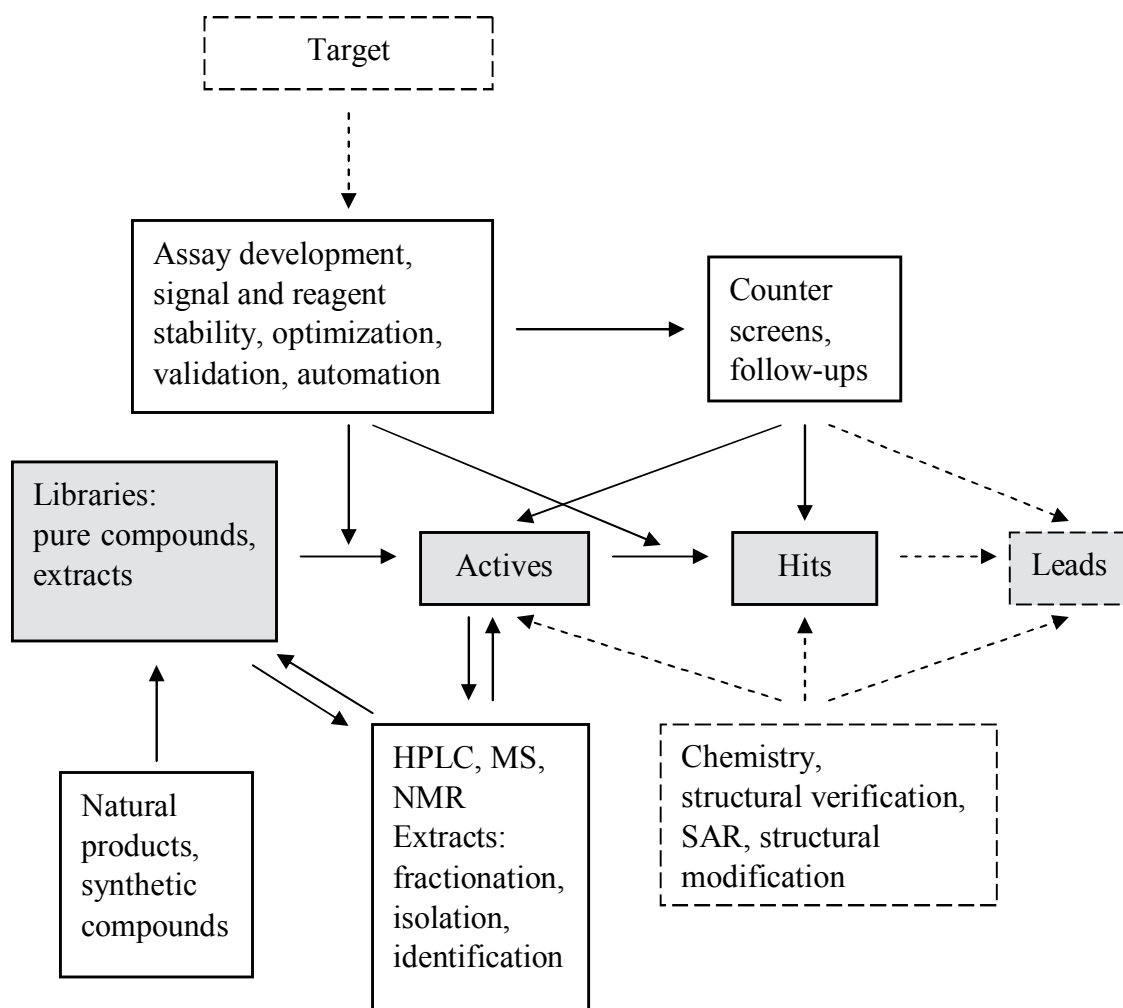


Figure 4. Schematic presentation of the drug discovery process represented from the perspective of this thesis. This thesis covers *in vitro* screening of the natural product and commercial synthetic compound libraries to create hits, as well as the assay development aspects for enzymatic targets. Target validation, medicinal chemistry and lead discovery parts were not dealt in this thesis (represented with dashed lines). General process was modified from Johnston and Johnston 2002, Potterat and Hamburger 2006, May and Bojanic 2009.

In the assay adaptation phase, the assay is optimized to the screening set-up and validated for the specific environment which usually includes miniaturization of the assay and validation of the automatic liquid handling robotics (Mayr and Bojanic 2009). Statistical parameters are used for assay validation to ensure robustness of the assay and discrimination of the signal from the background (Zhang et al. 1999, Bollini et al. 2002). The screening window coefficient (Z' -factor), signal-to-background (S/B) and signal-to-noise (S/N) ratios are common validations parameters.

In the primary screening phase the target is usually assayed using a single concentration of the compounds (Keseru and Makara 2006). A hit limit is set, e.g., $3 \times \text{SD}$

or 4xSD (SD, standard deviation) from the average to identify the compounds that cause an effect that significantly differs from the mean signal. In the secondary screening phase, several concentrations are used to evaluate dose-dependency of the hits in the primary screening. In addition, effects on other related targets can be assessed to evaluate selectivity. Preliminary toxicological evaluation in an *in vitro* set-up at this stage is wise because toxicological effects are among the main reasons for attritions in clinical phases (Paul et al. 2010). Prediction of drug-likeness is central in drug discovery. Selection of hits for lead optimization should be based on potency and drug-likeness because compounds entering the lead phase should have properties that ensure the bioavailability of the compound (Di et al. 2009). Solubility, permeability, metabolic stability and interactions with active transporters should be evaluated before any compound proceeds further along the pipeline as drug candidates. Rapid metabolism and high efflux by transporters can lead to short half-life, high clearance and inadequate efficacy which in turn lead to low bioavailability of the drug.

The lead discovery and optimization phase aims to improve the hit quality towards a lead and further a clinical candidate (Keseru and Makara 2009). The main goals are to improve, e.g., potency, biological availability and pharmacokinetic properties of the hits. In the analysis of Keseru and Makara (2009), several differences were found in the comparison of the hits in HTS screenings and corresponding leads. Lead potency, lipophilicity and molecular mass are increased compared to hits. In addition, lead structures are more complex and more flexible than hits. The leads derived from the hits discovered in HTS are not necessarily optimal drug candidates. In comparison, old leads published before year 2000 are less lipophilic. Increased lipophilicity has been postulated to be one of the reasons for high attritions in clinical trials. The optimal properties of each drug are a fine balance between multiple factors (Waring 2010). Lipophilicity is one of the key factors because it affects almost all aspects of drug behaviour within the body, e.g., solubility, toxicity, efficacy, bioavailability and metabolism. Chemical modifications of the hits affect also the metabolic stability of compounds which can be taken into account in drug development to increase bioavailability and the half-life of the drug candidates (Nassar et al. 2004). This can be achieved, e.g., by modifying metabolically active groups that are vulnerable to metabolic enzymes and by decreasing lipophilicity.

2.4.2 *In vitro* Screening of Cholinesterase Inhibitors

The Ellman's reagent based assay (Ellman et al. 1961) is by far the most widely used in the area of measuring cholinesterase activity. It is applicable for isolated AChE and BChE as well as for homogenated tissue samples. The assay has been used in cuvette and 96-well plate formats but also on thin layer chromatography (TLC) plates (e.g., Rhee et al. 2001) and in an on-line with high performance liquid chromatography (HPLC) coupled to a biological detection set-up (Ingkaninan et al. 2000). The substrate (ATCI, acetylthiocholine) reacts with the enzyme and the formed product further reacts with dithionitrobenzoate (DTNB, also called Ellman's reagent) to form a yellow anion.

Inhibitors decrease the formation of the coloured product which can be detected photometrically or visually on TLC. On TLC the yellow colour is not convenient because of the low contrast between the yellow colour and white plate. Marston et al. (2002) developed an assay on TLC that uses naphthyl acetate as a substrate and Fast blue salt B as a reagent. The product is purple which increases the contrast and eases visual detection of inhibitors in the sample. Both of these assays are compatible with natural products, either extracts or pure compounds, and synthetic compounds but the assay set-up can limit their usefulness in screening especially when increased throughput is required. Ellman's reagent based assay in micro-well plate format can be adapted to an automated environment which enables its usage when high throughput is required. The following paragraphs discuss and evaluate the pros and cons of different libraries used in the screening process of drug discovery.

2.4.2.1 Natural Products

Natural products have been an undeniable success story in drug discovery historically but also during the past three decades (Newman et al. 2003, Newman and Cragg 2007). Half of the drugs approved for the market during 1981–2006 are either direct isolates from nature or their semi-synthetic derivatives or proteins or molecules otherwise related to natural sources. In spite of this, problems in adapting natural products into HTS platforms like low throughput in screening and pitfalls in the handling of extracts are issues that have been resulted in a decreased interest in natural product research (Eldridge et al. 2002). In addition, natural compounds are often more challenging to produce in large quantities than synthetic compounds because extraction, separation and structure elucidation processes are considered laborious and time consuming. Development of high-throughput platforms for large libraries has led to the usage of synthetic compounds produced by combinatorial chemistry over natural products in industrial screens (Li and Vederas 2009). Despite the huge expectations loaded on large random screens of hundreds of thousands synthetic compounds, the number of approvals for synthetic entities has not increased with the increase in throughput. Development of analytical techniques like liquid chromatography coupled to nuclear magnetic resonance spectroscopy (LC-NMR) and multi-dimensional-NMR, in particular, and the down-scaling of the amount of sample needed, are elements that will attract pharmaceutical companies back to natural product research (Eldridge et al. 2002, Wolters et al. 2002, Lambert et al. 2005, Lambert et al. 2007, Lang et al. 2008, Lin et al. 2008, Queiroz et al. 2009).

As an example of the development of analytical techniques, amounts as low as 7 to 50 µg of a new compound is enough for structure elucidation by 1D and 2D proton-NMR analyses in new microcoil flow probes compared to several milligrams needed in traditional NMR analyses (Eldridge et al. 2002, Dalisay and Molinski 2010). At its best, fractionation of 700 µg of an extract using analytical scale HPLC run into microtiter plates has been shown to be sufficient for both structure elucidation and biological assay if spectral data are combined and analyzed with the help of database information (Lang et al.

2008). UV and MS analyses of fractions in combination with NMR analysis using a capillary NMR probe are powerful tools for quick structure elucidation. Quantitation of nanomole amounts of compounds by NMR is also possible which further stresses the importance of the new NMR techniques in natural product drug discovery (Dalisy and Molinski 2009). Quantitative nuclear magnetic resonance spectroscopy (qNMR) does not necessarily require analytical standards for quantitation which is a huge advantage over other quantitative techniques like HPLC-UV (UV, ultra violet) (Lubbe et al. 2010).

Natural product scaffolds are more complex in structure than synthetic drugs or compounds in synthetic compound libraries (Lee and Schneider 2001, Feher and Schmidt 2003, Ortholand and Ganesan 2004, Newman 2008, Rosen et al. 2009, Sukuru et al. 2009). The complexity of natural products is mostly due to polycyclic structures, heterocyclic substituents and multiple chiral centers but also the size and lipophilicity differ from compounds in synthetic libraries. High numbers of trade drugs are of natural origin (Newman et al. 2003, Newman and Cragg 2007) which can also be seen by comparing properties of natural products, trade drugs and synthetic compounds in libraries where trade drugs resemble more natural products than the combinatorial compounds do (Feher and Schmidt 2003). In addition, combinatorial compounds are more flexible than natural compounds which have been postulated to decrease their specificity and activity. The connections in combinatorial compound ring systems are established by single bonds between them whereas natural ring systems are rigid and have few routable bonds. Lee and Schneider (2001) analysed the ring scaffolds in a set of natural products and compared them to trade drugs and found that only 17% of the scaffolds found in natural products are present in trade drugs. Importantly, differentiation of biologically relevant chemical space from general chemical space gives more power to predictions of biological activity (Larsson et al. 2007). Natural product scaffolds are well represented in regions of biologically relevant chemical space that is rarely occupied by synthetic compounds (Rosen et al. 2009). There is a huge pharmaceutical potential in diverse ring systems of natural origin which is also acknowledged by industry (Drewry and Macarron 2010).

In general, nature has been a good source to find cholinesterase inhibitors. One of the three cholinesterase inhibitors on the market today, galantamine, is a direct isolate of plants belonging to Amaryllidaceae species such as *Leucojum* spp. L., *Narcissus* species or *Galanthus* spp. L. (Heinrich and Lee Teoh 2004). This drug was found based on ethnopharmacological knowledge from the Caucasus mountain range and Eastern Europe. The association to AChE inhibition was established in the 1960s but it was licensed only during the year 2000 for the treatment of AD.

Another well known cholinesterase inhibitor from a natural source is physostigmine isolated from *Physostigma venenosum* (Balf.) (Zhao et al. 2004). Its narrow therapeutic index and short half-life have limited its medicinal usage (Camps and Munoz-Torrero 2002, Coelho and Birks 2001) but the compound is extensively used as a reference compound in enzymatic assays. The carbamate analogue of physostigmine, rivastigmine, has been approved for the market for AD treatment (Polinsky 1998, Jann 2000).

Huperzine isolated from the Chinese plant *Huperzia serrata* (Thunb. ex Murray) Trev., is at the clinical phase of development (Ma et al. 2007). In addition to cholinesterase inhibition, huperzine has shown neuroprotective potential against apoptosis, beneficial actions on A β processing and a reduction of oxidative stress which makes this multi-targeted compound highly interesting as a future treatment option (Zhang and Tang 2006). The primary results are encouraging but large trials with standardized products still needs to be conducted (Desilets et al. 2009). Some of the clinical trials have estimated the effects of plant extracts that were not standardized.

2.4.2.2 Natural Product Libraries for Screening

Large and small scale natural product libraries consisting of either pure isolated compounds or extracts have been used successfully in screening campaigns against various isolated or cell-based targets (Eldridge et al. 2002, Wagenaar 2008, Jayasuriya et al. 2005, Shaneyfelt et al. 2006, Bugni et al. 2008, Lee et al. 2008, Salam et al. 2008a, Hashimoto et al. 2009, Hung et al. 2009).

The number of isolated compounds in natural pure compound libraries in the literature is most often in the region of hundreds, e.g., 280 compounds (Shaneyfelt et al. 2006), 200 compounds (Salam et al. 2008a) or 400 compounds (Hung et al. 2009) which diminishes the throughput in screening. In principle, there are no limitations in possible compound amounts in natural pure compound libraries due to the fact that thousands of compounds can be isolated within months (Bindseil et al. 2001). In practice, isolation procedures increase expenses of this approach extensively prior the actual bioactivity phase when the number of compounds increases to thousands. On the other hand, the following stages from hit to a validated lead are significantly easier and less expensive than working with extracts. For pharmaceutical companies that routinely screen the same library for tens of targets, this approach may well be cost-effective also at a larger scale. It is noteworthy that a natural pure compound library has been shown to produce significantly higher hit-rates when nine targets were evaluated compared to a synthetic compound library (Bindseil et al. 2001). Natural pure compounds are routinely included as a part of huge chemical libraries of millions of compounds in pharmaceutical companies from which a subset of compounds is selected for each screen (Sukuru et al. 2009). If structure diversity is a selection criterion for a certain screen, a large fraction of natural products is usually selected because of their inherent chemical diversity.

Screening of large crude extract libraries of thousands of extracts is another approach in the screening of natural products. Generally this is not considered the best option. The properties like viscosity can interfere with automatic liquid handling, compounds can mask each other's activities, and some of the compounds can interfere with assays and raise the false positive or false negative rates (Wagenaar 2008, Bindseil et al. 2001). These issues lower the quality of the hits. Nevertheless, screens of 1400 extracts (Lee et al.

2008), 64 000 extracts (Jayasuriya et al. 2005) and even over 110 000 extracts have been conducted in which target specific active constituents have been identified after bioactivity guided fractionation of the active extracts.

Pre-fractionation of extracts has been an option in natural product screening to simplify the complexity of the screened extracts and to avoid the above mentioned problems. Most often pre-fractionation is achieved through coupling reversed-phase HPLC (RP-HPLC) separation techniques to an automated fraction collector (Eldridge et al. 2002, Wagenaar 2008, Bugni et al. 2008). Typically, fractions contain several compounds e.g. 1–5 compounds per fraction, and before structure elucidation more detailed separations of the compounds in the active fraction is required. Alternatively, LC-MS (MS, mass spectroscopy) and LC-NMR techniques can be utilized on-line with fractionation while part of the eluent is split and directed to spectroscopic detection which gives preliminary data on the number of the compounds present and their structures. Bioactivity of each compound has to be assayed separately. Large pre-fractionated libraries have been conducted from microbial, plant and marine sources which can contain up to 200 000 fractions (Eldridge et al. 2002, Wagenaar 2008, Bugni et al. 2008).

As proven with synthetic compound libraries, high throughput and an increase in the number of compounds in screening campaigns do not necessarily result in higher numbers of validated leads. This also applies for natural products. The possibility to target the library based on a prior knowledge is an advantage for natural products. Natural products have been used in traditional healing for centuries which is a rich source of information that can be applied in screening strategies to narrow down the screening material in the library from random to a more targeted set of compounds or extracts. This approach is also known as ethopharmacological knowledge. Probability in detecting a certain bioactivity is higher if relative traditional use has been reported for the natural product, e.g., in case of antifungal activity (Svetaz et al. 2010). The probability to find antifungal activity was 40% if traditional use was reported for the assayed plant compared to 20% probability for random plant selection. In addition, detected potencies were higher in the group with an ethopharmacological background. Derived from a prior knowledge of bioactivity or a bioactive compound in a specific species, the screening set-up can be broadened to cover phylogenically related species. Furthermore, knowledge of biosynthetic routes, chemotaxonomy and similarity of compounds in unrelated species are means to target material.

2.4.2.3 Synthetic Compound Libraries for Screening

Design of synthetic compounds for synthetic compound libraries is directed to cover a broad chemical space and to make compounds drug-like. Drug-like traditionally translates into compound properties which fit with the Lipinski rule of five (Lipinski et al. 1997). This rule uses computational and experimental approaches to predict solubility and permeability of a compound through physical bi-layer membranes and ultimately the

human body after oral delivery. Prediction of permeation is crucial in drug discovery because even the most promising compound is worthless if it cannot reach the target in the body and cannot be used as a drug. Basic rules are as follows: i) number of H-donors should not exceed 5; ii) number of H-acceptors should not exceed 10; iii) molecular weight should not exceed 500; iii) calculated LogP should not exceed 5. The lipophilicity limit has been shown to be dependent on the molecular weight in a way that the lower the weight the lower lipophilicity limit should be for the compounds (Waring 2009).

Several errors may occur in screening if the compounds' drug-likeness is not considered early on (Di et al. 2009). It is an advantage in synthetic compound library design that these properties may be considered. Solubility is one of the most important issues because it affects results in *in vitro* assays and *in vivo*. *In vitro*, potency of poorly dissolving compounds may be artificially low which reflects structure-activity relationship (SAR) determinations and decision making when choosing compounds for further studies. Toxicity may also be underestimated. *In vivo*, poorly soluble compounds have poor bioavailability and variation between subjects and species may be substantial. Studies on compounds in the HTS libraries reveal that 30% of the compounds have solubility less than 10 μM in biological assays. The problem has been acknowledged in pharmaceutical companies and nowadays drug-likeness is assessed before *in vivo* studies.

Designing a library can be derived from several perspectives. Drug-likeness and preferable ADME properties are main strategies for creating synthetic compound libraries (Goodnow et al. 2003). A design that aims to create a more focused set of compounds based on the target dimensions can lead to better hit-rates. Computational technologies have emerged to make the library design more efficient and cost-effective (Balakin et al. 2006). Structure-based design, pharmacophore model and fragment based topological information combined to combinatorial chemistry can yield potential ligands for therapeutic targets. An example of a targeted approach in synthesizing a chemical library is to select a certain structural core that is known to have activity against a specific target or to have interaction with the target, and create derivatives or mimics of this structure (Birks 2006, Best et al. 2004, Bhatia et al. 2010, Shih et al. 2010). The goal is to cut down the size of the library and increase the hit rate. As an example, the β -lactam scaffold has been used to create a highly diverse combinatorial library of compounds that meet the criteria for drug-likeness (Steger et al. 2001).

Diversity-oriented synthesis of compounds aims to fill chemical space occupied by natural products which are inheritably diverse and "designed by evolution" to interact with three-dimensional natural surfaces, or to fill new unexplored areas of chemical space (Cordier et al. 2008, Spandl et al. 2008). Another important goal is to be able to synthesize compounds efficiently without compromising the diversity requirements. The realization that compounds created with traditional combinatorial chemistry lack the complexity and diversity of natural products has led to the new era of trying to combine the best of both by means of using natural templates in combinatorial chemistry set-ups or to mimic the

chemical space of natural compounds in combinatorial synthesis (Ortholand and Ganesan 2004, Nielsen 2002).

Natural product scaffolds have been characterized to form scores that allows the characterization of synthetic compounds for their “natural product-likeness” with the view to broaden the chemical space of combinatorial compounds (Lee and Schneider 2001, Ertl et al. 2008). Diversity-oriented synthesis differs from target oriented combinatorial synthesis in aiming to cover wider chemical space (Cordier et al. 2008). The aim is to keep synthesis of highly diverse compounds simple. Assembly of a small molecule library should have up to 5 synthetic steps. Extending the combinatorial chemistry approach to complex natural products creates highly diverse sets of compounds that have the scaffold from natural compounds but is substituted with additional groups. Further challenges arise from requirements to synthesize skeletally diverse compounds inspired by nature that would be structurally unique. It remains to be seen if these approaches will lead to higher hit rates in screening campaigns in the future. Better knowledge on synthesis of complex compounds will aid further in the synthesis of “real” natural compounds at an industrial scale because the reliable access and supply of rare natural materials for isolation of highly potent compounds directly from nature is often a problem that has to be solved before commercialization of the product (Li and Vederas 2009).

When creating chemical libraries by means of combinatorial chemistry lots of closely related compounds are created (Metzger et al. 2006). In the secondary phase of screening the activity data of these compounds can be used in quantitative structure activity relationship determinations (QSAR). Statistical analysis of bioactivities in modularly assembled libraries conducted with a computational program aid in creating trends between structures and activities (Seedhouse et al. 2010). Further, the data reveal features that are important for activity which can be used in the design of second generation compounds.

Synthetic chemical libraries have been screened successfully for loads of novel enzyme inhibitors with high potency (Batra et al. 2002). Carbonell et al. (2000) identified nine selective inhibitors of acetylcholinesterase from a small combinatorial library of 100 compounds. Also, other strategies to find new AChE inhibitors exist, e.g., Kapková et al. (2005) utilized random chemistry where tacrine derivatives were created by irradiating tacrine in different solvents which leads to radical recombination and formation of new derivatives.

2.4.3 *In vitro* Screening as a Basis for *In silico* Predictions

Virtual screening can be divided into two approaches: i) similarity based screening in which compounds known to bind to the target are used as structural queries for the model and ii) automated docking of compounds into the target binding site when the 3D structure of the target is known (Lengauer et al. 2004, Taft et al. 2008). In similarity based screens

no information on the structure of the target is needed. Quality of the similarity based *in silico* screen is based on the results from *in vitro* screens which highlights the importance of selecting the data. Data sets from the literature can also be used to test the predictive value of the docking model by comparison of the predicted potency values (e.g. IC₅₀ values) from automatic docking assay to observed ones (Bernard et al. 1999, Recanatini et al. 2000, Sippl et al. 2001). The computational model for biological activity can be as good as the quality of the observed biological data (Cronin and Schultz 2003).

Often the data sets are collected from several publications which increase the possibility of an error if the selection process is not done carefully (Cho et al. 1996, Lv and Xue 2010). Differences in results produced with different assays are acknowledged but not so much in modifications of the same assay. By taking a closer look into the screening of acetylcholinesterase inhibitors, the literature is full of “minor” modifications of the Ellman’s reagent based assay for the measurement of acetylcholinesterase activity (Ellman et al. 1961) (Appendix 1). Variations include enzyme, substrate and reagent concentrations and assay parameters, e.g., temperatures, measurement wavelengths, measurement times and incubation times.

In addition, acetylcholinesterase can be isolated from humans, invertebrates and mammals. The amino acid sequence of acetylcholinesterase is dependent on the origin of the enzyme (Wiesner et al. 2007, Khan 2009) which also affects the kinetic constants of acetylcholine and other substrates as well as inhibitors (e.g., Ellman et al. 1961, Vellom et al. 1993, Contreras et al. 1999, Sauvaitre et al. 2007). The sequence similarity is high which makes, e.g., AChE isolated from the eel a good model system to screen AChE inhibitors. Nevertheless, the differences should be taken into consideration when creating an *in silico* prediction. AChE isolated from human erythrocytes and human recombinant AChE have been shown to have the same affinity for acetylthiocholine which makes results from these two enzymes being comparable when using this substrate (Darvesh et al. 2003b). When creating an *in silico* model, the data from several enzyme sources can be separately evaluated in their own data sets after which a comparison of the alignment of active compounds can give additional information on selectivity (Dimoglo et al. 2001). An example on the effect of the variant of the same assay is potency of physostigmine for AChE: the observed IC₅₀ value for physostigmine was reported to be 0.69 nM with pre-incubation and 340 nM without pre-incubation when a mouse brain homogenate was used as the enzyme source (Sugimoto et al. 1990) whereas with 20 min pre-incubation of physostigmine with human erythrocyte AChE led to an IC₅₀ of 19 nM (Villalobos et al. 1994). Based on these observations, the minor modification of the assay can barely be called “minor” if it causes major changes in the potency evaluation in the order of a 500-fold difference.

Attempts to ensure, if even possible, compatibility of the data sets collected from several publications have been made by several means. Comparison of activity values of the reference compounds assayed under the same conditions is probably the most obvious way (Bernard et al. 1999, Cho et al. 1996). This leads to a problem in comparing the assay

conditions if each group modifies the assay (Appendix 1). Using results from papers published by a certain group which have screened lots of compounds probably ensures the most similarity in terms of the assay conditions (Dimoglo et al. 2001, Saracoglu and Kandemirli 2008, Gupta and Mohan 2010). Ideally high quality data should be generated by the same lab and the same person with exactly the same protocol (Cronin and Schultz 2003, Scior et al. 2009). At the worst, the combined data from several publications are not comparable and even at the best are subject to significant variation.

Many times confirmation of the assay conditions in publications is not possible because the authors may just refer to the original publication but do not specify which variant of the assay in the original paper was used or leave out information concerning assay conditions (Atta-ur-Rahman et al. 2001, Kang et al. 2001, Cardoso et al. 2004, Zhan et al. 2010). In the original paper for assaying acetylcholinesterase activity, Ellman et al. (1961) investigated kinetic profiles of several substrates, initiated the reaction with both enzyme preparation and substrate, and utilized tissue homogenates and purified enzymes as sources for cholinesterases. If authors refer to the original publication without specification of the conditions the information is incomplete and misleading. Lv and Xue (2010) classified AChE inhibitors and non-inhibitors according to potencies collected from 21 literature reports and characterized molecular descriptors by machine learning methods. A closer look at the data collected from the literature reveals that IC_{50} values are defined mostly with Ellman's assay but the assay conditions vary substantially. The enzyme sources vary from human and bovine erythrocytes, mouse and rat brain homogenates to eel, whereas the substrate concentrations vary from 0.2 mM to 4.8 mM and also other conditions are not the same. One reference included in the data set even uses another assay for determination of AChE inhibitory effect. The predictive accuracy of the created model was low (76.3 – 88.0%) which could be explained, at least partly, with the poor quality of the original data set due to incomparable IC_{50} values.

When creating high quality *in vitro* data the assay should be validated by means of statistical parameters that measure variation and signal window width to ensure discrimination of background deviation from the signal (Zhang et al. 1999, Bollini et al. 2002). None of the references mentioned in Appendix 1 include validation data in spite of the numerous modifications mentioned. Nevertheless, the data have been used to create computational models to predict biological activity which raises the question of the quality of these *in silico* models (Dimoglo et al. 2001, Saracoglu and Kandemirli 2008, Gupta and Mohan 2010).

Virtual screening and HTS can be used separately but at the best they complement each other. A preliminary *in silico* screen can substantially diminish the number of data points needed in *in vitro* screens and at the same time increase the hit rate (Entzeroth 2003, Bajorath 2002, Stahura and Bajorath 2004, Han et al. 2008). Natural product libraries are also used in virtual screening to limit the number of compounds which are then evaluated in *in vitro* assays (Salam et al. 2008b). Also, acetylcholinesterase inhibitors

have been successfully screened using combination of virtual screening, docking and *in vitro* studies (Rollinger et al. 2005, Schuster et al. 2010).

Iterative similarity based screen utilizes a small set of *in vitro* data to create a docking model (Lengauer et al. 2004, Plewczynski et al. 2009). A second small set of compounds similar to the most active ones in the first cycle is then assayed and docked in the model to train it. The same cycle can be repeated as long as necessary to create actives working in the nanomolar range. In one-shot screening, a large chemical library is first assayed *in vitro* for activity and the data are used for creating the docking model (Gupta et al. 2011).

Established ligand-protein complexes can be a basis for creating a pharmacophore based docking model that uses information on biologically active conformation of the known inhibitors (Sopkova-de Oliveira Santos et al. 2010, Zaheer-ul-Haq et al. 2010). Combination of results from several docking algorithms can create a representative model for screening (Zaheer-ul-Haq et al. 2010). The top scored conformations from the first algorithm are rescored with a second algorithm to increase the predictivity of the model and so on. Pharmacophore model based virtual screening has proven to be a powerful tool for finding new AChE inhibitors (Sopkova-de Oliveira Santos et al. 2010). The top score compounds in virtual screening had low-nanomolar potency when assayed *in vitro*. AChE is a complex and flexible enzyme system which in turn complicates the *in silico* modelling of substrate and inhibitor interactions with the enzyme (Khan 2009, Lushington et al. 2006). The best predictions and detailed factors affecting binding are from hybrid methods that combine several approaches to compensate for each other's shortcomings. As an example, including data on ligand-protein crystallographic complexes to the docking algorithm improves the correlation between observed and predicted data (Guo et al. 2004).

3 Aims of the Study

The main aim of the study was to assess the applicability of in-house chemical libraries for screening purposes. The libraries consisted of plant and fungal extracts, natural coumarins with their synthetic derivatives, and synthetic compounds. Further, to achieve the main aim, two cholinesterase inhibitory assays were adapted to appropriate formats for the screening of each library.

The specific aims were:

- 1) to critically evaluate the applicability of the two assays used in this contribution as screening tools for cholinesterase inhibitors (I–V). Assay performance was assessed using controls and statistical parameters which work as indicators for the quality of the assays.
- 2) to assess the fungal and plant extract libraries as sources for cholinesterase inhibitors (I, II). Special features of extract library screening were addressed, mostly through coupling of analytical separation techniques to biological assays.
- 3) to assess the small coumarin and large synthetic compound libraries as sources for cholinesterase inhibitors (III, IV). Adaptation of the assay formats to low-throughput screening and to high-throughput screening set-ups were evaluated.

4 Experimental

4.1 Materials

4.1.1 Fungal Cultures and Extraction (I)

Mycelia of studied fungi were grown in low nitrogen liquid media for 8 weeks at +28°C. The growth media (600 ml) was extracted with ethyl acetate (3 x 200 ml). Ethyl acetate fractions were collected, combined and evaporated to dryness. All screened fungi belonged to the group of wood rotting fungi (Table 1 *in I*).

4.1.2 Plant Material and Extraction (II)

Mentha longifolia L., *M. pulegium* L., *M. arvensis* L., *M. × gentilis* L., *M. × piperita* L. var. *officinalis*, *M. spicata* L. and *M. spicata* L. var. *crispa* Benth (Lamiaceae) were cultivated in Mikkeli, Finland and harvested in August 2000 after a growth period of two years (*M. longifolia*, *M. pulegium*) or three years (*M. arvensis*, *M. × gentilis*, *M. × piperita* var *officinalis*, *M. spicata*, *M. spicata* var *crispa*) and identified by Bertalam Galambosi, senior research scientist at the Agrifood Research Centre, Finland (II).

Air-dried plant material was extracted in 80% methanol (MeOH) (aq) under sonication according to Rauha et al. (2000). Different species as well as different parts of plants i.e. flowers, leaves, stems and roots were extracted separately.

4.1.3 Coumarin Library (III)

From the studied coumarin library (Fig. 5, Table 1) coumarins **1–24** are of natural origin whereas coumarins **25–29** are synthetic derivatives of coumarins.

Table 1. The coumarin library and sources of the compounds.

Compound	Source
1 4-methyl-7-hydroxycoumarin (4-methylumbelliferone)	Extrasynthèse (Genay, France)
2 6,7-dimethoxycoumarin (scoparone)	Extrasynthèse (Genay, France)
3 6-Hydroxy-7-methoxycoumarin (isoscopoletin)	Extrasynthèse (Genay, France)
4 7,8-dihydroxy-6-methoxycoumarin (fraxetin)	Extrasynthèse (Genay, France)
5 6,7-dimethoxy-8-hydroxycoumarin (fraxidin)	Extrasynthèse (Genay, France)
6 7,8-dihydroxycoumarin (daphnetin)	Extrasynthèse (Genay, France)
7 8-hydroxy-7-methoxycoumarin (7-methyetherdaphnetin)	Extrasynthèse (Genay, France)
8 7-hydroxy-6-methoxycoumarin (scopoletin)	Sigma–Aldrich (St. Louis, MO, USA)
9 7- hydroxycoumarin (umbelliferone)	Sigma–Aldrich (St. Louis, MO, USA)
10 6-methylcoumarin (toncarine)	Extrasynthèse (Genay, France)
11 4-hydroxycoumarin	Sigma–Aldrich (St. Louis, MO, USA)
12 3-(1'-phenyl-2'-acetyلهyl)-4-hydroxycoumarin (warfarin)	Sigma–Aldrich (St. Louis, MO, USA)
13 6-methoxy-4-methylcoumarin	Extrasynthèse (Genay, France)
14 7-methoxy-4-methylcoumarin (4-methylherniarin)	Extrasynthèse (Genay, France)
15 coumarin	Sigma–Aldrich (St. Louis, MO, USA)
16 5,7-dihydroxy-4-methylcoumarin	Extrasynthèse (Genay, France)
17 6,7-dihydroxy-4-methylcoumarin (4-methylesculetin)	Extrasynthèse (Genay, France)
18 7,8-dihydroxy-4-methylcoumarin (4-methydaphnetin)	Extrasynthèse (Genay, France)
19 7-methoxycoumarin (herniarin)	Carl Roth (Karlsruhe, Germany)
20 6,7-dibromocoumarin-3-carboxylate	Research Chemicals LTD (Lancs, UK)
21 esculetin 6-β-D-glucoside (esculin)	Sigma–Aldrich (St. Louis, MO, USA)
22 6,7-dihydroxycoumarin (esculetin)	Sigma–Aldrich (St. Louis, MO, USA)
23 5-methoxypsoralen (bergapten)	Carl Roth (Karlsruhe, Germany)
24 8-methoxypsoralen (xanthotoxin)	Extrasynthèse (Genay, France)
25 coumarin 153	AcrosOrganics (New Jersey, USA)
26 coumarin 106	AcrosOrganics (New Jersey, USA)
27 coumarin 102	AcrosOrganics (New Jersey, USA)
28 coumarin 30	AcrosOrganics (New Jersey, USA)
29 coumarin 7	AcrosOrganics (New Jersey, USA)

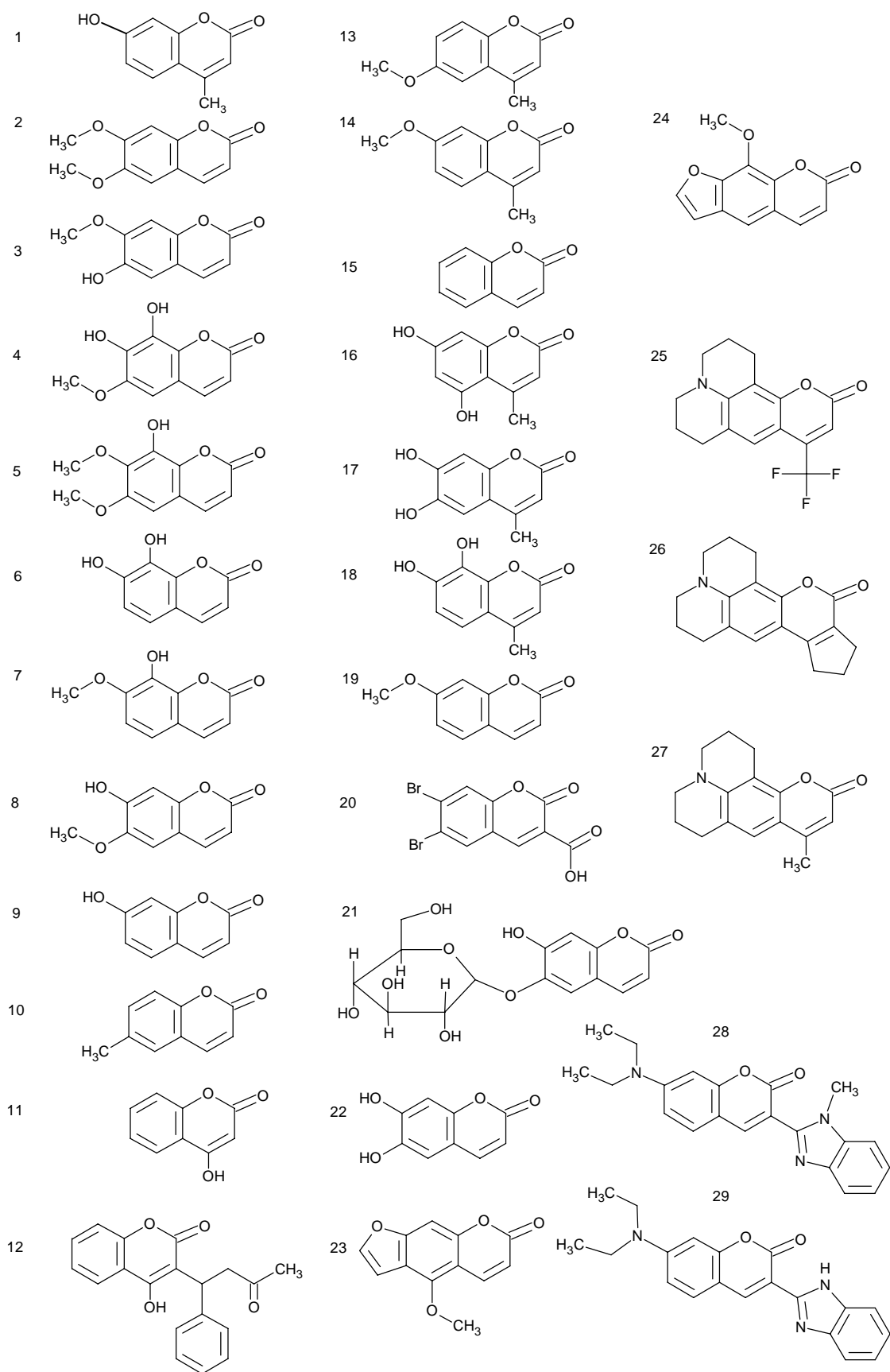


Figure 5. The structures of the screened coumarins in the coumarin library.

4.1.4 Synthetic Compound Library (IV)

Compounds used during the screening campaign of the synthetic compound library belonged to the commercial libraries of ChemDiv Inc (San Diego, US), ChemBridge Corporation (San Diego, US) and Tripos (St Louis, US). Altogether, 56,320 compounds were assayed for AChE inhibitory effect.

4.2 Enzymatic Methods

4.2.1 Ellman's Reagent Based Photometric Microplate Assay (II, III, IV, V)

Ellman et al. (1961) developed an assay for measuring AChE activity in a photometric assay using cuvettes and micro-cells. They applied it to isolated enzymes, blood samples and other tissues. In this assay, acetylthiocholine is used as a substrate. The hydrolyzed product, thiocholine, further reacts with dithionitrobenzoate (also called Ellman's reagent) to form a yellow anion detectable at 412 nm. This assay has further been applied for BChE with butyrylthiocholine being the substrate. Ellman et al. (1961) utilized butyrylthiocholine as a substrate to compare several tissues for non-specific esterase activity.

We used the AChE inhibitory assay in 96-well (II, III) and 384-well plate format (IV, V). HPLC micro-fractionated extracts (II), and pure compounds (III) were assayed in a 96-well plate. For screening of the large synthetic chemical library in an automated environment, the AChE inhibitory assay needed to be miniaturized and validated for a 384-well plate format (IV).

The BChE inhibitory assay was used in a 96-well plate format (III, IV). Hits in the large synthetic chemical library screening for AChE inhibition were assayed also against BChE.

Ellman's method is the most applied method for detecting acetylcholinesterase activity (e.g., Ingkaninan et al. 2000, Di Giovanni et al. 2008). Several modifications of the assay has been introduced which vary in substrate concentration, reagent concentration, enzyme source, enzyme concentration, detection wavelength, detection time, incubation time, incubation temperature and so on (e.g., Munoz-Ruiz et al. 2005a, Adersen et al. 2007, Lee et al. 2007, Vinutha et al. 2007, Jang et al. 2008, Pan et al. 2008, Pereira et al. 2009, Tang et al. 2009). We compared different assay variants to see how the change in pipeting order and substrate concentration affects the potency evaluation (V).

Screening window coefficient (Z' -factor), signal to background ratio (S/B) (Zhang et al. 1999) and signal to noise ratio (S/N) (Bollini et al. 2002) were used to evaluate the quality of the assay performance (II, III, IV):

$$Z' = 1 - [(3 \times SD_s + 3 \times SD_b) / |X_s - X_b|]$$

$$S/B = X_s / X_b$$

$$S/N = (X_s - X_b) / (SD_s^2 + SD_b^2)^{1/2}$$

X_s and SD_s = average and standard deviation of the maximum signal i.e. absorption detected at the last measurement point in blank wells after initiation of the reaction, X_b and SD_b = average and standard deviation of the background i.e. the absorption at the first measurement point in blank wells after initiating the reaction. These parameters were utilized in the assay validation process and also as acceptance criteria to monitor changes in assay performance during the screening campaigns.

4.2.2 Bioautographical Assay on TLC (I, II, III)

Bioautography is a technique to couple a biological assay to TLC (Potterat and Hamburger 2006). The developed TLC plate is sprayed with an enzyme solution. After an incubation period, the substrate and reagent are applied to the plate and inhibition zones are detected. The same technique is applicable also for detection of anti-microbial compounds where a developed TLC plate is dipped into a microbial suspension, and incubated after which microbial growth can be detected.

For screening of AChE and BChE inhibitors the method developed by Marston et al. (2002) was followed. Briefly, cholinesterases catalyze the hydrolysis of 1-naphthyl acetate (substrate) after which the degradation product reacts with Fast Blue Salt B to form a purple azo dye. Inhibitors are detected as white spots against a purple background. The assay can be utilized to screen both acetylcholinesterase and butyrylcholinesterase inhibitors. Detection limits for the known AChE inhibitors physostigmine and galantamine as well as for the fungal compound isolated from the extract of *Piptoporus betulinus* (Bull.: Fr.) P.Karst were calculated from the densitograms created using VideoScan software (Camag, Muttenz, Switzerland). Usually evaluation of the detection limit has been done visually as the lowest detectable inhibitory band.

This bioautographic assay was utilized for primary screening of fungal extracts (I), plant extracts (II), and coumarins (III) for AChE and BChE inhibition.

4.3 Separation and Analytical Methods

4.3.1 HPLC Micro-Fractionation of the Extracts (II)

An automated fraction collector (Gilson FC 204, Middleton, WI, USA) coupled with HPLC equipment (Perkin Elmer, Norwalk, CT, USA) was used to separate and collect compounds from extract directly into 96-well plates (Nunc, Roskilde, Denmark) with a collection time of 0.3 min/well (II). HPLC separation was achieved using a Supelcosil™ LC-18 column (250×4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA). The mobile phase, consisting of acetonitrile (ACN) (B) and water, was used at a flow rate of 1 ml/min. The gradient profile applied was: 0–30 min linear gradient from 5% to 95% B and 30–50 min isocratic 95% B. Injection volume was 20 μl, sample concentration 10 mg/ml and detection wavelength 255 nm. The positive control for the acetylcholinesterase assay, physostigmine in MeOH (0.5 μg/well, $n = 12$), was added to the plate and the solvents were lyophilized (HETO LyoPro 3000, Heto-Holten A/S, Denmark). For identification the compounds were collected from the plate before lyophilisation.

4.3.2 TLC (I, II, III)

TLC plates (DC-Alufolien Kieselgel 60 F254 Silica, Merck) were eluted first with acetone in order to wash them. The separation was carried out using CHCl₃: MeOH: 2.5% acetic acid (aq) (65:35:5) (I, II, III) or CHCl₃: MeOH: 0.1% acetic acid (aq) (65:35:5) (I) as an eluent.

4.3.3 LC-MS (I, II)

I) The HPLC-DAD-ESI-HRMS instrument (HRMS, high-resolution mass spectroscopy) used was an Agilent 1200 HPLC-system (Agilent Technologies, Waldbronn, Germany) connected to a Bruker micrOTOF-Q instrument (Bruker Daltonics, Bremen, Germany). The column used was a LiChrocart Purospher RP-18 (250×4.0 mm, 5 μm, Merck, Darmstadt, Germany). The mobile phases in the HPLC gradient consisted of 10 mM NH₄COOH in water and MeOH. The flow rate 0.7 ml/min was split 1:4 (0.14 ml/min to the MS instrument). The UV/VIS DAD data was recorded with an Agilent G1315C Starlight instrument (Agilent Technologies) in the wavelength range 190–950 nm. Negative ionization mode was used.

II) The samples were separated first with an Agilent 1100 Series LC equipped with a diode array detector and directly connected to MS detector (Agilent MSD Trap XCT Plus mass spectrometer). A Zorbax SB-C18 column (50×2.1 mm, 3.5 μm, Agilent Technologies, CA, USA) was used and eluted with ACN and water at a flow rate of 0.3

ml/min. 0.1% formic acid in ACN was added to the eluent before ionization at a flow rate of 0.05 ml/min. Electrospray ionization (ESI) was performed in positive ion mode.

4.3.4 NMR (I)

NMR spectra were acquired using a Bruker Avance 500 spectrometer (equipped with BBO-5mm-Zgrad probe) operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C . Spectra were recorded at 25°C using methanol- d_4 as a solvent. Proton and carbon spectra were referenced internally to tetramethylsilane (TMS) signal using value 0.00 ppm.

^1H and ^{13}C proton-decoupled NMR spectra were acquired with single-pulse excitation and 30° flip angle. Number of scans for carbon spectrum was 21200. Gradient selected ^1H - ^{13}C HSQC spectrum was acquired with hsqcetgpsisp.2 pulse program using shaped pulses (pulse programs refer to original ones installed by Bruker) with 34 scans. Gradient selected ^1H - ^{13}C HMBC spectrum was acquired with hmbcgp1pndqf pulse program with 260 scans.

5 Results and Discussion

5.1 Ellman's Reagent Based Photometrical Microplate Assay

Ellman's photometric assay to measure AChE activity was utilized in both 96-well plate (II, III) and 384-well plate formats (IV, V) in this contribution. The assay is applicable in miniaturized form for large scale high-throughput screening purposes for pure compounds but it is also a suitable platform for fractionated and crude extracts in 96-well plates. Possibility to modify the assay is an advantage in assay adaptation to different screening platforms but at the same time several modifications make comparison of the results in the literature close to impossible. The modifications in the assay variables are evaluated and discussed.

5.1.1 Adaptation of the Assay for Screening of AChE Inhibitors

The assay performance was validated and monitored using the statistical parameters Z' , S/N and S/B (Zhang et al. 1999, Bollini et al. 2002). The same parameters were utilized during screening with acceptance limits of $Z' \geq 0.5$, $S/N \geq 10$ and $S/B \geq 4$ (II, III, IV). The limits were set to ensure robustness and reproducibility of the assay as well as reliable identification of true hits. If conditions changed during screening for any reason it could be detected as a decrease in the values. Such changes could be technical, chemical or biological, e.g., due to incorrect pipeting volumes, errors in plate reading or mistakes in the concentrations of assay reagents. The screening window coefficient (Z') and signal-to-noise value (S/N) are more reliable indicators of assay conditions than S/B because the values are affected if variation increases between individual wells but also if the error is more systematic and occurs on the whole plate. Drift in side wells of a microplate would be detected if assay design was such that control wells were located on both side wells and middle wells. In the large screening campaign of a chemical library two columns on each side of a 384 plate were left for solvent controls and positive control, in this case DMSO (in 23 and 24 columns) and a known AChE inhibitor physostigmine (in 1 and 2 columns) (IV).

The selected variables – mostly measurement time, reaction order and substrate concentration – were evaluated in this contribution. The effect of measurement time on assay reliability was evaluated by means of statistical parameters (Table 2). According to the set acceptance limits for Z' , S/B and S/N, the assay was reliable five minutes after starting the reaction if the reaction was initiated with the enzyme (at ATCI concentration 1.5 mM). In the literature, measurement times are often shorter than that: e.g. 2 minutes (Chattipakorn et al. 2007, Langjae et al. 2007) or 1.7 minutes (Adersen et al. 2007) (Appendix 1). The question is: can their modification of the method discriminate background variation from the signal? The authors have not discussed this issue in their publications. Short measurement times reflect to low S/B values. The Z' value is directly

linked to S/B and the coefficient of variance (CV) (Sui and Wu 2007). Subsequently, in case of a low S/B value, a low CV is required for acceptable assay performance. A measurement time shorter than 2 minutes may be acceptable but great attention should be paid to the deviation. In our contributions, we used 8 minutes as the measurement interval to ensure good separation between the background noise and the signal. From a practical point of view, this measurement interval allowed reading of the whole 96- and 384-well plates at a time and dispensing of the solution that initiated the reaction using the microplate reader which was important for the kinetic measurements.

Table 2. The effect of measurement time on statistical parameters Z', S/B and S/N in a 96-well plate format. Assay conditions: reaction initiated with AChE (Reaction order 1), ATCI 1.5 mM, $n = 94$. Grey area represents values that would be accepted according to the acceptance limits: $Z' \geq 0.5$, $S/N \geq 10$ and $S/B \geq 4$.

Measurement time (min)	Average change in absorbance	sd	CV	Z'	S/B	S/N
1	0.35	0.03	0.081	-0.02	1.61	4.03
2	0.48	0.04	0.076	0.39	2.22	6.57
3	0.62	0.04	0.068	0.55	2.83	8.75
4	0.75	0.05	0.067	0.62	3.46	10.08
5	0.89	0.06	0.063	0.67	4.09	11.45
6	1.03	0.06	0.056	0.72	4.70	13.50
7	1.15	0.05	0.046	0.77	5.29	16.30
8	1.28	0.05	0.042	0.80	5.87	18.96
9	1.40	0.05	0.038	0.82	6.43	21.01

When conducting the Ellman's reagent based assay, the enzymatic reaction can be initiated either with the enzyme (AChE, reaction order 1) or the substrate (ATCI, reaction order 2). Both variants of the assay have been largely applied in the literature without further evaluation of the effect on results, e.g., reaction order 1 (e.g., Ingkaninan et al. 2000, Adsersen et al. 2007, Chattipakorn et al. 2007, Rhee et al. 2003, Papandreou et al. 2009) and reaction order 2 (e.g., Kwon et al. 2007, Zhu et al. 2009, Atta-ur-Rahman et al. 2001, Liston et al. 2004, Pietsch and Gutschow 2005, Girisha et al. 2009). We evaluated these variants in more detail to confirm good assay performance and reliable detection of true hits in screening (Fig. 6, Table 3, Fig. 1 *in IV* and Table 1 *in IV*).

Both reaction orders were validatable for screening (Table 3). Some differences were found that favoured the reaction order 2. The reaction order 2 had a wider signal window than reaction order 1. This is largely due to the spontaneous non-enzymatic hydrolysis of the substrate before initiating of the enzymatic reaction (Ellman et al. 1961) which

increases variation and background value if substrate solution and reagent solution are mixed before the enzyme is added (Fig. 6, Table 3).

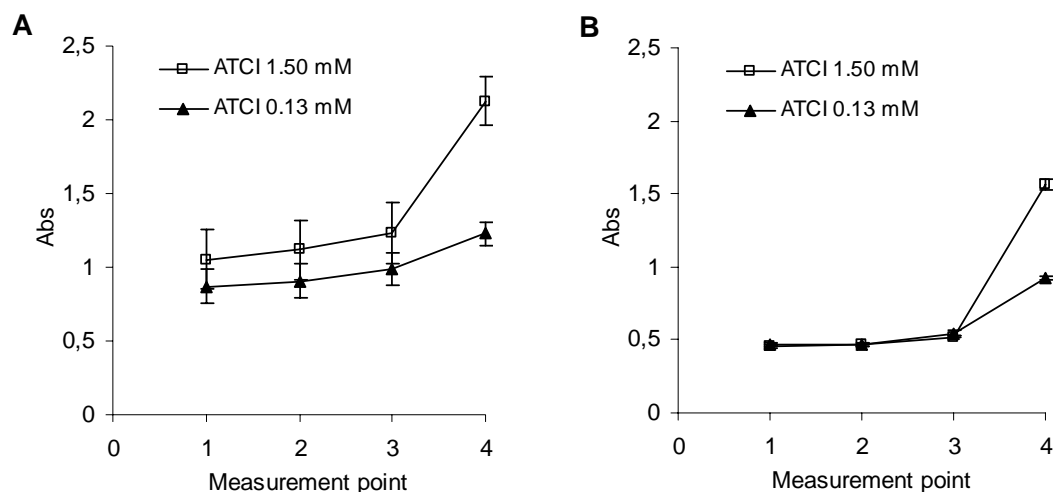


Figure 6. The effect of reaction order on spontaneous hydrolysis in a 384-well plate format. The first two measurement points are background measurements. The reaction was initiated just before the third measurement point either with AChE (reaction order 1) or ATCI (reaction order 2). The kinetic interval was 8 minutes. The values are means and standard deviations ($n = 72$) (Table 3). A) Reaction order 1: Spontaneous hydrolysis of substrate without enzymatic catalysis increase the measured background values but also variation if the substrate solution is mixed with the Ellman's reagent before addition of the enzyme. Standard deviation was up to 18.9%. B) Reaction order 2: The assay was robust using reaction order 2 because the standard deviation was less than 3% for all measurement points.

With a 1.5 mM substrate concentration, the change in absorbance and accordingly the signal window, is greater than with a substrate concentration of 0.13 mM which can be seen as an increase in Z' values in both variants (Table 3). Z' values for a substrate concentration of 0.13 mM are also above 0.5 which is considered as the limit for an excellent assay (Zhang et al. 1999). Nevertheless, percentual deviations for reaction order 1 and reaction order 2 at this substrate concentration (13.5 and 11.10%, respectively) are significantly higher than with the substrate concentration 1.5 mM (4.60 and 1.66%, respectively). If the signal window is narrow the assay becomes sensitive to deviation which can lead to high rejection rates of plates in a screening campaign.

Table 3. The effect of reaction order on signal window coefficient (Z') and measurements at several measurement points in a 384-well plate format. Reaction order 1: the reaction is initiated with the enzyme (AChE). Reaction order 2: the reaction is initiated with the substrate (ATCI). $n = 72$.

		Z'	Measurement point			
			b1	b2	s1	s2
Reaction order 1 (ATCI 1.5 mM)	average	0.81	1.05	1.12	1.23	2.13
	sd	0.04	0.20	0.20	0.21	0.17
	CV	0.046	0.189	0.179	0.170	0.078
Reaction order 1 (ATCI 0.13 mM)	average	0.60	0.87	0.91	0.99	1.23
	sd	0.08	0.12	0.11	0.11	0.08
	CV	0.135	0.133	0.125	0.115	0.064
Reaction order 2 (ATCI 1.5 mM)	average	0.89	0.46	0.46	0.52	1.57
	sd	0.01	0.01	0.01	0.01	0.04
	CV	0.017	0.022	0.018	0.013	0.023
Reaction order 2 (ATCI 0.13 mM)	average	0.71	0.46	0.47	0.55	0.92
	sd	0.08	0.01	0.01	0.02	0.01
	CV	0.111	0.029	0.023	0.039	0.015

Z' : signal window coefficient

b1: change in absorbance at the 1st background measurement point

b2: change in absorbance at the 2nd background measurement point

s1: change in absorbance at the 1st signal measurement point

s2: change in absorbance at the 2nd signal measurement point

5.1.2 Effect of Assay Variant on Potency of Inhibition

Assay modification was found to affect the potency evaluation of inhibitors with different kinetic properties and inhibitory mechanisms (Table 4, Fig. 1 *in V*). The structures of the studied compounds are presented in Fig. 7. When initiating the reaction with the substrate (reaction order 2), inhibitors are pre-incubated with the enzyme during the background measurements which is not the case with the reaction order 1. Therefore, reaction order 2 favours the binding of slow-binding inhibitors, which is detected as an increase in their potencies. In practice, pre-incubation means that the screened compound is in contact with

the enzyme before the substrate is added. Physostigmine forms a complex with AChE slowly even though its K_i value (K_i , dissociation constant for an inhibitor for binding to the enzyme) is small (Stojan and Zorko 1997). It has been defined as slow reversible or pseudo-irreversible inhibitor because it forms a carbamylated complex with the serine residue in the catalytic triad of AChE which is hydrolyzed slowly thus, pre-incubation increases its potency (Camps and Munoz-Torrero 2002, Darvesh et al. 2003b, Augustinsson and Nachmansohn 1949, Darvesh et al. 2008). Accordingly, the IC_{50} value for physostigmine decreased when the reaction order was changed from order 1 to order 2 at substrate concentration of 1.5 mM by 91-fold and at a substrate concentration of 0.13 mM by 21-fold (Table 4, Fig. 1 *in V*). Sugimoto et al. (1990) observed even more substantial change (decrease in IC_{50} nearly 500-fold) with 60 minutes pre-incubation time when utilizing mouse brain homogenate as the enzyme source with a substrate concentration of 0.5 mM. In addition, competitive inhibitors, such as physostigmine, bind to the same site as the substrate (the catalytic site) and therefore increasing the substrate concentration increases the IC_{50} value without pre-incubation. This lowering of physostigmine's potency was detected at the higher substrate concentration when reaction order 1 was applied.

Table 4. IC_{50} values (μM) for the three AChE inhibitors (tacrine, physostigmine and E017-0008) using two assay variants and two substrate concentrations. The reaction was initiated either with the enzyme (AChE) or the substrate (ATCI). The effect of substrate concentration was assessed using concentrations of 0.13 mM and 1.5 mM. The values are averages \pm standard deviations of three independent assays. For each compound, statistical comparisons of the potencies were made using one-way ANOVA and Tukey post test: statistical difference between the two substrate concentrations (a) and between the two reaction orders (b) ($p < 0.05$). The change in assay conditions did not affect IC_{50} of tacrine. Both changes – the substrate concentration and the reaction order – affected IC_{50} of physostigmine, whereas only the substrate concentration affected IC_{50} of E017-0008.

	ATCI concentration (mM)	Reaction initiated with:	
		AChE (IC_{50} , μM)	ATCI (IC_{50} , μM)
Tacrine	0.13	0.063 ± 0.013	0.065 ± 0.021
	1.50	0.071 ± 0.008	0.071 ± 0.003
Physostigmine	0.13	$0.581 \pm 0.060^{a,b}$	0.025 ± 0.002^b
	1.50	$1.809 \pm 0.175^{a,b}$	0.020 ± 0.0005^b
E017-0008	0.13	0.013 ± 0.004^a	0.008 ± 0.002^a
	1.50	0.022 ± 0.0008^a	0.021 ± 0.001^a

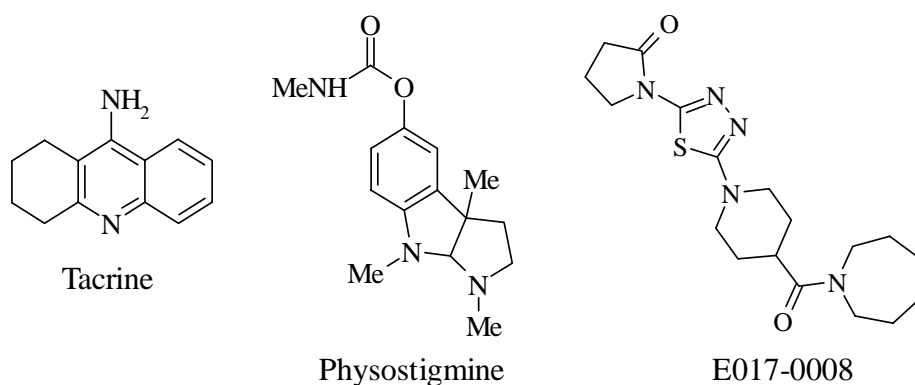


Figure 7. The structures of the AChE inhibitors used for potency assessments.

Potency of tacrine (Table 4, Fig. 1 *in V*) did not significantly change with the two substrate concentrations or the order of reagent addition which is in agreement with tacrine's kinetic mechanism of a reversible mixed-type inhibitor with a strong non-competitive component, i.e. the inhibitor binds with equal affinity to the free enzyme and enzyme-substrate complex (Berman and Leonard 1992). Nochi et al. (1995) and Pietsch et al. (2009) have determined α' values for tacrine to be 1.1 and 1.4, respectively. α' is a factor which indicates how one ligand affects another ligand's binding affinity. An α' value = 1 indicates to a pure non-competitive mode of action. For a non-competitive inhibitor, the IC_{50} is independent of the substrate concentration and corresponds to the K_i (Macarron and Hertzberg 2009). Tacrine is not regarded as a slow-binding inhibitor thus pre-incubation with the enzyme does not affect tacrine's inhibitory effect (Al-Jafari 1996, Ogura et al. 2000). Accordingly, the reaction order did not affect the potency of tacrine.

One of the hits in the large screening campaign (E017-0008, ChemDiv Inc, San Diego, US) was further investigated with both reaction orders to see if a similar pattern of inhibitory behaviour to physostigmine or tacrine could be detected (Table 4, Fig. 1 *in V*). The reaction order had no effect upon potency but a statistically significant difference was detected between substrate concentrations. This behaviour indicates to a reversible competitive inhibitor but most probably with mixed mode of inhibition. It would require more detailed study to make any further conclusions.

Knowing how substrate concentrations and the order of initiating the reaction affect the potency determination of AChE inhibitors allows the exercising of better judgement when selecting the optimal assay for large primary screening campaigns. In terms of substrate concentration, if the selected $[S]/K_m$ ($[S]$, substrate concentration; K_m , Michaelis constant, the substrate concentration at which the enzymatic reaction rate is half of the maximum velocity (V_{max})) ratio is lower than 1 the assay becomes more sensitive to competitive inhibitors, reaching an asymptotic limit in which IC_{50} would be equal to K_i (Cheng and Prusoff 1973). On the other hand, increasing the $[S]/K_m$ ratio makes the assay

more sensitive to uncompetitive inhibitors, which do not bind to the free enzyme. Thus, for screening purposes, the rationale of keeping the $[S]/K_m$ ratio close to 1 would be to guarantee the sensitivity of the assay to all types of inhibitors. Often, however, substrate concentrations need to be increased to achieve appropriated assay performance. In that regard, a high $[S]/K_m$ ratio (i.e. up to 10) may be necessary to improve the assay signal window. On the other hand, it is apparent that slow-binding inhibitors would need pre-incubation with the enzyme prior to the starting of the reaction to get comparable results with fast-binders, so to ensure as few false negative hits as possible. Thus, choosing to start the reaction with the substrate for large screening campaigns would favour the robustness and stability of the assay, while providing a more reliable hit rate for both slow- and fast- binding inhibitors.

Several other modifications of the assay including differences in temperatures, pre-incubation times, pHs and reagent concentrations are found in the literature (Appendix 1). In addition, the data were conducted with enzymes from human, invertebrate and mammalian sources, under different enzyme concentrations and not all the conditions were even mentioned, which makes comparison of the results in the literature close to impossible. The conditions for a certain screening campaign have to be validated to be able to discriminate hits from background noise. If the results are to be used for further *in silico* structure-activity determinations in combination with results from the literature careful consideration of the assay format should be paid to create comparable results. As an example a reversible competitive inhibitor of AChE, galantamine (Camps and Munoz-Torrero 2002), have been reported to have IC_{50} value of 3.18 μM (Lee et al. 2007), 0.88 μM (Papandreou et al. 2009) or 1.4 μM (Adersen et al. 2007) when initiating the reaction with the enzyme and 0.55 μM (Tang et al. 2009, Wen et al. 2008) or 1.82 μM (Berkov et al. 2008) when initiating the reaction with the substrate. Changes in assay conditions affect half-inhibitory concentration of inhibitors with various modes of inhibition but not to the extent of pseudo-irreversible carbamates.

Like physostigmine, rivastigmine belongs to carbamate family of inhibitors and has a pseudo-irreversible mode of inhibition on AChE (Darvesh et al. 2008, Groner et al. 2007) which at least partially explains the extensive variation in the reported IC_{50} values in the literature, e.g., 0.03 μM (Rizzo et al. 2008) and 1.535 μM (Bolognesi et al. 2004). Groner et al. (2007) addressed the question of comparing IC_{50} values for carbamates and pointed out that the relationship between structure and therapeutic efficacy correlates better to individual rate constants between inhibitor and enzyme than IC_{50} values. Nevertheless, structure-activity relationship studies of AChE inhibiting carbamates found in the literature are conducted by comparing IC_{50} values (e.g., Bolognesi et al. 2004, Yu et al. 2001, Sterling et al. 2002, Bartolucci et al. 2006, Takahashi et al. 2010a, Takahashi et al. 2010b). In general, a time-dependent inhibition mode is considered disadvantageous in biochemical screenings (McGovern et al. 2002, Rishton 2003). This mode of action leads to false-positive hits that most probably act on several targets. They are classified as non-lead-like compounds that should be assayed in biological assays and not in large scale biochemical assays. Many times the time-dependent activity is due to non-specific

interactions such as aggregation, covalent binding, or electrophilic protein-reaction. The mechanism by which small aggregating compounds inhibit enzymes non-specifically in aqueous solutions is by partial denaturation of the protein structure (Coan 2009). Rishton (2003, 2008) defined properties for a good lead-like binder to be: i) non-covalent binding with high affinity, ii) reversible, time-independent competitive binding and iii) tractability in structure-activity relationship of a series of structural analogues of the binder.

In case of AChE inhibitors, ruling out the time-dependent carbamates would leave out an important group of true hits. Consequently, as their mode of inhibition differs from fast binding compounds, they should be dealt with separately when conducting structure-activity relationship data. When an assay is designed to detect both fast and slow binding inhibitors, i.e., compounds are pre-incubated with the enzyme before initiation of the reaction, including all hits into the QSAR studies after determining IC_{50} values without further information on enzyme kinetics will most probably, decrease the predictive power of an *in silico* model. In other words, founding *in silico* predictions merely on IC_{50} values should be done cautiously and kinetic constants should be preferred if available. The time-dependent mode of inhibition cannot be evaluated based on IC_{50} values if just one reaction order is used which also highlights the importance of secondary studies.

When data are combined from several publications in the literature for *in silico* studies careful attention should be paid on the assay conditions because modifications of the same assay may produce a different order of activities for inhibitors with several modes of action. A poor correlation as determined from the Spearman's correlation coefficient for the reaction order 2 and both substrate concentrations is proof of a significant difference in assay modifications (Table 5). When using reaction order 1 changing the substrate concentration did not change the order of activities determined as IC_{50} values which correspond as Spearman's correlation coefficient = 1. The lowest correlation was detected between reaction order 1 and 2 with substrate concentration of 1.5 mM. If the order of potencies changed with the change in assay modifications it is obvious that the predictive power of the *in silico* model created using IC_{50} values determined with different modifications of the assay would be poor.

Table 5. Spearman's correlation coefficients for order of activities (IC_{50}) for tacrine, physostigmine and compound E017-0008, when comparing two reaction orders and two substrate concentrations. Correlation coefficient = 1 means that changes in variables does not change the order of activities for the three compounds i.e. when using reaction order 1 E017-0008 had the lowest IC_{50} with both substrate concentrations and so on. When using 1.5 mM substrate concentration and reaction order 1, E017-0008 had the lowest IC_{50} value whereas with reaction order 2, physostigmine had the lowest IC_{50} value which reflects as a poor Spearman's correlation coefficient (-0.5).

	X_i	Y_i	Spearman's correlation coefficient
Reaction order 1	ATCI 0.13	ATCI 1.5	1
Reaction order 2	ATCI 0.13	ATCI 1.5	0.5
ATCI 0.13	Reaction order 1	Reaction order 2	0.5
ATCI 1.5	Reaction order 1	Reaction order 2	-0.5

5.2 Bioautographical Assay on TLC

5.2.1 Evaluation of TLC Bioautography for the Screening of AChE and BChE Inhibitors

Bioautographical AChE and BChE inhibitory assays were successfully applied for the primary screening of fungal extracts (I) and *Mentha* extracts (II). The method is straight forward and relatively fast after the TLC separation of compounds within the extracts. TLC bioautography couples analytical separation with a bioactivity assay therefore it is a good method to study extracts. Different *Mentha* species and parts of *Mentha* plants were extracted and assayed separately (Table 1 in II). The separation of compounds on TLC before the enzymatic assay enables comparison of the active bands between species as well as between parts of plants. The assay is more sensitive than the Ellman's reagent based 96-well plate assay (Rhee et al. 2001). The detection limit for galantamine in the Ellman's reagent based assay for AChE in the 96-well plate format has been reported as 1 μ M which correlates to 250 pmol/well whereas the calculated detection limit on the TLC using method of Marston et al. (2002) was 280 fold lower (Fig. 3 in I). High detection capacity is fundamental for the screening of extracts because selective detection of active principles among mixtures even at low concentrations ensures good screening efficiency.

Most often in the TLC bioautography, the enzyme activity has been determined as a detection limit that is visually evaluated from the dilution series of concentration (e.g., Marston et al. 2002, Di Giovanni et al. 2008). As an example, linarin showed concentration dependent inhibitory effect for AChE with a detection limit of 16.8 pmol /band (II). No inhibitory effect for BChE was detected. The detection limit for linarin was evaluated visually. Dependency of the value on the selected concentrations makes this

visually detected value an estimate. Densitometric determination of enzyme inhibitory effect utilizing VideoScan software was developed to calculate detection limits more accurately for AChE and BChE inhibitors (I). With this method the detection limit is based on multiple concentration points. Logarithmic correlation of concentrations to inhibitory band intensities determined as an area of the peak in the densitogram was used to create a formula from which a more accurate value was calculated.

VideoScan software uses images captured by a CCD-camera (charge coupled device camera) and VideoStore software for creation of the densitograms. Image analysis is not as sensitive a method for quantitative TLC compared to the measured densitograms. For the evaluation of the intensity and size of the enzyme inhibitory band, this less sensitive method is more suitable because spraying of the enzyme, substrate and reagent solutions to the TLC-plate leads to an uneven background. VideoScan software enables the manual editing of the baseline which is critical for the detection of inhibitory bands against the uneven background colour. The same plates could be measured densitometrically before the solutions for the enzymatic reaction are sprayed, and quantitative measures could be used for dose-response determinations of known compounds. Efficacy evaluation of the inhibitors in the extract was not as straight forward because the compounds in the extracts were not known.

Evaluation of the kinetic parameters has to be conducted in a photometric assay, either using the Ellman's reagent based assay or Fast blue salt B assay in a micro-well plate format (Di Giovanni et al. 2008). In general, it is advantageous to use two methods to make sure that the shown activity is due to a real enzyme catalyzed reaction and not due to inhibition of the reaction between the hydrolyzed substrate and the reagent (Rhee et al. 2003). Some aldehydes and amines have been shown to give false positive results in the Ellman's reagent based assay on the TLC and in the 96-well plate.

5.3 Coupling Cholinesterase Inhibitory Assays and Analytical Techniques for the Screening of Extracts

5.3.1 Tracking of Bioactive Compounds in Extracts

The biggest advantage of coupling separation techniques to a bioactivity assays is the information that helps to associate the detected bioactivity with a peak in a chromatogram. Traditionally, the active compounds in the extracts have been tracked with bioactivity guided fractionation processes. The loss of activity during the isolation and purification processes is possible, particularly, if the concentration of the active compound in the extract is low (Hamburger 2003). If the activity is lost during the long process and no analytical data are available it is impossible to go backwards in the process. Coupling of separation to bioactivity assay enables usage of several detection methods on-line or off-

line which fastens the identification of interesting compounds in the extracts. Splitting of eluent after HPLC separation enables simultaneous detection of separated compounds in the extract using on-line UV detector as well as MS and NMR spectrometers (Potterat and Hamburger 2006). In addition, a part of the eluent is directed to a fraction collector. After assaying the fractions, the activity data of the most active fractions is combined with the analytical and spectroscopical data. Preliminary structural information on the active compounds is created during one run.

The crude extracts are complex mixtures of tens or even hundreds of compounds. A large part of the compounds in any extract are most probably known. A part of the compounds within an extract are totally new whereas a compound may be identified from the certain species for the first time even if it is known in another species. Fast identification of known compounds should be achieved without laborious separation steps. Using time and labour for the isolation of a compound that turns out to be a known compound has traditionally been one of the pitfalls in natural product screening (Hamburger 2003).

Even if a compound is known it still may be interesting because new activities may be found for known compounds. At best, the identification of the known compounds can be achieved without further isolation based on UV and MS detection methods and with the help of literature. This was the case in the identification of linarin in the *Mentha* extract (II). After HPLC separation and micro-fractionation of the *Mentha arvensis* flower extract, inhibitory effect was associated with one peak in the chromatogram which was identified as linarin in the following LC-MS-MS analysis conducted using the same separation conditions as in the fractionation. In addition, the compound was commercially purchased as a pure compound and the inhibitory effect was confirmed on the TLC. In addition, the fragmentation was confirmed in MS-MS experiments.

When the activity has been tracked to a certain peak in the chromatogram and the separation system is robust, repeated runs with a fraction collection can be utilized for the isolation of the compound. Optimization of the separation method should be done carefully because a robust method can be utilized in both the fractionation of the extract at the analytical scale and the isolation of the active compound (Wennberg et al. 2004). Optimization of the separation can be improved with the help of computational programs like DryLab (Molnar Institute) to achieve baseline separation that allows the increasing of the concentration of the injected sample and/or the injection volume to affect the efficacy of the isolation. An optimized separation can be scaled-up to a semi-preparative or preparative system.

Fractionation of the extract prior the bioactivity assay reduces the complexity of samples (Potterat and Hamburger 2006, Wagenaar 2008). The crude extracts are mixtures of tens of compounds which can mask each other's activities whereas viscosity and colours can interfere with the detection process. Fractionation lowers the viscosity of samples which results in improved physical properties making liquid handling more

accurate, especially at the microlitre scale. In general, fractionation increases the quality of the extract library which in turn improves the possibility to detect activity in the extracts.

In TLC bioautography, the activity is traced to a certain band in the separated extract. Further identification steps are often laborious especially if another separation system such as preparative HPLC needs to be optimized for isolation. To get past this step, the active compound in the *Piptoporus betulinus* extract was isolated directly from analytical TLC plates (I). Good separation of the compounds of the extract on TLC and high enough concentrations of the active compound in the extract enabled the isolation. Extraction of the compound from TLC was relative laborious because 100 plates were extracted to get 12 mg of pure compound for MS and NMR analyses. TLC-MS interface equipment could have been attached directly to the MS but further isolation was required for NMR analysis. GC-MS analysis is another technique that may be used for the identification of compounds on the TLC. As an example, the compounds possessing AChE inhibitory effect in the extract were scratched off the TLC plate and extracted from the silica with MeOH (Berkov et al. 2008). Dissolved compounds were analysed by GC-MS and identified as galantamine derivatives.

5.3.2 The Effect of Solvents on Fractionation and Enzyme Activity

Coupling an enzymatic assay to analytical techniques requires, most of all, studies on the effects of solvents upon the enzyme activity. Analytical solvents are evaporated before proceeding on to the bioactivity assay but some traces of the solvents can still be present especially on the TLC-plate but also in the wells of the micro-well plate. AChE and BChE were found to be sensitive to low pHs which narrows down the selection of useable eluents in HPLC and TLC when coupling separation techniques to bioassays (I, II). Formic acid could not be used even at low concentrations in the HPLC elution because the decrease in enzymatic activity could be detected when fractions were assayed. Solvents were evaporated from the microplate before the enzymatic assay but traces of acid in the wells led to increased deviation between wells. Consequently, gradient eluent used in the separation and fractionation of compounds in *Mentha* extracts consisted of water (A) and acetonitrile (B) (II). In this case, the peak of the active component, linarin, was relatively sharp with minimum tailing which enabled fraction collection according to time (0.3 min/well with flow rate of 1 ml/min). If peaks are wide and the compound disperses between several wells the concentration of the compound in each fraction decreases which may lead to difficulties in detecting bioactivity and correct hit identification. In this project, assaying the fractionations was a secondary method for identification of the active compound because it was already known based on the TLC bioautography that the *Mentha arvensis* flower extract contained a selective AChE inhibitor. Nevertheless, the same fractionation approach could be used to create a pre-fractionated extract library for primary screening purposes.

Gradient eluents consisting of e.g. 0.005% trifluoroacetic acid (TFA) (aq) (A) and 0.005% TFA in MeOH (B) have been used in the generation of pre-fractionated natural product libraries (Wagenaar 2008). The library plates were used in screening of compounds for kinase and ion channel activities and no effects of the solvents on the assays were reported. Similarly, 0.05 % TFA (aq) (A) and ACN (B) in the gradient elution did not affect an antimicrobial or cytotoxicity assay (Lang et al. 2006). Usage of acids or low pH buffers in eluents often sharpens the peak shape of ionizable elutes which is advantageous for fractionation (Wagenaar 2008). If an eluent contains any acid, the effect on the target should always be assessed, whether the target is an isolated enzyme or an intact cell. In an ideal situation, each fractionated compound would be in a separate well at a high enough concentration which may be achieved if peaks are sharp. In reality, the goal in natural product library generation for pre-fractionated extracts is 1-5 compounds per well which can be further analysed and separated after tracking down the bioactivity to a certain well (Eldridge et al. 2002). If the separation is compromised, a proper concentration can be assured by using preparative LC for fractionation (Wagenaar 2008). On the other hand, more fractions correlate to more data points in screening which increases the workload extensively if hundreds of extracts are to be screened. In large screens, rough fractionation at a rate of ten fractions per extract increases the throughput in screening.

In the bioautographical assay for AChE and BChE on TLC, usage of acids in the eluent decreased the enzyme activity on the plate. Consequently, poor contrast of inhibitory bands against the coloured background was detected. Lowering the concentration of acetic acid in the eluent allowed its use (I, II). The total percentage of acetic acid in the eluent mixture which did not affect the detection of enzyme inhibitory bands on TLC was 0.1%. This percentage was not exceeded in the assays (I, II).

5.4 Cholinesterase Inhibitors from In-House Libraries

5.4.1 Fungal and Plant Extract Libraries

Extract screens require special attention in assay set-ups because extracts are complex mixtures of compounds possibly with very different chemical structures. TLC bioautography was found to be a sensitive method with low detection limits for AChE and BChE inhibitors. Primary screening of fungal and *Mentha* extracts on TLC resulted in detection of a high number of inhibitory bands. In further analyses, two new cholinesterase inhibitors were identified (I, II). The active compound in *Piptoporus betulinus* extract was directly isolated from TLC plates (I). The requirement of high compound amount for NMR resulted in identification of only one of the inhibitory compounds from fungal extracts. Isolation directly from the TLC plates was achieved because the inhibitory effect was associated to a large peak in the densitogram.

Consequently, the inhibitory effect was lower than hoped-for. Isolation of a minor peak would have been even more laborious but at the same time the potency of the compound might have been higher. It is likely that the isolated and identified compound, 2,5-dihydroxy- α -oxo-benzeneacetic acid, was not the most potent cholinesterase inhibitor in the screened fungal extracts.

Micro-fractionation of an extract into 96-well plate using HPLC leads to a set of low volume fractions that have a low number of compounds per fraction. Tracking the active principle in the *Mentha arvensis* extract was successfully executed after HPLC micro-fractionation (II). Tracking of the active compound to one peak in the chromatogram requires small fractions which were achieved by collecting the compounds according to time (0.3 min/well). Detection limits for AChE and BChE inhibitors are higher in micro-plates compared to TLC which limits the use of this approach.

Different *Mentha* species as well as different parts of plants were extracted and analysed for AChE and BChE inhibitory effect separately (II). The same active band was detected in several samples but the concentration varied which was detected as varying intensities on TLC. The comparison of related species reveals that there are differences in production of secondary metabolites between species. Also, the active compound was produced in all parts of plant. If the compound would be isolated from a plant in larger scale the selection of species as well as the part of plant for harvesting and isolation could be optimized for high yield. Natural supply of the bioactive compounds could be achieved.

5.4.1.1 2,5-Dihydroxy- α -oxo-benzeneacetic Acid Isolated from *Piptoporus betulinus* Extract

The dual cholinesterase inhibitor identified from *Piptoporus betulinus* (Bull.: Fr.) P.Karst was 2,5-dihydroxy- α -oxo-benzeneacetic acid (Fig. 8) (I). Only few literature reports of this compound exist. Crowden et al. (1967) have isolated it from basidiomycete fungus *Polyporus tumulosus* (Cooke). The compound showed dose-dependent inhibitory effect for AChE and BChE on TLC (Fig. 3 in I). The related structure, hydroquinone, inhibits AChE with an IC_{50} of 89 μ M (Wang et al. 2007). Its inhibitory effect was shown to be a true enzymatic effect because the compound did not inhibit the reaction between the substrate and the reagent in the assay for a false-positive effect.

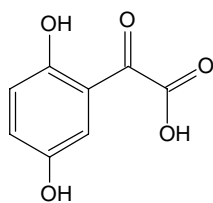


Figure 8. The structure of 2,5-dihydroxy- α -oxo-benzeneacetic acid, an AChE and BChE inhibitor isolated from a wood-rotting fungus *Piptoporus betulinus*.

Fungal extracts were rich sources of cholinesterase inhibitors because 115 of the assayed extracts showed an AChE inhibitory effect and 72 of the extracts showed a BChE inhibitory effect (I). Most of the detected inhibitory bands were selective for either of the enzymes but also 17 dual inhibitory bands were detected (Fig 1 *in I* and Table 2 *in I*). Comparison of the relative R_f values (R_f , retardation factor) of detected inhibitory bands in different extracts revealed that 2,5-dihydroxy- α -oxo-benzeneacetic acid is not a common secondary metabolite in the fungi. The inhibitory effect was moderate but the structure allows chemical modifications of the side chains that could be conducted to enhance efficacy against both AChE and BChE. The structure would be a good starting point for the creation of series of derivatives.

5.4.1.2 Linarin from *Mentha arvensis* Extract

Linarin (Fig. 9) identified from the *M. arvensis* flower extract inhibited AChE selectively because no BChE inhibition was detected on TLC (II). Linarin is a known natural compound isolated earlier from, e.g., *Valeriana officinalis* L. (Fernandez et al. 2004), *Calamintha grandulosa* (Req.) Benth (Marin et al. 2001), *Foeniculum vulgare* Mill. Var. *vulgare* (Parejo et al. 2004), *Chrysanthemum zawadskii* var. *latilobum* Kitamura (Compositae) (Singh et al. 2005) and *Buddleia cordata* HBK (Martinez-Vazquez et al. 1996, Martinez-Vazquez et al. 1998). It has been connected to biological activities, i.e., sedative and sleep enhancing properties (Fernandez et al. 2004), anti-cancer effect on prostate carcinoma cells (Singh et al. 2005), anti-inflammatory and antipyretic activities (Martinez-Vazquez et al. 1996, Martinez-Vazquez et al. 1998).

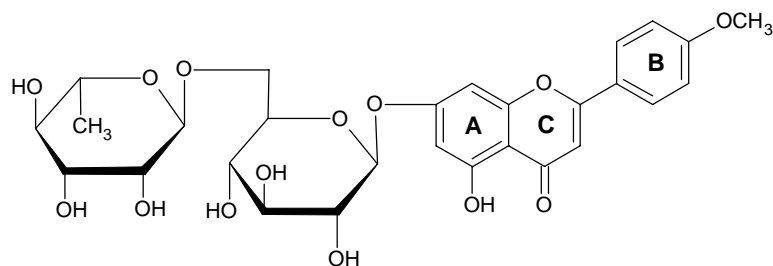


Figure 9. The structure of linarin showing the annotation of flavonoid rings (A-C).

Fan *et al.* (2008) studied 16 structurally related flavonoids to linarin and found that only tilianin is active against AChE in addition to linarin. The detection limit for tilianin for AChE on TLC is 90 pmol /band whereas the detection limit for linarin was 17 pmol /band (II). Tilianin's structure is similar to linarin except it lacks the rhamnoside moiety. Interestingly, fortunellin which differ from linarin only by an inter-glycosidic linkage is not active. Together with our findings it can be stated that a methoxy group in position 4' in the B-ring and a 7-*O*-sugar are essential structures for the observed AChE inhibitory effect.

Pectolarin acetates also inhibit AChE (Loizzo *et al.* 2008). Structurally, the pectolarin aglycone has an additional methoxy group in position 6 in the A-ring compared to linarin. Pectolarin acetates have an acetate group attached to the rhamnoside moiety through one of the OH groups at the position 2''', 3''' or 4'''. On the contrary, linarin acetate was not active (I). Linarin did not inhibit BChE (I) but some other flavonoids have been shown to have moderate BChE inhibitory effect such as quercetin with K_i of 3.8 μ M (Khan *et al.* 2009) and galangin with K_i of 6.9 μ M (Katalinic *et al.* 2010).

The active band in the *Mentha* extracts had the same R_f value (0.81) but different intensities which indicated to one active compound with varying concentrations in the extracts (II). Intensive inhibitory bands were detected in all parts of the plants but there were differences between species. An intensive inhibitory band could be detected in five out of seven flower extracts whereas only one of the root extracts showed the same intensive band. The varying concentrations between the parts of the plant was expected but not between species at the extent detected. All parts of *M. x gentiles* showed the clear inhibitory band whereas only the flower extract of *M. pulegium* showed any detectable inhibitory effect.

5.4.2 Compound Libraries

The pure compound libraries varied in size and origin (III, IV). Based on the literature, some of the coumarins were known to have cholinesterase inhibitory effects. Consequently, a selection of compounds having a coumarin scaffold to create a targeted coumarin library for screening of cholinesterase inhibitors was a straight forward strategy (III). Most of the compounds were of natural origin but also their synthetic derivatives were included in the library. Targeted libraries require low throughput in screening which leaves room for the assay selection and format. The hit rate for the coumarin library was 17.2% for AChE inhibitory effects. The likelihood of finding hits in a targeted library screening is higher than in a randomly selected compound library because the selection of compounds into the library is based on a prior knowledge and a defined criterion.

The random screen of a large library of synthetic compounds requires higher throughput in screening for which the Ellman's reagent based assay was miniaturized for 384-well plates and validated for an automated environment (IV). Overall hit rate was 0.67% of the screened 56,320 compounds. Hit rates for the two screens are not totally comparable because of the differences in concentrations and hit limits. The comparison of the hit-rates gives a general idea of the power of a targeted compound selection over random selection. The concentration of compounds in the assay plates in the synthetic compounds screen was 2.5 μM and hit limit 4 SDs from the average whereas in coumarin screen concentration was 5 μM and hit limit 3 SDs from the average. The hit limit should be strict for the large scale screens because even a low hit rate may result in tens or hundreds of hits. Hit rate as low as 0.67% resulted in 350 hits in the synthetic compound screen which led in a high number of data points in the secondary screening. In the large screening campaign, the compounds were transferred from the library plates to the assay plates using a pin-tool which is not as precise tool as a pipet. The pins are dipped into a solvent (DMSO) containing the compounds and then dipped into the assay buffer. The amount of the sample that is transferred from one well to another was assessed to be 0.14 μl . The variation between wells was 7–10%. The pins are washed and re-used which makes it a cost-effective technique when thousands of compounds are screened. The variance in transfer was another reason why the hit limit was set 4 SDs from the average instead of 3 SDs.

5.4.2.1 Hits in the Coumarin Library

The coumarin library that contained natural coumarins and their synthetic derivatives was screened for AChE and BChE inhibitory activities (III). Synthetic coumarin derivatives were identified as hits at 5 μM concentration against AChE with coumarin 106 being the most active one (compounds 25-29, Fig. 1 *in* III) (Fig. 10). The IC_{50} value for coumarin 106 was 10.4 μM and K_i 2.36 ± 0.17 μM . The mode of AChE inhibition was of the mixed type. Coumarin 106 also inhibited BChE but with a slightly lower potency of IC_{50} 27.8 μM . A compound aimed to act in the CNS should have properties enabling the penetration

through the blood-brain-barrier (BBB). The potency in an *in vitro* experiment alone should not be overestimated because of the compounds pharmacokinetic properties may affect its activity *in vivo* substantially. Passive BBB transport can be predicted by calculating the logBB value for the compound (Vilar et al. 2010). The logBB for coumarin 106 is higher than 0.3 which is the limit for good passive penetration to the CNS, therefore the structure is favourable for passive BBB transport. In addition, the number of hydrogen acceptors (3) and donors (0), the clogP value (4.48) and the molecular mass (281.34 g/mol) of coumarin 106 predict favourable oral absorption properties (Lipinski et al. 1997).

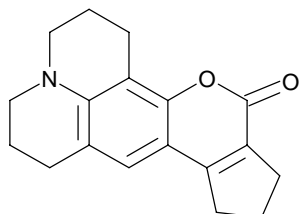


Figure 10. The structure of coumarin 106.

The coumarins' inhibitory effect for AChE has been studied previously in many papers in the literature. Some of the natural coumarins inhibit AChE in the micromolar range but semi-synthetic coumarin derivatives are significantly more potent. Scopoletin has been shown to inhibit AChE in a dose-dependent manner with IC_{50} of 168 μ M (Rollinger et al. 2004). The effect *in vivo* is comparable to galantamine although galantamine is much more potent *in vitro*. It is noteworthy that the 7-O-glucoside of scopoletin, scopolin, gave the highest response *in vivo* which can be considered surprising because the IC_{50} value *in vitro* is in the millimolar range. In our screening, at the concentration of 30 μ M, scopoletin showed inhibition just above the hit limit which is in agreement with the literature. Potency of scopoletin can be altered by chemical modification. A scopoletin derivative with a 3-{4-[(benzylethylamino)methyl]-phenyl} -substituent at position 3 has substantially lower IC_{50} value (18.3 nM) (Piazzi et al. 2008).

Zhou et al. (2008) synthesized a series of coumarin derivatives to investigate the structure-activity relationships of the compounds for AChE. Substitutions in the positions 3 and/or 4 of coumarin ring were shown to increase AChE inhibitory potency where as the substitution in the position 6 was not considered important for the interaction. The 4-phenylpiperazine substituted coumarin compound has an IC_{50} of 4.5 μ M. On the other hand, Pisani et al (2010) created a series of AChE inhibitors with different coumarin scaffolds attached to a edrophonium-like fragment and found that the 6,7-dimethoxycoumarin scaffold with an edrophonium fragment attached to position 3 is the best scaffold for creating potent AChE inhibitors acting in the picomolar range. In our screening, 6,7-dimethoxycoumarin was just above the hit limit at a concentration of 30 μ M which indicates that the additional substitution is essential for increasing the potency.

In addition, coumarin has been a scaffold for creating dual inhibitors. Some of the dual inhibitors are designed to inhibit AChE's active and peripheral sites (Piazzini et al. 2003, Shen et al. 2005) but dual inhibitors can be designed to inhibit two different enzymes like AChE and β -secretase (BASE) (Piazzini et al. 2008) or AChE and monoamine oxidase (MAO) (Brühlmann et al. 2001). These strategies aim to affect amyloid aggregation to create disease modifying therapies for AD.

5.4.2.2 Hits in the Synthetic Compound Library

Three of the hits in the synthetic compound library had potencies in the low nanomolar range (Table 6, compounds 1-3) (IV). The most potent hit ((N-[3-(3,5-dimethyl-1-piperidinyl)propyl]-5-ethyl-2-methyl-8-oxo-thieno[2',3':4,5]pyrrolo[1,2-d][1,2,4] triazine-7(8H)-acetamide)) had an IC_{50} value of 0.019 μ M. The second most potent hit turned out to be the known cholinesterase inhibitor tacrine which unexpectedly served as a reference for the screening. Altogether, 42 of the hits had IC_{50} value between 0.1 μ M and 1 μ M (Fig. 11).

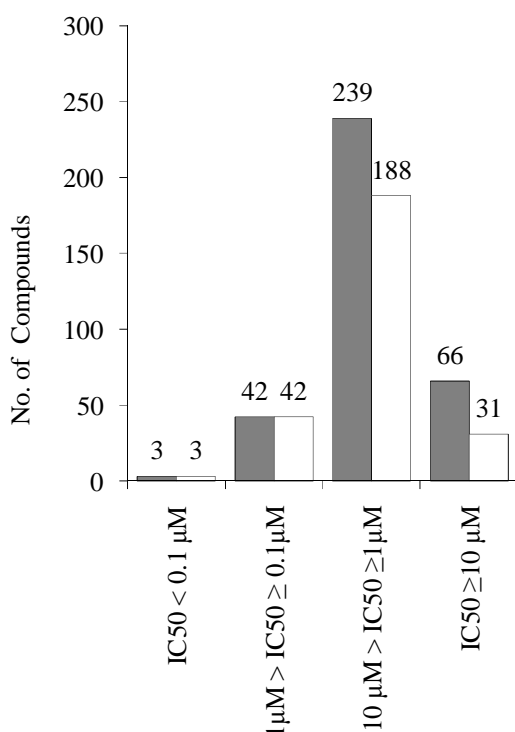


Figure 11. IC_{50} values of the hits for AChE in the synthetic compound library. The values were measured using the kinetic approach (grey bars). In comparison, the results were re-analysed as only the endpoint measurement was used (white bars). Altogether, 51 of the compounds in the category of 10 μ M > IC_{50} > 1 μ M would have been false negatives if only the end point measurement was used. Most of the hits had an IC_{50} in the micromolar range but the three best hits were in the nanomolar range.

Table 6. The structures and IC₅₀ values for the most potent hits in the screen of the synthetic compound library for AChE, as well as their IC₅₀ values for BChE determined in the secondary phase of screening.

		AChE IC ₅₀ (μM)	BChE IC ₅₀ (μM)
1		0.019	13.270
2		0.063	0.0108
3		0.069	23.580
4		0.152	nd
5		0.204	10.850
6		0.229	nd, x
7		0.236	nd
8		0.315	nd
9		0.332	nd

nd: IC₅₀ not determined, BChE inhibition < 50% at concentration 3.3 μM; x BChE inhibition 86.8 % at 3.3 μM but IC₅₀ not determined

In the large screening of the synthetic chemical library, the kinetic approach was utilized in both primary and secondary screening phases. The time consumed on this approach is undeniably long but if the results were re-analysed with only the endpoint being measured, 51 of the compounds in the category of $10\ \mu\text{M} > \text{IC}_{50} > 1\ \mu\text{M}$ would have been false negatives (Fig. 11). The most potent false negative would have been a compound displaying an IC_{50} of $1.35\ \mu\text{M}$. The most active compounds in categories $\text{IC}_{50} < 1\ \mu\text{M}$ would have been detected but if follow-up studies are considered among others *in silico* predictions a large set of data would have been miss-interpreted which can lead to low predictive values, e.g., in QSAR studies. The quality of the created dataset is essential if the results are to be used in follow-up studies.

The primary screening phase was conducted as a single point analysis using one concentration of compound (IV). The analysis of the data was qualitative because all hits below the set hit limit ($4\times\text{SD}$) were subjected to the secondary screening in which the dose-dependency was evaluated using eight concentrations. The single concentration analysis creates a possibility for false negatives in this phase. The primary screening can also be conducted as a quantitative multipoint analysis. Inglese et al. (2006) utilized 7 concentration points in the primary screening for 60,000 compounds in a 1536-well plate format. They also analysed the data as if only one concentration point was used and realized that up to 75% false negative rate was possible when a $6\times\text{SD}$ threshold was used for the detection limit. The false negative rate was 40% when a $3\times\text{SD}$ threshold was the hit limit. Quantitative screening would always be preferable if appropriate liquid handling equipment is available and the assay set-up is miniaturizable to 1536-well plate format because the solvent consumption does not increase compared to a single point assay in a 384-well plate format. The consumption of compound may even decrease. Instead, the number of plates and the number of data points increase which sets limits if the detection is the rate limiting step. Data created in quantitative screening is based on pharmacology whereas evaluation of data in qualitative single point screening is based on statistical analysis. The potency is often dependent on assay design which further highlights the importance of high data quality and favour quantitative over qualitative analysis.

All hits in the AChE screening were subjected to a follow-up screening for BChE to investigate selectivity of inhibition. Compounds were primarily assayed at a single concentration of $3.3\ \mu\text{M}$ after which the compounds showing more than 50 % inhibition were subjected for IC_{50} determinations. Tacrine was more potent for BChE over AChE as would be expected but otherwise the eight most active AChE inhibitors were more potent for AChE over BChE (Table 6).

Five of the hits in the AChE screening were found to inhibit BChE with substantially higher potencies (IC_{50} values against BChE were in the nanomolar range). The lowest detected IC_{50} against BChE was $6.1\ \text{nM}$. The IC_{50} of the same compound against AChE was $1.78\ \mu\text{M}$ which makes the compound highly selective for BChE over AChE. The active site of AChE is located in the bottom of the tight gorge whereas the active site of BChE is more accessible to compounds which could partly explain why some of the

moderate AChE inhibitors were more potent BChE inhibitors (Saxena et al. 1997). Selectivity is mostly based on interactions of a specific group or atom in the compound with a specific amino acid residue in the enzyme. The amino acids in the active sites of the two enzymes differ which makes size of the compound just one of the factors affecting the selectivity.

The possible interactions of the most active compound in the screen for AChE and BChE were evaluated in *in silico* docking studies (IV). This hit apparently binds at the entrance of the gorge site of AChE, due to the π - π stacking interactions between thiophene and the pyrrole ring of the compound and the aromatic Trp279 in AChE (Fig. 4 in IV). This residue belongs to the PAS region of AChE and suggests the possibility that it could interfere with amyloid fibrillogenesis in addition to inhibition of the catalytic function of the enzyme.

5.4.3 Chemical Evaluation of the Hits in the Libraries

ChemGPS-NP is a model that maps compounds into a chemical space that is relevant to biology (Larsson et al. 2007, Rosén et al. 2009). The model has been created based on the compounds in the Dictionary of Natural Products which is comprised of 167,169 natural compounds. Later on, more than 1 million unique compounds have been compiled for the subsequent validation of the model. Natural compounds occupy a larger and different space than compounds traditionally dealt with in medicinal chemistry. The fact that the model has been created using natural compounds makes it more predictive for biologically relevant chemical space than other chemical space models. The model uses all together 35 descriptors. The dimensionality has been reduced using principal component analysis leading to eight principal components (PC) describing molecular properties in the final model. The first four PCs explain 77% of the variance. PC1 represents size and shape; PC2 represents aromaticity and conjugation-related properties; PC3 corresponds to lipophilicity, polarity, and H-bond capacity, whereas PC4 expresses flexibility and rigidity. The size increases in the positive direction of PC1; the degree of aromaticity increases in the positive direction of PC2; hydrophobic compounds are situated in the positive directions of PC3 and hydrophilic compounds in the negative direction of PC3; and compounds are increasingly flexible in the positive direction of PC4 and more rigid in the negative direction. The ChemGPS model for medicinal compounds or drug-like compounds differs from ChemGPS-NP model in the order of explained properties. In the ChemGPS model, lipophilicity descriptors are the second most important descriptors explaining the variance, whereas flexibility and rigidity parameters are the third most important descriptors. Medicinal compounds tend to be more lipophilic than natural compounds which explain the change in order of descriptors. Natural compounds are evolutionarily optimized to function optimally in hydrophilic environments, e.g., inside cells and solutions meant for defence.

The chemical properties of the hits in the extract libraries, linarin and 2,5-dihydroxy- α -oxo-benzeneacetic acid, differed from each other in size, aromaticity and rigidity which can be seen clearly when comparing the PC1, PC2, and PC4 dimensions (Figs. 12 and 14). Nevertheless, the values describing lipophilicity (PC3) were close to each other, -2.00 and -2.32, respectively. Linarin is a diglycoside which makes it very different from any other compound mapped on the charts (Figs. 12-14). Size and aromaticity of 2,5-dihydroxy- α -oxo-benzeneacetic acid mapped close to the region of natural coumarins which was seen in the PC1 to PC2 chart (natural coumarins have closed symbols without numbers in Fig. 13).

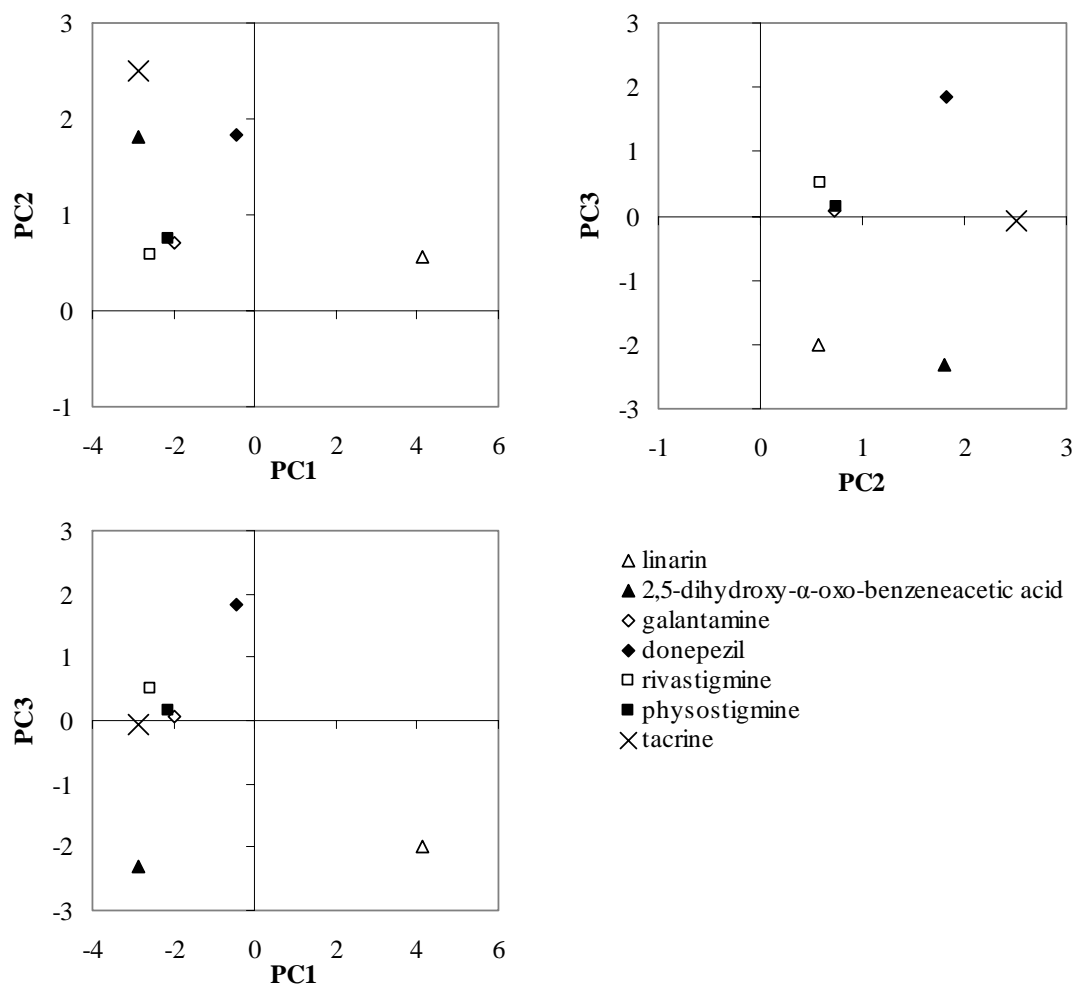


Figure 12. The hits identified from the natural extract libraries (linarin and 2,5-dihydroxy- α -oxo-benzeneacetic acid), and known cholinesterase inhibitors (galantamine, donepezil, rivastigmine, physostigmine and tacrine) mapped into the biologically relevant chemical space using the ChemGPS-NP model (Larsson et al. 2007, Rosén et al. 2009).

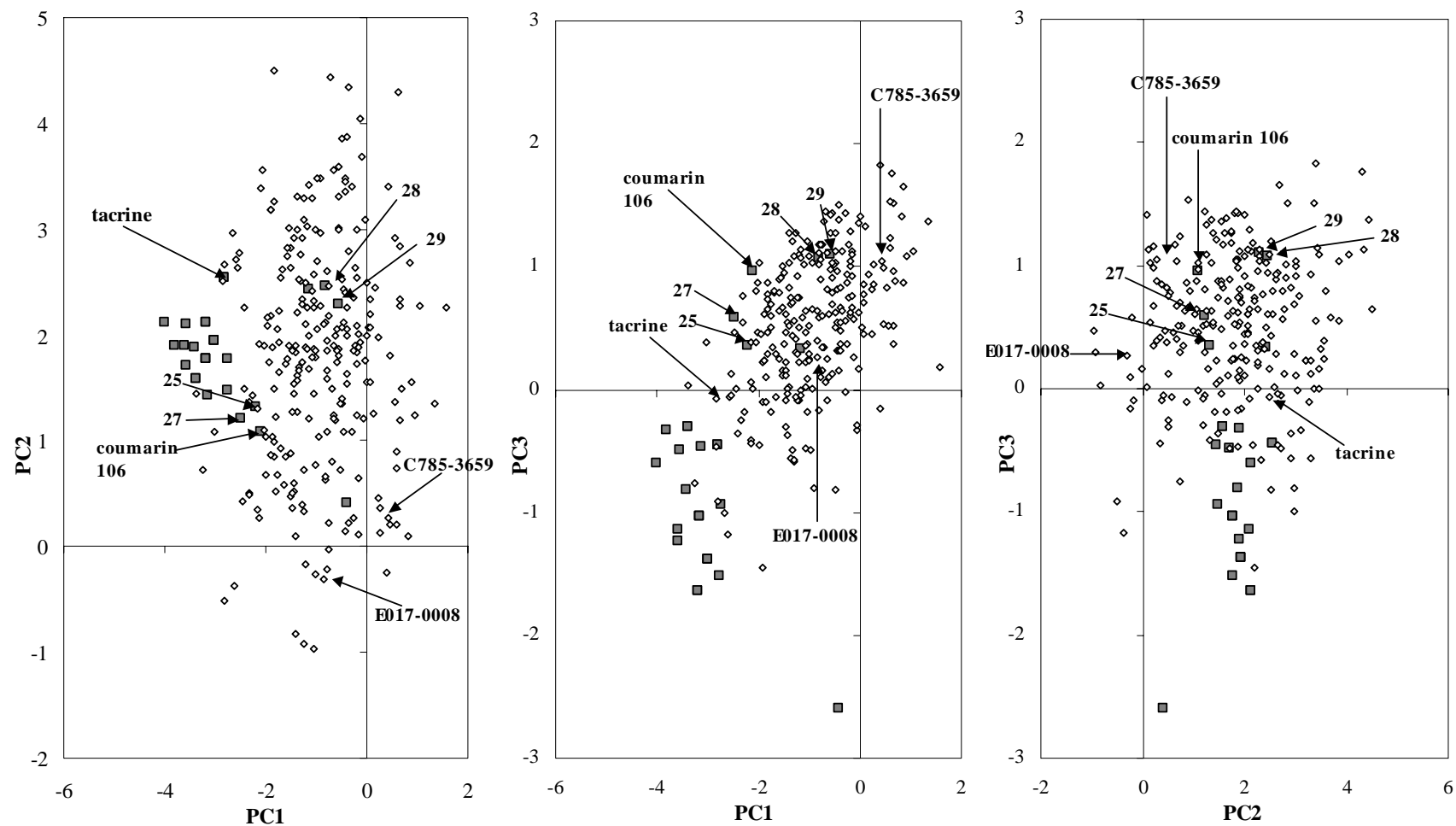


Figure 13. Mapping of the hits in the synthetic compound library (open symbols), and all coumarins in the coumarin library (closed symbols), using the ChemGPS-NP model (Larsson et al. 2007, Rosén et al. 2009). The most active compounds in the synthetic compound library were C785-3659, tacrine, and E017-0008 (compounds 1-3 in Table 6). The synthetic coumarins are marked with numbers. (Fallarero et al., unpublished data)

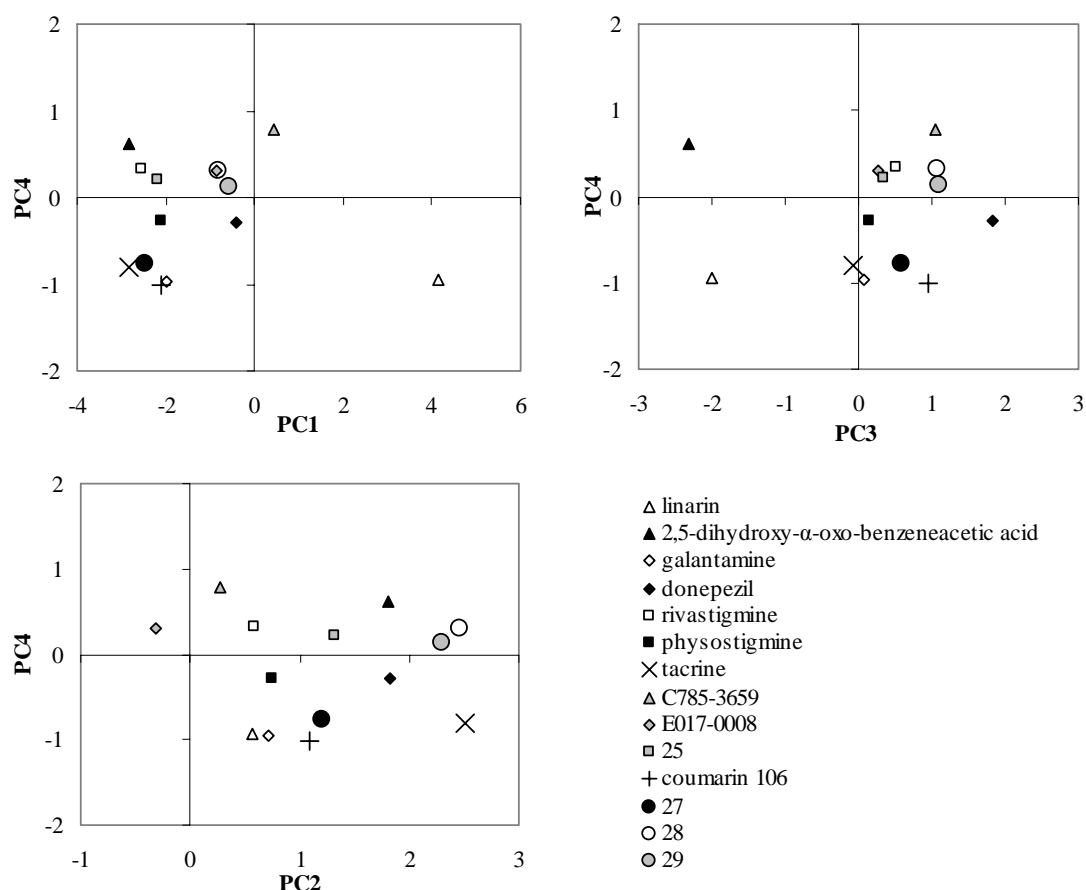


Figure 14. Mapping of the three most active hits in the large synthetic compound screening (C785-3659, tacrine, and E017-0008), the hits in the extract libraries (linarin and 2,5-dihydroxy- α -oxo-benzeneacetic acid), the synthetic coumarins (coumarin 106 and compounds 25, 27-29) as well as known cholinesterase inhibitors (galantamine, donepezil, rivastigmine, and physostigmine) using the ChemGPS-NP model. Each of the first three dimensions (PC1-3) was compared to rigidity (PC4).

The synthetic hits occupied a large area on the PC1 to PC2 chart (Fig. 13). Even so, the coverage of chemical space was limited mostly to the negative range of PC1 and the positive range of PC2. Most of the natural coumarins (closed symbols without numbers) were even at a lower region on the PC1 scale than synthetic compounds (Fig. 12). The synthetic compounds were mostly in the positive region on the lipophilicity scale (PC3) whereas all the natural compounds including the natural coumarins, linarin and 2,5-dihydroxy- α -oxo-benzeneacetic acid were in the negative region on the PC3 scale. The finding supports the general view that synthetic compounds in HTS libraries are more lipophilic than natural compounds which was also noted by the authors when creating the ChemGPS-NP model (Larsson et al. 2007 and Rosén et al. 2009). It is worth keeping in mind that the synthetic compounds mapped on the charts (Figs. 13 and 14) were all hits in

the primary screening phase. General comparison between natural compounds vs. synthetic compounds based on these results may be biased. Nevertheless, it can be stated that the discovered synthetic AChE inhibitors, including synthetic coumarins, were more lipophilic than the two discovered natural AChE inhibitors.

The ChemGPS-NP model has also been used to compare drugs and natural compounds (Rosén et al. 2009). It has been shown that some of the natural compounds that map close to a certain drug possess similar activities, e.g., against targets like angiotensin-converting enzyme, calcium channels blockers, and several viral enzymes. To some extent, chemical properties seem to correlate to activity. Consequently, the ChemGPS-NP model can be applied to find new active compounds or at least narrow down the number of compounds selected for screening. The known cholinesterase inhibitors, galantamine, donepezil, rivastigmine, physostigmine, and tacrine, were mapped on the charts with the new cholinesterase inhibitors to see if the active compounds would map with close proximity to each other (Figs. 12 and 14). Further detailed calculations need to be conducted before any final conclusions could be drawn.

Some preliminary observations were made for cholinesterase inhibitors. The five known inhibitors mapped on the negative scale of PC1 and on the positive scale of PC2 (Figs. 12-14). The values on the PC3 scale were close to zero except for donepezil which was 1.84. Similarly, most of the hits in the synthetic compound library and the synthetic coumarins which were above the hit limit at the concentration of 5 μ M mapped on the negative scale of PC1, to the positive scale of PC2, and to the scale of -0.5 –1.5 on PC3. In general, most of the active compounds were: i) rather small in size and shape, ii) they were highly aromatic, and iii) were to some extent lipophilic. Interestingly, none of these generalizations applied to linarin, whereas 2,5-dihydroxy- α -oxo-benzeneacetic acid was an outlier for lipophilicity.

5.5 Production and Management of the Libraries for Screening

5.5.1 Extract Libraries

Natural products have been shown to contain a large variety of structurally diverse compounds (Lee and Schneider 2001, Newman 2008). Natural compounds are synthesized by biological organisms which means that the compounds are likely to interact with biological targets and they are “designed by evolution”. On the other hand, biological environment is a challenging producer which cannot be as much controlled as chemical synthesis. When natural extracts are included in libraries for screening, the supply of the extract has to be secured at least until the active compound has been identified. The same species do not produce the same compounds in all conditions and not even with the same conditions (Samuelsson and Bohlin 2009). Also, it is possible that cultures that are derived

from the same cultivar and grow in the same incubator next to each other vary in secondary metabolite production. Several factors, partly unknown ones, affect this equation.

Plant or fungal material may be isolated from the nature, may be grown in a greenhouse or on a field or may be grown in a culture. Each approach may be valid but may also create problems. The process of creating a natural compound library has to be well documented from the collection of the species, the identification of the species, to the handling and storage of the material (Samuelsson and Bohlin 2009). Samples from nature are identified by an expert on the specific field and collected in a *herbarium* which can be used for later verification if needed. Documentation reduces the effect of unknown factors. *Mentha* samples screened in this contribution for AChE and BChE inhibitory effects were extracts of different species and variants but also different parts of the plant (II). A part of dry plant material for each sample was reduced in size and extracted to make an extract library which leaves an opportunity to go back to the extraction phase if more of the extract is needed later on.

The fungi screened in this contribution belong to a large culture collection which is maintained at the Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki. The samples are mainly collected from nature during years of research and identified by numerous experts. The cultures are maintained on agar plates. For screening, fungi were inoculated in liquid cultures, grown for eight weeks after which the liquor was extracted with ethyl acetate (I). In principle, it is possible to grow more cultures if the supply of the extract runs out. In practice, during the growth period which is relatively long due to the slow growth rate of the polypore fungi, there is a risk that not the same secondary metabolites are produced in the repeated cultures. At least, the concentrations and rates of the metabolite may vary. To minimize this effect, three parallel cultures were grown at the same time and the liquors were pooled before extraction.

To avoid re-culturing the material, the use of increased sizes of the initial culture to create two parallel sets of the extract is wise: a working batch for fractionation and crude extract screening and a frozen batch as a back-up may be considered (Wagenaar 2008). The back-up crude extract can be utilized for isolation and identification of the compound. There is a possibility that the organism does not produce the same metabolites if it is cultured again. Even if the same metabolites would be produced much time is saved if repeating the growth period and chemical and biological analyses are avoided.

5.5.2 Compound Libraries

The compounds in the synthetic compound libraries are designed to be drug-like according to the rule of five (Lipinski et al. 1997). Several rules have been defined for compounds in HTS chemical libraries which aim to exclude non-lead-like chemical structures that contain reactive functional groups which potentially bind covalently to proteins from

libraries (Rishton 2008, Baell and Holloway 2010). Lead-likeness can be achieved by designing the chemical library in the way that it discriminates structures that are likely to produce false-positive results in large scale biochemical screens (Baell and Holloway 2010). The following rules are designed for this goal: i) molecular weight 200–350, ii) $\text{clogP} < 3$, iii) single charge present, iiiii) exclusion of chemically reactive functional groups, promiscuous inhibitors, frequent hitters, and “warheads” like covalent binders and iiiiii) exclude peptides (Rishton 2008). As a result, the discrimination of structures decreases the structural diversity of the library but also decreases the workload in the following phase of the drug discovery pipeline because lead-like compounds should “behave properly” in, e.g., *in silico* models.

In addition, certain structural elements are more likely to lead to coloured or fluorescent compounds that interfere with assays and should therefore be eliminated from chemical libraries as non-lead-like structures (Baell and Holloway 2010). Assay development and validation are essential steps to decrease the possibility of assay interference. Assay designs aiming to detect effects of real target-ligand interactions increase the rate of true hits. Addition of a nonionic detergent to an enzyme assay prevents nonspecific inhibition by aggregating compounds (Ryan et al. 2003) or the use of time-resolved fluorescent detection to decrease the effect of compound fluorescent (Thorne et al. 2010) are examples of the matters that can be addressed in the assay design phase.

False negatives and positives create a real problem for screening and data applicability for which different means have been used to minimize this effect. Posner et al. (2009) introduced a method to enhance hit selection via a local hit rate analysis. This method evaluates true hits in screening by comparing a compound’s activity to the activity of their structural analogs. The probability of finding a true hit increases if the analogs also show activity. This approach can be applied for selection of hits for secondary screening and also for the finding of false negatives.

The management of the good quality of pure compound libraries is essential for screening. The quality of the library, determined as high purity of the compounds, correct identity of compounds and implementation of information technologies in compound management, reflects directly to the quality of the data created (Houston et al. 2008). The flexible format of the compound deck allows adding of new compounds into it, adding of structural and biological information about the compound into the databases and managing the inventory of the existing samples. The inventory includes compound stability and integrity studies of the dissolved library samples (Janzen and Popa-Burke 2009). In the early days of HTS when compounds produced by combinatorial chemistry were included in the screens for the first time, the purity of the samples was low. Nowadays, the integrity of the samples included in the libraries is prioritised because it is acknowledged that chemical errors decrease the quality of data which leads, e.g., to errors in SAR analyses.

The storage and handling of dissolved samples, usually in DMSO, over long periods of time is a risk for compound integrity (Matson et al. 2009). Storage of the plates in low

temperatures and therefore repetitive freezing and thawing cycles of the plates for each screen may lead to degradation of compounds. Some compounds are more susceptible for the temperature changes than others. Also, DMSO is a solvent that absorbs water easily which is more profound during freezing and thawing (Janzen and Popa-Burke 2009). Increase in water content changes the physicochemical properties of DMSO, including solvation properties. There are different practices between the pharmaceutical companies to store the compounds. In any case, the best practice to evaluate structural integrity is to include analytical methods to the screening process and the library inventory.

5.6 Selection of Compounds in a Library for Screening

The implementation of a prior knowledge and information technologies in the screening are shifting the HTS screens from completely random to more focused direction which should be seen in the future as a maximized chances for success. Natural and synthetic compounds should both be implemented in screening platforms to increase the efficacy in finding good hits and future clinical candidates. As proven in the screens of this contribution, both natural sources and a commercial synthetic compound library were shown to contain compounds able to inhibit cholinesterases with various selectivities and potencies.

Including natural pure compounds in chemical decks is a possibility for screening facilities to increase the diversity of compound structures in libraries if they do not want to handle extracts (Drewry and Macarron 2010, Sukuru et al. 2009). If structure diversity is a selection criterion for a certain screen in the industrial set-ups, a large fraction of natural products is usually selected because of their inherent chemical diversity. On the other hand, the production of pre-fractionated extract libraries into a format compatible with HTS platforms have made screening of extracts easier (Wagenaar 2008). Improvements in sensitivity of analytical technologies enable screening and identification of natural compounds without laborious isolation steps of compounds from extracts (Dalisay and Molinski 2010).

Commercial companies offer large compound libraries for screening in which the compounds are designed to fulfil certain criteria, e.g., to be drug-like or contain diverse set of compounds spread evenly in the chemical space. The library of 56,320 compounds screened in this contribution included commercially available compounds from three companies (ChemDiv Inc (San Diego, US), ChemBridge Corporation (San Diego, US) and Tripos (St Louis, US). The screening was conducted on random basis and all the compounds were assayed in the primary screening phase (IV). In the secondary screening phase hits were cherry picked from the plates and serially diluted for the dose response determination. The number of compounds and data points was in the range that was manageable for the random screening approach in 384-well plate format.

The pharmaceutical companies have compound decks that may contain over million compounds. The increase in number of compounds per screen does not increase the quality of data. On the contrary, focused selection of subset of compounds for a specific screen does (Houston et al. 2008). The number of compounds in these subset libraries is in the range of 30-60,000 compounds. Selection based on the structural requirement for a compound to interact with the specific target class increases the probability in finding potent compounds. Highly automated and flexible libraries based on vial storage enable creation of subset libraries for each screen separately. When structural information is available in the format that computational methods may be utilized for pre-analysis and creation of a focused library, the number of compounds needed to be assayed in an *in vitro* set-ups decrease accordingly without decreasing the chance in finding hits. Combinatorial chemistry creates lots of closely related compounds which may be utilized to study the QSAR of a certain structure in the secondary screening phase (Metzger et al. 2006).

6 Summary and Conclusions

High quality of data created in screening is essential. The assays used should be validated and quality parameters should be determined to monitor the assay performance during screening. The data created in validated assay set-ups can be utilized for the discrimination of an inhibitory effect from the average signal as well as for quantitative analysis such as the potency evaluation of an inhibitor.

In this contribution, two AChE inhibitory assays were evaluated as screening tools: TLC bioautography and a micro-plate assay based on the Ellman's reagent. The variants of the Ellman's reagent based assay were studied in more detail to assess how the changes of assay conditions affect the potency of inhibitors. The main variants studied were measurement time, substrate concentration and the order in which the reaction was initiated. The reaction was initiated either with the enzyme (AChE, reaction order 1) or the substrate (ATCI, reaction order 2). The quality parameters Z' , S/B and S/N were utilized.

All studied variants of the Ellman's reagent based assay were validatable when measurement time was long enough and variation was low. Z' value ≥ 0.5 , indicating an excellent assay, was achieved at three minutes after initiating the reaction. Also, S/N and S/B were above the set limits, ≥ 10 and ≥ 4 , respectively, for 5 minutes after the initiation of the reaction. Some advantages were detected to favour the reaction order 2 for screening. A part of the substrate hydrolyzes spontaneously. The spontaneous hydrolysis increases the background value and the variation between wells when reaction order 1 is used because the hydrolysed substrate reacts with the reagent before the enzyme is added. Thus, the reaction order 2 had greater a signal window than reaction order 1. In addition, with 1.5 mM substrate concentration, the change in absorbance and accordingly the signal window is greater than with a substrate concentration of 0.13 mM which can be seen as an increase in Z' values in both variants.

Each library has its pros and cons which makes the selection of the library for screening a challenge. When screening extracts for AChE inhibitory effects, separation beforehand was conducted either on TLC or HPLC. The TLC separation was coupled with bioautography for the detection of AChE and BChE inhibitors in *Mentha* and fungal extracts. HPLC micro-fractionation of the flower extract of *Mentha arvensis* L. was coupled to an off-line AChE assay to track the inhibitory effect to a certain peak in the chromatogram. The active compound in *Mentha* was identified as a known compound, linarin, using MS spectroscopy without further isolation of the compound. The active compound in one fungal extract, *Piptoporus betulinus* (Bull.: Fr.) P.Karst extract, was isolated directly from the TLC plates and identified as 2,5-dihydroxy- α -oxo-benzeneacetic acid using NMR and MS spectroscopy. The fungi were found to be rich sources of cholinesterase inhibitors because 115 of the assayed extracts showed AChE inhibitory effect and 72 of the extracts showed BChE inhibitory effect. Isolation and identification of the active compounds was laborious so only one of the active compounds in the fungal extracts was identified.

Two pure compound libraries were screened for AChE inhibitory effects: a small coumarin library of 29 compounds and a large synthetic compound library of 56,320 compounds. The hit rate in the coumarin library was substantially higher than in the synthetic compound library because this targeted library was created based on a prior knowledge on AChE inhibitory effect of the coumarin scaffold. Furthermore, the potencies of hits within the synthetic compound library were much higher than the potencies of the coumarins.

Both natural and synthetic compound libraries were shown to contain cholinesterase inhibitors with various potencies and selectivities, and therefore complement each other as sources of compounds for screening. It is acknowledged that natural compounds differ structurally from compounds in synthetic compound libraries which further support the view of complementation especially if a high diversity of structures is the criterion for selection of compounds in a library. The structural difference between natural and synthetic compounds was also noted in the analysis of chemical properties of the hits in the libraries. The drug discovery process is more demanding and time consuming if natural extracts are included in the screening campaign compared to pure compounds due to the isolation and identification phases of active compounds. Even so, identification of cholinesterase inhibitors in extracts was achieved using LC-MS detection in combination with literature search or after isolation of the compound combined with MS and NMR analyses. Isolated natural pure compounds, in addition to synthetic compounds, are suitable as such for compound libraries for HTS screening. Also, pre-fractionated extracts in 96-well plates are compatible with HTS screening platforms which make pre-fractionation of extracts an alternative way to form natural product libraries. Recent improvements in analytical and spectroscopic techniques facilitate following these processes.

Appendix 1.

Examples of modification of Ellman's assay found in the literature and observed half inhibitory concentrations (IC₅₀) for reference compounds. The examples were randomly selected from the literature to show wide variety of assay modifications.

AChE source	AChE concentr./ amount	ATCI concentr. (mM)	DTNB concentr. (mM)	Reaction initiated with enzyme (E) or substrate (S)	Incubation time (min)	Incubation temperature (°C)	Measurement wavelength (nm)	Measurement time	Reference compound(s)	IC ₅₀ (AChE) (µM)	Reference
-	0.028 U/ml	1.5	1.5	E	-	-	405	2min, 5s intervals	galantamine	0.77	Chattipakorn et al. 2007
eel	0.02 U	0.5	0.3	S	10	37	405	5 min	tacrine; donepezil	0.15; 0.08	Kwon et al. 2007
eel	0.022 U/ml	1.5	1.5	E	-	-	405	13s x 8	galantamine	1.4	Adsersen et al. 2007
eel	0.028 U/ml	0.15	1.5	E	-	-	405	2min, 5s intervals	galantamine	0.59	Langjae et al. 2007
eel	-	-	-	S	15	25	412	-	galantamine	-	Orhan et al. 2007
eel	-	-	-	S	30	25	412	-	physost.	0.04	Atta-ur-Rahman et al. 2001
eel	0.022 U/ml	1.5	1.5	E	-	-	405	after 5 min incubation	galantamine	0.88	Papandreou et al. 2009
eel	-	-	-	According to Ellman 1961	-	-	-	-	galantamine	0.55	Tang et al. 2007
eel	0.056 U/ml	0.3	0.2	S	-	25	412	after 5 min	physost.	0.073	Vinutha et al. 2007
eel	0.06 U/ml	0.3	250	S	30	25	405	measured after 3 min	galantamine	1.82	Berkov et al. 2008
eel	-	-	-	According to Ellman 1961	-	-	-	-	huperzine A; huperzine B	0.8; 8.0	Choo et al. 2007

AChE source	AChE concentr./ amount	ATCI concentr. (mM)	DTNB concentr. (mM)	Reaction initiated with enzyme (E) or substrate (S)	Incubation time (min)	Incubation temperature (°C)	Measurement wavelength (nm)	Measurement time	Reference compound(s)	IC ₅₀ (AChE) (µM)	Reference
eel	0.0286 U/ml	1.95	1.92	E	-	-	405	12 min	galantamine	3.18	Lee et al. 2007
eel	0.01 U/ml	0.5	0.5	S	15	37	412	1 min intervals	tacrine	0.1	Pan et al. 2008
eel	-	-	-	S	15	25	412	-	physost.; tacrine; galantamine	0.041; 0.021; 0.45	Khalid et al. 2004
eel, T.c., bovine erythr.; human recomb.	0.055 U/ml	0.6	0.27	S	-	25	412	2 min	tacrine	0.074 (eel); 0.157 (T.c.); 0.446 (bovine erythr.); 1.03 (human recomb.)	Sauvatre et al. 2007
eel, human erythr.	0.05 U/ml	0.2	0.28	S	10	25	412	1 min	tacrine; donepezil	0.039; 0.049 (eel); 0.096; 0.016 (human erythr.)	Contreras et al. 2001
human brain	0.02 U	0.5	0.3	S	10	30	405	5 min	tacrine	0.167	Munoz-Ruiz et al. 2005b
human erythr.	-	0.3	0.2 %	E	-	37	440	reaction stopped after 8 min	tacrine	0.44	Wang et al. 2007
human erythr.	0.0024 U/ml / 0.03 U/ml	0.04	0.024	S	30	4	412	after 20 min, 37 °C	galantamine	0.93	Jang et al. 2008
human erythr.	0.035 U/ml	0.55	0.34	S	20	37	412	5 min	physost.	0.014	Rampa et al. 2001
human erythr.	0.035 U/ml	0.55	0.34	S	20	37	412	5 min	tacrine	0.25	Recanati et al. 2000
human erythr.	0.02 U/ml	0.2	0.1	S	20	-	412	5 min	physost.	0.019	Villalobos et al. 1994

AChE source	AChE concentr./ amount	ATCI concentr. (mM)	DTNB concentr. (mM)	Reaction initiated with enzyme (E) or substrate (S)	Incubation time (min)	Incubation temperature (°C)	Measurement wavelength (nm)	Measurement time	Reference compound(s)	IC ₅₀ (AChE) (µM)	Reference
human recomb.	0.035U/ml	0.55	0.34	S	20	37	-	-	donepezil	0.0079	Zhu et al. 2009
human recomb.	0.02 U/ml	0.55	0.34	S	20	37	-	-	donepezil	0.02	Rizzo et al. 2010
human recomb.	0.02 U/ml	0.55	0.34	S	20	37	412	-	rivastigmine	0.03	Rizzo et al. 2008
human recomb.		0.55	0.34	S	20	37	412	5 min	donepezil; tacrine; physost.	0.023; 0.424; 0.013	Bartolini et al. 2003
human recomb.	0.1 AU/min	0.55	1.34	S	20-60	37	412	6 min	rivastigmine	1.54	Bolognesi et al. 2004
rat brain; eel	-	54	0.036	S	10	37	412	10 min, 1 min intervals	neostigmine	0.04 (rat brain); 0.053 (eel)	Girisha et al. 2009
rat brain	-	-	-	According to Ellman 1961	-	-	-	-	physost.	0.001	Zhan et al. 2010
rat brain	-	-	-	According to Ellman 1961	-	-	-	-	galantamine	0.92	Cardoso et al. 2004
rat cortex homog.	-	0.3	1.2%	E/S	15	37	450	reaction stopped after 15 min	galantamine	1.17	Jia et al. 2009
rat cortex homog.	-	-	-	E	-	37	440	reaction stopped after 8 min	huperzine A; huperzine B	0.072; 0.019	He et al. 2007
mouse brain homog.	-	0.5		E	2 or 60	25	412	-	physost.	0.00069 with 60 min preincub.; 0.34 without preincub.	Sugimoto et al. 1990
T.c.	0.028 U/ml	0.15	1.5	E	-	-	405	2min, 5s intervals	galantamine	0.6	Sangnoi et al. 2008

T.c. *Torpedo californica*; eel *Electrophorus electricus*, physost. physostigmine

References

- Adersen, A., Kjolbye, A., Dall, O., Jager, A.K. (2007). Acetylcholinesterase and butyrylcholinesterase inhibitory compounds from *Corydalis cava* Schweigg. & Kort. *J. Ethnopharmacol.* 113:179-182.
- Al-Jafari, A.A. (1996). The nature of the inhibition of camel retina acetylcholinesterase (EC 3.1.1.7) activity by tetrahydroaminoacridine. *J. Ocul. Pharmacol. Ther.* 12:503-514.
- Alvarez, A., Alarcon, R., Opazo, C., Campos, E.O., Munoz, F.J., Calderon, F.H., Dajas, F., Gentry, M.K., Doctor, B.P., De Mello, F.G., Inestrosa, N.C. (1998). Stable complexes involving acetylcholinesterase and amyloid-beta peptide change the biochemical properties of the enzyme and increase the neurotoxicity of Alzheimer's fibrils. *J. Neurosci.* 18:3213-3223.
- Atta-ur-Rahman, Parveen, S., Khalid, A., Farooq, A., Choudhary, M.I. (2001). Acetyl and butyrylcholinesterase-inhibiting triterpenoid alkaloids from *Buxus papillosa*. *Phytochemistry* 58:963-968.
- Augustinsson, K.B., Nachmansohn, D. (1949). Studies on Cholinesterase: VI. Kinetics of the Inhibition of Acetylcholine Esterase. *J. Biol. Chem.* 179:543-559.
- Baell, J.B., Holloway, G.A. (2010). New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* 53:2719-2740.
- Bajorath, J. (2002). Integration of virtual and high-throughput screening. *Nat. Rev. Drug Discov.* 1:882-894.
- Balakin, K.V., Kozintsev, A.V., Kiselyov, A.S., Savchuk, N.P. (2006). Rational design approaches to chemical libraries for hit identification. *Curr. Drug Discov. Technol.* 3:49-65.
- Bartolini, M., Bertucci, C., Cavrini, V., Andrisano, V. (2003). beta-Amyloid aggregation induced by human acetylcholinesterase: inhibition studies. *Biochem. Pharmacol.* 65:407-416.
- Bartolucci, C., Siotto, M., Ghidini, E., Amari, G., Bolzoni, P.T., Racchi, M., Villetti, G., Delcanale, M., Lamba, D. (2006). Structural determinants of *Torpedo californica* acetylcholinesterase inhibition by the novel and orally active carbamate based anti-alzheimer drug ganstigmine (CHF-2819). *J. Med. Chem.* 49:5051-5058.
- Bartus, R.T., Dean, R.L., 3rd, Beer, B., Lippa, A.S. (1982). The cholinergic hypothesis of geriatric memory dysfunction. *Science* 217:408-414.
- Batra, S., Srinivasan, T., Rastogi, S.K., Kundu, B. (2002). Identification of enzyme inhibitors using combinatorial libraries. *Curr. Med. Chem.* 9:307-319.
- Berkov, S., Codina, C., Viladomat, F., Bastida, J. (2008). N-Alkylated galanthamine derivatives: Potent acetylcholinesterase inhibitors from *Leucojum aestivum*. *Bioorg. Med. Chem. Lett.* 18:2263-2266.
- Berman, H.A., Leonard, K. (1992). Interaction of tetrahydroaminoacridine with acetylcholinesterase and butyrylcholinesterase. *Mol. Pharmacol.* 41:412-418.

- Bernard, P., Kireev, D.B., Chretien, J.R., Fortier, P.L., Coppet, L. (1999). Automated docking of 82 N-benzylpiperidine derivatives to mouse acetylcholinesterase and comparative molecular field analysis with 'natural' alignment. *J. Comput. Aided Mol. Des.* 13:355-371.
- Berr, C., Wancata, J., Ritchie, K. (2005). Prevalence of dementia in the elderly in Europe. *Eur. Neuropsychopharmacol.* 15:463-471.
- Best, M.D., Brik, A., Chapman, E., Lee, L.V., Cheng, W.C., Wong, C.H. (2004). Rapid discovery of potent sulfotransferase inhibitors by diversity-oriented reaction in microplates followed by in situ screening. *Chem. Bio. Chem.* 5:811-819.
- Bhatia, M.S., Zarekar, B.E., Choudhari, P.B., Ingale, K.B., Bhatia, N.M. (2010). Combinatorial approach: identification of potential antifungals from triazole minilibraries. *Med. Chem. Res.* DOI 10.1007/s00044-009-9283-8.
- Bindseil, K.U., Jakupovic, J., Wolf, D., Lavayre, J., Leboul, J., van der Pyl, D. (2001). Pure compound libraries; a new perspective for natural product based drug discovery. *Drug Discov. Today* 6:840-847.
- Birks, J. (2006). Cholinesterase inhibitors for Alzheimer's disease. *Cochrane Database Syst. Rev.* DOI: 10.1002/14651858.CD005593.
- Bollini, S., Herbst, J.J., Gaughan, G.T., Verdoorn, T.A., Ditta, J., Dubowchik, G.M., Vinitsky, A. (2002). High-throughput fluorescence polarization method for identification of FKBP12 ligands. *J. Biomol. Screen.* 7:526-530.
- Bolognesi, M.L., Andrisano, V., Bartolini, M., Banzi, R., Melchiorre, C. (2005). Propidium-based polyamine ligands as potent inhibitors of acetylcholinesterase and acetylcholinesterase-induced amyloid-beta aggregation. *J. Med. Chem.* 48:24-27.
- Bolognesi, M.L., Bartolini, M., Cavalli, A., Andrisano, V., Rosini, M., Minarini, A., Melchiorre, C. (2004). Design, synthesis, and biological evaluation of conformationally restricted rivastigmine analogues. *J. Med. Chem.* 47:5945-5952.
- Bolognesi, M.L., Bartolini, M., Mancini, F., Chiriano, G., Ceccarini, L., Rosini, M., Milelli, A., Tumiatti, V., Andrisano, V., Melchiorre, C. (2010). Bis(7)-tacrine derivatives as multitarget-directed ligands: Focus on anticholinesterase and anti-amyloid activities. *Chem. Med. Chem.* 5:1215-1220.
- Bolognesi, M.L., Cavalli, A., Melchiorre, C. (2009). Memoquin: a multi-target-directed ligand as an innovative therapeutic opportunity for Alzheimer's disease. *Neurotherapeutics* 6:152-162.
- Braak, E., Griffing, K., Arai, K., Bohl, J., Bratzke, H., Braak, H. (1999). Neuropathology of Alzheimer's disease: what is new since A. Alzheimer? *Eur. Arch. Psychiatry Clin. Neurosci.* 249 Suppl 3:14-22.
- Braak, H., Braak, E., Bohl, J., Reintjes, R. (1996). Age, neurofibrillary changes, A β -amyloid and the onset of Alzheimer's disease. *Neurosci. Lett.* 210:87-90.
- Brühlmann, C., Ooms, F., Carrupt, P.A., Testa, B., Catto, M., Leonetti, F., Altomare, C., Carotti, A. (2001). Coumarins derivatives as dual inhibitors of acetylcholinesterase and monoamine oxidase. *J. Med. Chem.* 44:3195-3198.

- Bugni, T.S., Richards, B., Bhoite, L., Cimborá, D., Harper, M.K., Ireland, C.M. (2008). Marine natural product libraries for high-throughput screening and rapid drug discovery. *J. Nat. Prod.* 71:1095-1098.
- Camps, P., Formosa, X., Galdeano, C., Gomez, T., Munoz-Torrero, D., Ramirez, L., Viayna, E., Gomez, E., Isambert, N., Lavilla, R., Badia, A., Clos, M.V., Bartolini, M., Mancini, F., Andrisano, V., Bidon-Chanal, A., Huertas, O., Dafni, T., Luque, F.J. (2010). Tacrine-based dual binding site acetylcholinesterase inhibitors as potential disease-modifying anti-Alzheimer drug candidates. *Chem. Biol. Interact.* 187:411-415.
- Camps, P., Formosa, X., Galdeano, C., Gomez, T., Munoz-Torrero, D., Scarpellini, M., Viayna, E., Badia, A., Clos, M.V., Camins, A., Pallas, M., Bartolini, M., Mancini, F., Andrisano, V., Estelrich, J., Lizondo, M., Bidon-Chanal, A., Luque, F.J. (2008). Novel donepezil-based inhibitors of acetyl- and butyrylcholinesterase and acetylcholinesterase-induced beta-amyloid aggregation. *J. Med. Chem.* 51:3588-3598.
- Camps, P., Munoz-Torrero, D. (2002). Cholinergic drugs in pharmacotherapy of Alzheimer's disease. *Mini Rev. Med. Chem.* 2:11-25.
- Carbonell, T., Masip, I., Sanchez-Baeza, F., Delgado, M., Araya, E., Llorens, O., Corcho, F., Perez, J.J., Perez-Paya, E., Messeguer, A. (2000). Identification of selective inhibitors of acetylcholinesterase from a combinatorial library of 2,5-piperazinediones. *Mol. Divers.* 5:131-143.
- Cardoso, C.L., Castro-Gamboa, I., Silva, D.H., Furlan, M., Epifanio Rde, A., Pinto Ada, C., Moraes de Rezende, C., Lima, J.A., Bolzani Vda, S. (2004). Indole glucoalkaloids from *Chimarrhis turbinata* and their evaluation as antioxidant agents and acetylcholinesterase inhibitors. *J. Nat. Prod.* 67:1882-1885.
- Chattipakorn, S., Pongpanparadorn, A., Pratchayasakul, W., Pongchaidacha, A., Ingkaninan, K., Chattipakorn, N. (2007). *Tabernaemontana divaricata* extract inhibits neuronal acetylcholinesterase activity in rats. *J. Ethnopharmacol.* 110:61-68.
- Cheng, Y., Prusoff, W.H. (1973). Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem. Pharmacol.* 22:3099-3108.
- Cho, S.J., Garsia, M.L., Bier, J., Tropsha, A. (1996). Structure-based alignment and comparative molecular field analysis of acetylcholinesterase inhibitors. *J. Med. Chem.* 39:5064-5071.
- Choo, C.Y., Hirasawa, Y., Karimata, C., Koyama, K., Sekiguchi, M., Kobayashi, J., Morita, H. (2007). Carinatumins A-C, new alkaloids from *Lycopodium carinatum* inhibiting acetylcholinesterase. *Bioorg. Med. Chem.* 15:1703-1707.
- Coelho, F., Birks, J. (2001). Physostigmine for Alzheimer's disease. *Cochrane Database Syst. Rev.* DOI: 10.1002/14651858.CD001499.
- Coan, K.E., Maltby, D.A., Burlingame, A.L., Shoichet, B.K. (2009). Promiscuous aggregate-based inhibitors promote enzyme unfolding. *J. Med. Chem.* 52:2067-2075.
- Contreras, J.M., Parrot, I., Sippl, W., Rival, Y.M., Wermuth, C.G. (2001). Design, synthesis, and structure-activity relationships of a series of 3-[2-(1-benzylpiperidin-4-yl)ethylamino]pyridazine derivatives as acetylcholinesterase inhibitors. *J. Med. Chem.* 44:2707-2718.

- Contreras, J.M., Rival, Y.M., Chayer, S., Bourguignon, J.J., Wermuth, C.G. (1999). Aminopyridazines as acetylcholinesterase inhibitors. *J. Med. Chem.* 42:730-741.
- Cordier, C., Morton, D., Murrison, S., Nelson, A., O'Leary-Steele, C. (2008). Natural products as an inspiration in the diversity-oriented synthesis of bioactive compound libraries. *Nat. Prod. Rep.* 25:719-737.
- Costantini, C., Kolasani, R.M., Puglielli, L. (2005). Ceramide and cholesterol: possible connections between normal aging of the brain and Alzheimer's disease. Just hypotheses or molecular pathways to be identified? *Alzheimers Dement.* 1:43-50.
- Cronin, M.T.D., Schultz, T.W. (2003). Pitfalls in QSAR. *J. Mol. Struct.* 622:39-51.
- Crowden, R.K. (1967). Biosynthesis of the polyphenolic acid metabolites of *Polyporus tumulosus* Cooke. *Can. J. Microbiol.* 13:181-197.
- Dalisay, D.S., Molinski, T.F. (2010). Structure elucidation at the nanomole scale. 3. Phorbosides G-I from *Phorbas* sp. *J. Nat. Prod.* 73:679-682.
- Dalisay, D.S., Molinski, T.F. (2009). NMR quantitation of natural products at the nanomole scale. *J. Nat. Prod.* 72:739-744.
- Darvesh, S., Darvesh, K.V., McDonald, R.S., Mataija, D., Walsh, R., Mothana, S., Lockridge, O., Martin, E. (2008). Carbamates with differential mechanism of inhibition toward acetylcholinesterase and butyrylcholinesterase. *J. Med. Chem.* 51:4200-4212.
- Darvesh, S., Hopkins, D.A., Geula, C. (2003a). Neurobiology of butyrylcholinesterase. *Nat. Rev. Neurosci.* 4:131-138.
- Darvesh, S., Walsh, R., Kumar, R., Caines, A., Roberts, S., Magee, D., Rockwood, K., Martin, E. (2003b). Inhibition of human cholinesterases by drugs used to treat Alzheimer disease. *Alzheimer Dis. Assoc. Disord.* 17:117-126.
- Decker, M., Kraus, B., Heilmann, J. (2008). Design, synthesis and pharmacological evaluation of hybrid molecules out of quinazolinimines and lipoic acid lead to highly potent and selective butyrylcholinesterase inhibitors with antioxidant properties. *Bioorg. Med. Chem.* 16:4252-4261.
- Desilets, A.R., Gickas, J.J., Dunican, K.C. (2009). Role of huperzine a in the treatment of Alzheimer's disease. *Ann. Pharmacother.* 43:514-518.
- Di Giovanni, S., Borloz, A., Urbain, A., Marston, A., Hostettmann, K., Carrupt, P.A., Reist, M. (2008). In vitro screening assays to identify natural or synthetic acetylcholinesterase inhibitors: thin layer chromatography versus microplate methods. *Eur. J. Pharm. Sci.* 33:109-119.
- Di, L., Kerns, E.H., Carter, G.T. (2009). Drug-like property concepts in pharmaceutical design. *Curr. Pharm. Des.* 15:2184-2194.
- Diamant, S., Podoly, E., Friedler, A., Ligumsky, H., Livnah, O., Soreq, H. (2006). Butyrylcholinesterase attenuates amyloid fibril formation in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 103:8628-8633.
- Dickerson, T.J., Beuscher, A.E., 4th, Rogers, C.J., Hixon, M.S., Yamamoto, N., Xu, Y., Olson, A.J., Janda, K.D. (2005). Discovery of acetylcholinesterase peripheral anionic site ligands through computational refinement of a directed library. *Biochemistry* 44:14845-14853.

- Dimoglo, A.S., Shvets, N.M., Tetko, I.V., Livingstone, D.J. (2001). Electronic-Topological Investigation of the Structure - Acetylcholinesterase Inhibitor Activity Relationship in the Series of N-Benzylpiperidine Derivatives. *Quant. Struct.-Act. Relat.* 20:31-45.
- Dinamarca, M.C., Arrazola, M., Toledo, E., Cerpa, W.F., Hancke, J., Inestrosa, N.C. (2008). Release of acetylcholinesterase (AChE) from β -amyloid plaques assemblies improves the spatial memory impairments in APP-transgenic mice. *Chem. Biol. Interact.* 175:142-149.
- Drewry, D.H., Macarron, R. (2010). Enhancements of screening collections to address areas of unmet medical need: an industry perspective. *Curr. Opin. Chem. Biol.* 14:289-298.
- Dubois, B., Feldman, H.H., Jacova, C., Dekosky, S.T., Barberger-Gateau, P., Cummings, J., Delacourte, A., Galasko, D., Gauthier, S., Jicha, G., Meguro, K., O'Brien, J., Pasquier, F., Robert, P., Rossor, M., Salloway, S., Stern, Y., Visser, P.J., Scheltens, P. (2007). Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. *Lancet Neurol.* 6:734-746.
- Dvir, H., Silman, I., Harel, M., Rosenberry, T.L., Sussman, J.L. (2010). Acetylcholinesterase: from 3D structure to function. *Chem. Biol. Interact.* 187:10-22.
- Eichler, H.G., Aronsson, B., Abadie, E., Salmonson, T. (2010). New drug approval success rate in Europe in 2009. *Nat. Rev. Drug Discov.* 9:355-356.
- Eldridge, G.R., Vervoort, H.C., Lee, C.M., Cremin, P.A., Williams, C.T., Hart, S.M., Goering, M.G., O'Neil-Johnson, M., Zeng, L. (2002). High-throughput method for the production and analysis of large natural product libraries for drug discovery. *Anal. Chem.* 74:3963-3971.
- Ellman, G.L., Courtney, K.D., Andres, V., Jr, Feather-Stone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7:88-95.
- Entzeroth, M. (2003). Emerging trends in high-throughput screening. *Curr. Opin. Pharmacol.* 3:522-529.
- Ertl, P., Roggo, S., Schuffenhauer, A. (2008). Natural product-likeness score and its application for prioritization of compound libraries. *J. Chem. Inf. Model.* 48:68-74.
- Fan, P., Hay, A., Marston, A., Hostettmann, K. (2008). Acetylcholinesterase-Inhibitory Activity of Linarin from *Buddleja davidii*, Structure-Activity Relationships of Related Flavonoids, and Chemical Investigation of *Buddleja nitida*. *Pharm. Biol.* 46:596-601.
- Fang, L., Jumpertz, S., Zhang, Y., Appenroth, D., Fleck, C., Mohr, K., Trankle, C., Decker, M. (2010). Hybrid molecules from xanomeline and tacrine: enhanced tacrine actions on cholinesterases and muscarinic M1 receptors. *J. Med. Chem.* 53:2094-2103.
- Farlow, M.R., Cummings, J.L. (2007). Effective pharmacologic management of Alzheimer's disease. *Am. J. Med.* 120:388-397.
- Feher, M., Schmidt, J.M. (2003). Property distributions: differences between drugs, natural products, and molecules from combinatorial chemistry. *J. Chem. Inf. Comput. Sci.* 43:218-227.
- Fernandez, S., Wasowski, C., Paladini, A.C., Marder, M. (2004). Sedative and sleep-enhancing properties of linarin, a flavonoid-isolated from *Valeriana officinalis*. *Pharmacol. Biochem. Behav.* 77:399-404.

- Ferri, C.P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., Hall, K., Hasegawa, K., Hendrie, H., Huang, Y., Jorm, A., Mathers, C., Menezes, P.R., Rimmer, E., Scazufca, M., Alzheimer's Disease International (2005). Global prevalence of dementia: a Delphi consensus study. *Lancet* 366:2112-2117.
- Fimea: Finnish Medicines Agency (2010). Medicines, FimeaWeb. (<http://www.fimea.fi/medicines/fimeaweb>).
- Fratiglioni, L., Launer, L.J., Andersen, K., Breteler, M.M., Copeland, J.R., Dartigues, J.F., Lobo, A., Martinez-Lage, J., Soininen, H., Hofman, A. (2000). Incidence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology* 54:S10-5.
- Geula, C., Greenberg, B.D., Mesulam, M.M. (1994). Cholinesterase activity in the plaques, tangles and angiopathy of Alzheimer's disease does not emanate from amyloid. *Brain Res.* 644:327-330.
- Geula, C., Nagykerly, N. (2007). Butyrylcholinesterase activity in the rat forebrain and upper brainstem: postnatal development and adult distribution. *Exp. Neurol.* 204:640-657.
- Giacobini, E. (2004). Cholinesterase inhibitors: new roles and therapeutic alternatives. *Pharmacol. Res.* 50:433-440.
- Giacobini, E. (2003a). Cholinergic function and Alzheimer's disease. *Int. J. Geriatr. Psychiatry.* 18:S1-5.
- Giacobini, E. (2003b). Cholinesterases: new roles in brain function and in Alzheimer's disease. *Neurochem. Res.* 28:515-522.
- Girisha, H.R., Narendra Sharath Chandra, J.N., Boppana, S., Malviya, M., Sadashiva, C.T., Rangappa, K.S. (2009). Active site directed docking studies: synthesis and pharmacological evaluation of cis-2,6-dimethyl piperidine sulfonamides as inhibitors of acetylcholinesterase. *Eur. J. Med. Chem.* 44:4057-4062.
- Glennner, G.G., Wong, C.W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* 120:885-890.
- Golde, T.E., Petrucelli, L., Lewis, J. (2010). Targeting A β and tau in Alzheimer's disease, an early interim report. *Exp. Neurol.* 223:252-266.
- Goodnow, R.A., Jr, Guba, W., Haap, W. (2003). Library design practices for success in lead generation with small molecule libraries. *Comb. Chem. High Throughput Screen.* 6:649-660.
- Greenberg, D.S., Toiber, D., Berson, A., Soreq, H. (2010). Acetylcholinesterase variants in Alzheimer's disease: from neuroprotection to programmed cell death. *Neurodegener. Dis.* 7:60-63.
- Greig, N.H., Utsuki, T., Ingram, D.K., Wang, Y., Pepeu, G., Scali, C., Yu, Q.S., Mamczarz, J., Holloway, H.W., Giordano, T., Chen, D., Furukawa, K., Sambamurti, K., Brossi, A., Lahiri, D.K. (2005). Selective butyrylcholinesterase inhibition elevates brain acetylcholine, augments learning and lowers Alzheimer beta-amyloid peptide in rodent. *Proc. Natl. Acad. Sci. U. S. A.* 102:17213-17218.

- Groner, E., Ashani, Y., Schorer-Apelbaum, D., Sterling, J., Herzig, Y., Weinstock, M. (2007). The kinetics of inhibition of human acetylcholinesterase and butyrylcholinesterase by two series of novel carbamates. *Mol. Pharmacol.* 71:1610-1617.
- Guo, J., Hurley, M.M., Wright, J.B., Lushington, G.H. (2004). A docking score function for estimating ligand-protein interactions: application to acetylcholinesterase inhibition. *J. Med. Chem.* 47:5492-5500.
- Gupta, S., Fallarero, A., Järvinen, P., Karlsson, K., Johnson, M.S., Vuorela, P., Mohan, G.C. (2011). Discovery of dual binding site acetylcholinesterase inhibitors identified by pharmacophore modeling and sequential virtual screening techniques. *Bioorg. Med. Chem. Lett.* 21:1105-1112.
- Gupta, S., Mohan, C.G. (2010). 3D-pharmacophore model based virtual screening to identify dual-binding site and selective acetylcholinesterase inhibitors. *Med. Chem. Res.* DOI 10.1007/s00044-010-9373-7.
- Hamburger, M. (2003). Tracking Bioactivity in Plant Extracts - New Concepts and Approaches, in: Sprecher, E., Caesar, W. (Eds.), Society for Medicinal Plant Research 50 Years 1953-2003, A Jubilee Edition. Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Germany, pp. 109-122.
- Han, L.Y., Ma, X.H., Lin, H.H., Jia, J., Zhu, F., Xue, Y., Li, Z.R., Cao, Z.W., Ji, Z.L., Chen, Y.Z. (2008). A support vector machines approach for virtual screening of active compounds of single and multiple mechanisms from large libraries at an improved hit-rate and enrichment factor. *J. Mol. Graph. Model.* 26:1276-1286.
- Hardy, J., Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297:353-356.
- Hardy, J.A., Higgins, G.A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256:184-185.
- Hashimoto, J., Watanabe, T., Seki, T., Karasawa, S., Izumikawa, M., Seki, T., Iemura, S., Natsume, T., Nomura, N., Goshima, N., Miyawaki, A., Takagi, M., Shin-Ya, K. (2009). Novel *in vitro* protein fragment complementation assay applicable to high-throughput screening in a 1536-well format. *J. Biomol. Screen.* 14:970-979.
- He, X.C., Feng, S., Wang, Z.F., Shi, Y., Zheng, S., Xia, Y., Jiang, H., Tang, X.C., Bai, D. (2007). Study on dual-site inhibitors of acetylcholinesterase: Highly potent derivatives of bis- and bifunctional huperzine B. *Bioorg. Med. Chem.* 15:1394-1408.
- Heinrich, M., Lee Teoh, H. (2004). Galanthamine from snowdrop--the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *J. Ethnopharmacol.* 92:147-162.
- Houston, J.G., Banks, M.N., Binnie, A., Brenner, S., O'Connell, J., Petrillo, E.W. (2008). Case study: impact of technology investment on lead discovery at Bristol-Myers Squibb, 1998-2006. *Drug Discov. Today.* 13:44-51.
- Hughes, B. (2010). 2009 FDA drug approvals. *Nat.Rev.Drug Discov.* 9:89-92.
- Hung, M.S., Xu, Z., Lin, Y.C., Mao, J.H., Yang, C.T., Chang, P.J., Jablons, D.M., You, L. (2009). Identification of hematein as a novel inhibitor of protein kinase CK2 from a natural product library. *BMC Cancer.* 9:135.

- Inestrosa, N.C., Alvarez, A., Perez, C.A., Moreno, R.D., Vicente, M., Linker, C., Casanueva, O.I., Soto, C., Garrido, J. (1996). Acetylcholinesterase accelerates assembly of amyloid- β -peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron* 16:881-891.
- Inestrosa, N.C., Dinamarca, M.C., Alvarez, A. (2008). Amyloid-cholinesterase interactions. Implications for Alzheimer's disease. *FEBS J.* 275:625-632.
- Inghaninan, K., de Best, C.M., van der Heijden, R., Hofte, A.J., Karabatak, B., Irth, H., Tjaden, U.R., van der Greef, J., Verpoorte, R. (2000). High-performance liquid chromatography with on-line coupled UV, mass spectrometric and biochemical detection for identification of acetylcholinesterase inhibitors from natural products. *J. Chromatogr. A.* 872:61-73.
- Inglese, J., Auld, D.S., Jadhav, A., Johnson, R.L., Simeonov, A., Yasgar, A., Zheng, W., Austin, C.P. (2006). Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc. Natl. Acad. Sci. U. S. A.* 103:11473-11478.
- Jang, M.H., Piao, X.L., Kim, J.M., Kwon, S.W., Park, J.H. (2008). Inhibition of cholinesterase and amyloid-beta aggregation by resveratrol oligomers from *Vitis amurensis*. *Phytother. Res.* 22:544-549.
- Jann, M.W. (2000). Rivastigmine, a new-generation cholinesterase inhibitor for the treatment of Alzheimer's disease. *Pharmacotherapy.* 20:1-12.
- Janzen, W.P., Popa-Burke, I.G. (2009). Advances in improving the quality and flexibility of compound management. *J. Biomol. Screen.* 14:444-451.
- Jayasuriya, H., Herath, K.B., Ondeyka, J.G., Guan, Z., Borris, R.P., Tiwari, S., de Jong, W., Chavez, F., Moss, J., Stevenson, D.W., Beck, H.T., Slattery, M., Zamora, N., Schulman, M., Ali, A., Sharma, N., MacNaul, K., Hayes, N., Menke, J.G., Singh, S.B. (2005). Diterpenoid, steroid, and triterpenoid agonists of liver X receptors from diversified terrestrial plants and marine sources. *J. Nat. Prod.* 68:1247-1252.
- Jia, P., Sheng, R., Zhang, J., Fang, L., He, Q., Yang, B., Hu, Y. (2009). Design, synthesis and evaluation of galanthamine derivatives as acetylcholinesterase inhibitors. *Eur. J. Med. Chem.* 44:772-784.
- Johnson, G., Moore, S.W. (2006). The peripheral anionic site of acetylcholinesterase: structure, functions and potential role in rational drug design. *Curr. Pharm. Des.* 12:217-225.
- Johnston, P.A., Johnston, P.A. (2002). Cellular platforms for HTS: three case studies. *Drug Discov. Today.* 7:353-363.
- Jönsson, L., Berr, C. (2005). Cost of dementia in Europe. *Eur. J. Neurol.* 12 Suppl 1:50-53.
- Jönsson, L., Wimo, A. (2009). The cost of dementia in Europe: a review of the evidence, and methodological considerations. *Pharmacoeconomics* 27:391-403.
- Kang, S.Y., Lee, K.Y., Sung, S.H., Park, M.J., Kim, Y.C. (2001). Coumarins isolated from *Angelica gigas* inhibit acetylcholinesterase: structure-activity relationships. *J. Nat. Prod.* 64:683-685.
- Kapková, P., Heller, E., Unger, M., Folkers, G., Holzgrabe, U. (2005). Random chemistry as a new tool for the generation of small compound libraries: development of a new acetylcholinesterase inhibitor. *J. Med. Chem.* 48:7496-7499.

- Katalinic, M., Rusak, G., Domacinovic Barovic, J., Sinko, G., Jelic, D., Antolovic, R., Kovarik, Z. (2010). Structural aspects of flavonoids as inhibitors of human butyrylcholinesterase. *Eur. J. Med. Chem.* 45:186-192.
- Kern, A., Behl, C. (2009). The unsolved relationship of brain aging and late-onset Alzheimer disease. *Biochim. Biophys. Acta* 1790:1124-1132.
- Keseru, G.M., Makara, G.M. (2009). The influence of lead discovery strategies on the properties of drug candidates. *Nat. Rev. Drug Discov.* 8:203-212.
- Keseru, G.M., Makara, G.M. (2006). Hit discovery and hit-to-lead approaches. *Drug Discov. Today* 11:741-748.
- Khalid, A., Zaheer-ul-Haq, Anjum, S., Khan, M.R., Atta-ur-Rahman, Choudhary, M.I. (2004). Kinetics and structure-activity relationship studies on pregnane-type steroidal alkaloids that inhibit cholinesterases. *Bioorg. Med. Chem.* 12:1995-2003.
- Khan, M.T. (2009). Molecular interactions of cholinesterases inhibitors using *in silico* methods: current status and future prospects. *N. Biotechnol.* 25:331-346.
- Khan, M.T., Orhan, I., Senol, F.S., Kartal, M., Sener, B., Dvorska, M., Smejkal, K., Slapetova, T. (2009). Cholinesterase inhibitory activities of some flavonoid derivatives and chosen xanthone and their molecular docking studies. *Chem. Biol. Interact.* 181:383-389.
- Kwon, Y.E., Park, J.Y., No, K.T., Shin, J.H., Lee, S.K., Eun, J.S., Yang, J.H., Shin, T.Y., Kim, D.K., Chae, B.S., Leem, J.Y., Kim, K.H. (2007). Synthesis, *in vitro* assay, and molecular modeling of new piperidine derivatives having dual inhibitory potency against acetylcholinesterase and A β 1-42 aggregation for Alzheimer's disease therapeutics. *Bioorg. Med. Chem.* 15:6596-6607.
- Lambert, M., Staerk, D., Hansen, S.H., Jaroszewski, J.W. (2005). HPLC-SPE-NMR hyphenation in natural products research: optimization of analysis of *Croton membranaceus* extract. *Magn. Reson. Chem.* 43:771-775.
- Lambert, M., Wolfender, J.L., Staerk, D., Christensen, S.B., Hostettmann, K., Jaroszewski, J.W. (2007). Identification of natural products using HPLC-SPE combined with CapNMR. *Anal. Chem.* 79:727-735.
- Lang, G., Mayhudin, N.A., Mitova, M.I., Sun, L., van der Sar, S., Blunt, J.W., Cole, A.L., Ellis, G., Laatsch, H., Munro, M.H. (2008). Evolving trends in the dereplication of natural product extracts: new methodology for rapid, small-scale investigation of natural product extracts. *J. Nat. Prod.* 71:1595-1599.
- Lang, G., Mitova, M.I., Ellis, G., van der Sar, S., Phipps, R.K., Blunt, J.W., Cummings, N.J., Cole, A.L., Munro, M.H. (2006). Bioactivity profiling using HPLC/microtiter-plate analysis: application to a New Zealand marine alga-derived fungus, *Gliocladium* sp. *J. Nat. Prod.* 69:621-624.
- Langjae, R., Bussarawit, S., Yuenyongsawad, S., Ingkaninan, K., Plubrukarn, A. (2007). Acetylcholinesterase-inhibiting steroidal alkaloid from the sponge *Corticium* sp. *Steroids* 72:682-685.
- Larsson, J., Gottfries, J., Muresan, S., Backlund, A. (2007). ChemGPS-NP: tuned for navigation in biologically relevant chemical space. *J. Nat. Prod.* 70:789-794.

- Lee, M.L., Schneider, G. (2001). Scaffold architecture and pharmacophoric properties of natural products and trade drugs: application in the design of natural product-based combinatorial libraries. *J. Comb. Chem.* 3:284-289.
- Lee, S.I., Kim, B.S., Kim, K.S., Lee, S., Shin, K.S., Lim, J.S. (2008). Immune-suppressive activity of punicalagin via inhibition of NFAT activation. *Biochem. Biophys. Res. Commun.* 371:799-803.
- Lee, S.S., Venkatesham, U., Rao, C.P., Lam, S.H., Lin, J.H. (2007). Preparation of secologorins against acetylcholinesterase. *Bioorg. Med. Chem.* 15:1034-1043.
- Lengauer, T., Lemmen, C., Rarey, M., Zimmermann, M. (2004). Novel technologies for virtual screening. *Drug Discov. Today* 9:27-34.
- Leon, R., de los Rios, C., Marco-Contelles, J., Huertas, O., Barril, X., Luque, F.J., Lopez, M.G., Garcia, A.G., Villarroya, M. (2008). New tacrine-dihydropyridine hybrids that inhibit acetylcholinesterase, calcium entry, and exhibit neuroprotection properties. *Bioorg. Med. Chem.* 16:7759-7769.
- Li, B., Stribley, J.A., Ticu, A., Xie, W., Schopfer, L.M., Hammond, P., Brimijoin, S., Hinrichs, S.H., Lockridge, O. (2000). Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. *J. Neurochem.* 75:1320-1331.
- Li, J.W., Vederas, J.C. (2009). Drug discovery and natural products: end of an era or an endless frontier? *Science* 325:161-165.
- Lin, Y., Schiavo, S., Orjala, J., Vouros, P., Kautz, R. (2008). Microscale LC-MS-NMR platform applied to the identification of active cyanobacterial metabolites. *Anal. Chem.* 80:8045-8054.
- Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J. (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 23:3-25.
- Liston, D.R., Nielsen, J.A., Villalobos, A., Chapin, D., Jones, S.B., Hubbard, S.T., Shalaby, I.A., Ramirez, A., Nason, D., White, W.F. (2004). Pharmacology of selective acetylcholinesterase inhibitors: implications for use in Alzheimer's disease. *Eur. J. Pharmacol.* 486:9-17.
- Lleo, A., Greenberg, S.M., Growdon, J.H. (2006). Current pharmacotherapy for Alzheimer's disease. *Annu. Rev. Med.* 57:513-533.
- Loizzo, M.R., Tundis, R., Menichini, F., Menichini, F. (2008). Natural products and their derivatives as cholinesterase inhibitors in the treatment of neurodegenerative disorders: an update. *Curr. Med. Chem.* 15:1209-1228.
- Lubbe, A., Pomahacova, B., Choi, Y.H., Verpoorte, R. (2010). Analysis of metabolic variation and galanthamine content in *Narcissus* bulbs by ¹H NMR. *Phytochem. Anal.* 21:66-72.
- Lushington, G.H., Guo, J.X., Hurley, M.M. (2006). Acetylcholinesterase: molecular modeling with the whole toolkit. *Curr. Top. Med. Chem.* 6:57-73.
- Lv, W., Xue, Y. (2010). Prediction of acetylcholinesterase inhibitors and characterization of correlative molecular descriptors by machine learning methods. *Eur. J. Med. Chem.* 45:1167-1172.
- Ma, X., Tan, C., Zhu, D., Gang, D.R., Xiao, P. (2007). Huperzine A from *Huperzia* species--an ethnopharmacological review. *J. Ethnopharmacol.* 113:15-34.

- Macarron, R., Hertzberg, R.P. (2009). Design and implementation of high-throughput screening assays. *Methods Mol. Biol.* 565:1-32.
- Mancini, F., Naldi, M., Cavrini, V., Andrisano, V. (2007). Multiwell fluorometric and colorimetric microassays for the evaluation of β -secretase (BACE-1) inhibitors. *Anal. Bioanal. Chem.* 388:1175-1183.
- Marco-Contelles, J., Leon, R., de los Rios, C., Samadi, A., Bartolini, M., Andrisano, V., Huertas, O., Barril, X., Luque, F.J., Rodriguez-Franco, M.I., Lopez, B., Lopez, M.G., Garcia, A.G., Carreiras Mdo, C., Villarroya, M. (2009). Tacripyrines, the first tacrine-dihydropyridine hybrids, as multitarget-directed ligands for the treatment of Alzheimer's disease. *J. Med. Chem.* 52:2724-2732.
- Marin, P.D., Grayer, R.J., Veitch, N.C., Kite, G.C., Harborne, J.B. (2001). Acacetin glycosides as taxonomic markers in *Calamintha* and *Micromeria*. *Phytochemistry* 58:943-947.
- Marston, A., Kissling, J., Hostettmann, K. (2002). A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants. *Phytochem. Anal.* 13:51-54.
- Martinez-Vazquez, M., Ramirez Apan, T.O., Aguilar, H., Bye, R. (1996). Analgesic and antipyretic activities of an aqueous extract and of the flavone linarin of *Buddleia cordata*. *Planta Med.* 62:137-140.
- Martinez-Vazquez, M., Ramirez Apan, T.O., Lastra, A.L., Bye, R. (1998). A comparative study of the analgesic and anti-inflammatory activities of pectolarin isolated from *Cirsium subcoriaceum* and linarin isolated from *Buddleia cordata*. *Planta Med.* 64:134-137.
- Masson, P., Carletti, E., Nachon, F. (2009). Structure, activities and biomedical applications of human butyrylcholinesterase. *Protein Pept. Lett.* 16:1215-1224.
- Masson, P., Froment, M.T., Gillon, E., Nachon, F., Darvesh, S., Schopfer, L.M. (2007). Kinetic analysis of butyrylcholinesterase-catalyzed hydrolysis of acetanilides. *Biochim. Biophys. Acta.* 1774:1139-1147.
- Matson, S.L., Chatterjee, M., Stock, D.A., Leet, J.E., Dumas, E.A., Ferrante, C.D., Monahan, W.E., Cook, L.S., Watson, J., Cloutier, N.J., Ferrante, M.A., Houston, J.G., Banks, M.N. (2009). Best practices in compound management for preserving compound integrity and accurately providing samples for assays. *J. Biomol. Screen.* 14:476-484.
- Mayr, L.M., Bojanic, D. (2009). Novel trends in high-throughput screening. *Curr. Opin. Pharmacol.* 9:580-588.
- McGovern, S.L., Caselli, E., Grigorieff, N., Shoichet, B.K. (2002). A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J. Med. Chem.* 45:1712-1722.
- McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., Stadlan, E.M. (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 34:939-944.
- Mesulam, M. (2004). The cholinergic lesion of Alzheimer's disease: pivotal factor or side show? *Learn. Mem.* 11:43-49.

- Mesulam, M., Guillozet, A., Shaw, P., Quinn, B. (2002a). Widely spread butyrylcholinesterase can hydrolyze acetylcholine in the normal and Alzheimer brain. *Neurobiol. Dis.* 9:88-93.
- Mesulam, M.M., Guillozet, A., Shaw, P., Levey, A., Duysen, E.G., Lockridge, O. (2002b). Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. *Neuroscience* 110:627-639.
- Metzger, A., Diller, D.J., Lin, T.H., Henderson, I., Webb, M.L. (2006). Successful screening of large encoded combinatorial libraries leading to the discovery of novel p38 MAP kinase inhibitors. *Comb. Chem. High Throughput Screen.* 9:351-358.
- Moran, M.A., Mufson, E.J., Gomez-Ramos, P. (1993). Colocalization of cholinesterases with β amyloid protein in aged and Alzheimer's brains. *Acta Neuropathol.* 85:362-369.
- Mosconi, L., Rinne, J.O., Tsui, W.H., Berti, V., Li, Y., Wang, H., Murray, J., Scheinin, N., Nagren, K., Williams, S., Glodzik, L., De Santi, S., Vallabhajosula, S., de Leon, M.J. (2010). Increased fibrillar amyloid- β burden in normal individuals with a family history of late-onset Alzheimer's. *Proc. Natl. Acad. Sci. U. S. A.* 107:5949-5954.
- Munos, B. (2009). Lessons from 60 years of pharmaceutical innovation. *Nat. Rev. Drug Discov.* 8:959-968.
- Munoz, F.J., Inestrosa, N.C. (1999). Neurotoxicity of acetylcholinesterase amyloid β -peptide aggregates is dependent on the type of Abeta peptide and the AChE concentration present in the complexes. *FEBS Lett.* 450:205-209.
- Munoz-Ruiz, P., Rubio, L., Garcia-Palomero, E., Dorronsoro, I., del Monte-Millan, M., Valenzuela, R., Usan, P., de Austria, C., Bartolini, M., Andrisano, V., Bidon-Chanal, A., Orozco, M., Luque, F.J., Medina, M., Martinez, A. (2005a). Design, synthesis, and biological evaluation of dual binding site acetylcholinesterase inhibitors: new disease-modifying agents for Alzheimer's disease. *J. Med. Chem.* 48:7223-7233.
- Munoz-Ruiz, P., Rubio, L., Garcia-Palomero, E., Dorronsoro, I., del Monte-Millan, M., Valenzuela, R., Usan, P., de Austria, C., Bartolini, M., Andrisano, V., Bidon-Chanal, A., Orozco, M., Luque, F.J., Medina, M., Martinez, A. (2005b). Design, synthesis, and biological evaluation of dual binding site acetylcholinesterase inhibitors: new disease-modifying agents for Alzheimer's disease. *J. Med. Chem.* 48:7223-7233.
- Nassar, A.E., Kamel, A.M., Clarimont, C. (2004). Improving the decision-making process in the structural modification of drug candidates: enhancing metabolic stability. *Drug Discov. Today.* 9:1020-1028.
- Newman, D.J. (2008). Natural products as leads to potential drugs: an old process or the new hope for drug discovery? *J. Med. Chem.* 51:2589-2599.
- Newman, D.J., Cragg, G.M. (2007). Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* 70:461-477.
- Newman, D.J., Cragg, G.M., Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.* 66:1022-1037.
- Nielsen, J. (2002). Combinatorial synthesis of natural products. *Curr. Opin. Chem. Biol.* 6:297-305.

- Nochi, S., Asakawa, N., Sato, T. (1995). Kinetic study on the inhibition of acetylcholinesterase by 1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidine hydrochloride (E2020). *Biol. Pharm. Bull.* 18:1145-1147.
- Näslund, J., Haroutunian, V., Mohs, R., Davis, K.L., Davies, P., Greengard, P., Buxbaum, J.D. (2000). Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *JAMA* 283:1571-1577.
- Ogura, H., Kosasa, T., Kuriya, Y., Yamanishi, Y. (2000). Comparison of inhibitory activities of donepezil and other cholinesterase inhibitors on acetylcholinesterase and butyrylcholinesterase in vitro. *Methods Find. Exp. Clin. Pharmacol.* 22:609-613.
- Orhan, I., KKartal, M., Naz, Q., Ejaz, A., Yilmaz, G., Kan, Y., Konuklugil, B., Sener, B., Choudhary, M.I. (2007). Antioxidant and anticholinesterase evaluation of selected Turkish *Salvia* species. *Food Chem.* 103:1247-1254.
- Ortholand, J.Y., Ganesan, A. (2004). Natural products and combinatorial chemistry: back to the future. *Curr. Opin. Chem. Biol.* 8:271-280.
- Pan, L., Tan, J.H., Hou, J.Q., Huang, S.L., Gu, L.Q., Huang, Z.S. (2008). Design, synthesis and evaluation of isaindigotone derivatives as acetylcholinesterase and butyrylcholinesterase inhibitors. *Bioorg. Med. Chem. Lett.* 18:3790-3793.
- Papandreou, M.A., Dimakopoulou, A., Linardaki, Z.I., Cordopatis, P., Klimis-Zacas, D., Margarity, M., Lamari, F.N. (2009). Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. *Behav. Brain Res.* 198:352-358.
- Parejo, I., Jauregui, O., Sanchez-Rabaneda, F., Viladomat, F., Bastida, J., Codina, C. (2004). Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. *J. Agric. Food Chem.* 52:3679-3687.
- Paul, S.M., Mytelka, D.S., Dunwiddie, C.T., Persinger, C.C., Munos, B.H., Lindborg, S.R., Schacht, A.L. (2010). How to improve R&D productivity: the pharmaceutical industry's grand challenge. *Nat. Rev. Drug Discov.* 9:203-214.
- Pepeu, G., Giovannini, M.G. (2009). Cholinesterase inhibitors and beyond. *Curr. Alzheimer Res.* 6:86-96.
- Pereira, D.M., Ferreres, F., Oliveira, J., Valentao, P., Andrade, P.B., Sottomayor, M. (2009). Targeted metabolite analysis of *Catharanthus roseus* and its biological potential. *Food Chem. Toxicol.* 47:1349-1354.
- Petersen, R.C., Smith, G.E., Waring, S.C., Ivnik, R.J., Tangalos, E.G., Kokmen, E. (1999). Mild cognitive impairment: clinical characterization and outcome. *Arch. Neurol.* 56:303-308.
- Piazzzi, L., Cavalli, A., Colizzi, F., Belluti, F., Bartolini, M., Mancini, F., Recanatini, M., Andrisano, V., Rampa, A. (2008). Multi-target-directed coumarin derivatives: hAChE and BACE1 inhibitors as potential anti-Alzheimer compounds. *Bioorg. Med. Chem. Lett.* 18:423-426.
- Piazzzi, L., Rampa, A., Bisi, A., Gobbi, S., Belluti, F., Cavalli, A., Bartolini, M., Andrisano, V., Valenti, P., Recanatini, M. (2003). 3-(4-[[Benzyl(methyl)amino] methyl]phenyl)-6,7-dimethoxy-2H-2-chromenone (AP2238) inhibits both acetylcholinesterase and

- acetylcholinesterase-induced β -amyloid aggregation: a dual function lead for Alzheimer's disease therapy. *J. Med. Chem.* 46:2279-2282.
- Pietsch, M., Christian, L., Inhester, T., Petzold, S., Gutschow, M. (2009). Kinetics of inhibition of acetylcholinesterase in the presence of acetonitrile. *FEBS J.* 276:2292-2307.
- Pietsch, M., Gutschow, M. (2005). Synthesis of tricyclic 1,3-oxazin-4-ones and kinetic analysis of cholesterol esterase and acetylcholinesterase inhibition. *J. Med. Chem.* 48:8270-8288.
- Pimplikar, S.W. (2009). Reassessing the amyloid cascade hypothesis of Alzheimer's disease. *Int. J. Biochem. Cell Biol.* 41:1261-1268.
- Pisani, L., Catto, M., Giangreco, I., Leonetti, F., Nicolotti, O., Stefanachi, A., Cellamare, S., Carotti, A. (2010). Design, synthesis, and biological evaluation of coumarin derivatives tethered to an edrophonium-like fragment as highly potent and selective dual binding site acetylcholinesterase inhibitors. *Chem. Med. Chem.* 5:1616-1630.
- Plewczynski, D., Spieser, S.A., Koch, U. (2009). Performance of machine learning methods for ligand-based virtual screening. *Comb. Chem. High Throughput Screen.* 12:358-368.
- Polinsky, R.J. (1998). Clinical pharmacology of rivastigmine: a new-generation acetylcholinesterase inhibitor for the treatment of Alzheimer's disease. *Clin. Ther.* 20:634-647.
- Posner, B.A., Xi, H., Mills, J.E. (2009). Enhanced HTS hit selection via a local hit rate analysis. *J. Chem. Inf. Model.* 49:2202-2210.
- Potterat, O., Hamburger, M. (2006). Natural Products in Drug Discovery - Concepts and Approaches for Tracking Bioactivity. *Curr. Org. Chem.* 10:899-920.
- Qiu, C., De Ronchi, D., Fratiglioni, L. (2007). The epidemiology of the dementias: an update. *Curr. Opin. Psychiatry.* 20:380-385.
- Queiroz, E.F., Wolfender, J.L., Hostettmann, K. (2009). Modern approaches in the search for new lead antiparasitic compounds from higher plants. *Curr. Drug Targets.* 10:202-211.
- Quinn, D. (1987). Acetylcholinesterase: Enzyme Structure, Reaction Dynamics, and Virtual Transition States. *Chem. Rev.* 87:955-979.
- Rampa, A., Piazzzi, L., Belluti, F., Gobbi, S., Bisi, A., Bartolini, M., Andrisano, V., Cavrini, V., Cavalli, A., Recanatini, M., Valenti, P. (2001). Acetylcholinesterase inhibitors: SAR and kinetic studies on omega-[N-methyl-N-(3-alkylcarbamoyloxyphenyl)methyl]aminoalkoxyaryl derivatives. *J. Med. Chem.* 44:3810-3820.
- Rauha, J.P., Remes, S., Heinonen, M., Hopia, A., Kahkonen, M., Kujala, T., Pihlaja, K., Vuorela, H., Vuorela, P. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiol.* 56:3-12.
- Recanatini, M., Cavalli, A., Belluti, F., Piazzzi, L., Rampa, A., Bisi, A., Gobbi, S., Valenti, P., Andrisano, V., Bartolini, M., Cavrini, V. (2000). SAR of 9-amino-1,2,3,4-tetrahydroacridine-based acetylcholinesterase inhibitors: synthesis, enzyme inhibitory activity, QSAR, and structure-based CoMFA of tacrine analogues. *J. Med. Chem.* 43:2007-2018.

- Reitz, C., Honig, L., Vonsattel, J.P., Tang, M.X., Mayeux, R. (2009). Memory performance is related to amyloid and tau pathology in the hippocampus. *J. Neurol. Neurosurg. Psychiatry* 80:715-721.
- Reyes, A.E., Chacon, M.A., Dinamarca, M.C., Cerpa, W., Morgan, C., Inestrosa, N.C. (2004). Acetylcholinesterase-A β complexes are more toxic than A β fibrils in rat hippocampus: effect on rat β -amyloid aggregation, laminin expression, reactive astrocytosis, and neuronal cell loss. *Am. J. Pathol.* 164:2163-2174.
- Reyes, A.E., Perez, D.R., Alvarez, A., Garrido, J., Gentry, M.K., Doctor, B.P., Inestrosa, N.C. (1997). A monoclonal antibody against acetylcholinesterase inhibits the formation of amyloid fibrils induced by the enzyme. *Biochem. Biophys. Res. Commun.* 232:652-655.
- Rhee, I.K., van de Meent, M., Ingkaninan, K., Verpoorte, R. (2001). Screening for acetylcholinesterase inhibitors from *Amaryllidaceae* using silica gel thin-layer chromatography in combination with bioactivity staining. *J. Chromatogr. A* 915:217-223.
- Rhee, I.K., van Rijn, R.M., Verpoorte, R. (2003). Qualitative determination of false-positive effects in the acetylcholinesterase assay using thin layer chromatography. *Phytochem. Anal.* 14:127-131.
- Rishton, G.M. (2008). Molecular diversity in the context of leadlikeness: compound properties that enable effective biochemical screening. *Curr. Opin. Chem. Biol.* 12:340-351.
- Rishton, G.M. (2003). Nonleadlikeness and leadlikeness in biochemical screening. *Drug Discov. Today* 8:86-96.
- Rizzo, S., Bartolini, M., Ceccarini, L., Piazzzi, L., Gobbi, S., Cavalli, A., Recanatini, M., Andrisano, V., Rampa, A. (2010). Targeting Alzheimer's disease: Novel indanone hybrids bearing a pharmacophoric fragment of AP2238. *Bioorg. Med. Chem.* 18:1749-1760.
- Rizzo, S., Riviere, C., Piazzzi, L., Bisi, A., Gobbi, S., Bartolini, M., Andrisano, V., Morroni, F., Tarozzi, A., Monti, J.P., Rampa, A. (2008). Benzofuran-based hybrid compounds for the inhibition of cholinesterase activity, β amyloid aggregation, and a β neurotoxicity. *J. Med. Chem.* 51:2883-2886.
- Rollinger, J.M., Hornick, A., Langer, T., Stuppner, H., Prast, H. (2004). Acetylcholinesterase inhibitory activity of scopolin and scopoletin discovered by virtual screening of natural products. *J. Med. Chem.* 47:6248-6254.
- Rollinger, J.M., Mocka, P., Zidorn, C., Ellmerer, E.P., Langer, T., Stuppner, H. (2005). Application of the in combo screening approach for the discovery of non-alkaloid acetylcholinesterase inhibitors from *Cichorium intybus*. *Curr. Drug Discov. Technol.* 2:185-193.
- Rosen, J., Gottfries, J., Muresan, S., Backlund, A., Oprea, T.I. (2009). Novel chemical space exploration via natural products. *J. Med. Chem.* 52:1953-1962.
- Rosenberry, T.L. (2010). Strategies to resolve the catalytic mechanism of acetylcholinesterase. *J. Mol. Neurosci.* 40:32-39.
- Rountree, S.D., Chan, W., Pavlik, V.N., Darby, E.J., Siddiqui, S., Doody, R.S. (2009). Persistent treatment with cholinesterase inhibitors and/or memantine slows clinical progression of Alzheimer disease. *Alzheimers Res. Ther.* DOI: 10.1186/alzrt7

- Ryan, A.J., Gray, N.M., Lowe, P.N., Chung, C.W. (2003). Effect of detergent on "promiscuous" inhibitors. *J. Med. Chem.* 46:3448-3451.
- Salam, N.K., Huang, T.H., Kota, B.P., Kim, M.S., Li, Y., Hibbs, D.E. (2008a). Novel PPAR-gamma agonists identified from a natural product library: a virtual screening, induced-fit docking and biological assay study. *Chem. Biol. Drug Des.* 71:57-70.
- Salam, N.K., Huang, T.H., Kota, B.P., Kim, M.S., Li, Y., Hibbs, D.E. (2008b). Novel PPAR-gamma agonists identified from a natural product library: a virtual screening, induced-fit docking and biological assay study. *Chem. Biol. Drug Des.* 71:57-70.
- Samuelsson, G., Bohlin, L. (2009). Drugs of natural origin: a treatise of pharmacognosy. 6th revised edition. Swedish Pharmaceutical Press, Stockholm, Sweden. pp. 776.
- Sangnoi, Y., Sakulkeo, O., Yuenyongsawad, S., Kanjana-opas, A., Ingkaninan, K., Plubrukarn, A., Suwanborirux, K. (2008). Acetylcholinesterase-inhibiting activity of pyrrole derivatives from a novel marine gliding bacterium, *Rapidithrix thailandica*. *Mar. Drugs.* 6:578-586.
- Saracoglu, M., Kandemirli, F. (2008). The investigation of structure-activity relationships of tacrine analogues: electronic-topological method. *Open Med. Chem. J.* 2:75-80.
- Sauvatre, T., Barlier, M., Herlem, D., Gresh, N., Chiaroni, A., Guenard, D., Guillou, C. (2007). New potent acetylcholinesterase inhibitors in the tetracyclic triterpene series. *J. Med. Chem.* 50:5311-5323.
- Savva, G.M., Wharton, S.B., Ince, P.G., Forster, G., Matthews, F.E., Brayne, C., Medical Research Council Cognitive Function and Ageing Study (2009). Age, neuropathology, and dementia. *N. Engl. J. Med.* 360:2302-2309.
- Saxena, A., Redman, A.M., Jiang, X., Lockridge, O., Doctor, B.P. (1997). Differences in active site gorge dimensions of cholinesterases revealed by binding of inhibitors to human butyrylcholinesterase. *Biochemistry* 36:14642-14651.
- Schliebs, R., Arendt, T. (2006). The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. *J. Neural Transm.* 113:1625-1644.
- Schuster, D., Kern, L., Hristozov, D.P., Terfloth, L., Bienfait, B., Laggner, C., Kirchmair, J., Grienke, U., Wolber, G., Langer, T., Stuppner, H., Gasteiger, J., Rollinger, J.M. (2010). Applications of integrated data mining methods to exploring natural product space for acetylcholinesterase inhibitors. *Comb. Chem. High Throughput Screen.* 13:54-66.
- Scior, T., Medina-Franco, J.L., Do, Q.T., Martinez-Mayorga, K., Yunes Rojas, J.A., Bernard, P. (2009). How to recognize and workaroud pitfalls in QSAR studies: a critical review. *Curr. Med. Chem.* 16:4297-4313.
- Seedhouse, S.J., Labuda, L.P., Disney, M.D. (2010). The Privileged Chemical Space Predictor (PCSP): a computer program that identifies privileged chemical space from screens of modularly assembled chemical libraries. *Bioorg. Med. Chem. Lett.* 20:1338-1343.
- Selkoe, D.J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* 399:A23-31.
- Shafferman, A., Kronman, C., Flashner, Y., Leitner, M., Grosfeld, H., Ordentlich, A., Gozes, Y., Cohen, S., Ariel, N., Barak, D. (1992). Mutagenesis of human acetylcholinesterase.

- Identification of residues involved in catalytic activity and in polypeptide folding. *J. Biol. Chem.* 267:17640-17648.
- Shaneyfelt, M.E., Burke, A.D., Graff, J.W., Jutila, M.A., Hardy, M.E. (2006). Natural products that reduce rotavirus infectivity identified by a cell-based moderate-throughput screening assay. *Viol. J.* 3:68.
- Shen, Q., Peng, Q., Shao, J., Liu, X., Huang, Z., Pu, X., Ma, L., Li, Y.M., Chan, A.S., Gu, L. (2005). Synthesis and biological evaluation of functionalized coumarins as acetylcholinesterase inhibitors. *Eur. J. Med. Chem.* 40:1307-1315.
- Shih, H.W., Chen, K.T., Chen, S.K., Huang, C.Y., Cheng, T.J., Ma, C., Wong, C.H., Cheng, W.C. (2010). Combinatorial approach toward synthesis of small molecule libraries as bacterial transglycosylase inhibitors. *Org. Biomol. Chem.* 8:2586-2593.
- Silman, I., Sussman, J.L. (2005). Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. *Curr. Opin. Pharmacol.* 5:293-302.
- Singh, R.P., Agrawal, P., Yim, D., Agarwal, C., Agarwal, R. (2005). Acacetin inhibits cell growth and cell cycle progression, and induces apoptosis in human prostate cancer cells: structure-activity relationship with linarin and linarin acetate. *Carcinogenesis* 26:845-854.
- Sippl, W., Contreras, J.M., Parrot, I., Rival, Y.M., Wermuth, C.G. (2001). Structure-based 3D QSAR and design of novel acetylcholinesterase inhibitors. *J. Comput. Aided Mol. Des.* 15:395-410.
- Sopkova-de Oliveira Santos, J., Lesnard, A., Agondanou, J.H., Dupont, N., Godard, A.M., Stiebing, S., Rochais, C., Fabis, F., Dallemagne, P., Bureau, R., Rault, S. (2010). Virtual screening discovery of new acetylcholinesterase inhibitors issued from CERMN chemical library. *J. Chem. Inf. Model.* 50:422-428.
- Soreq, H., Seidman, S. (2001). Acetylcholinesterase--new roles for an old actor. *Nat. Rev. Neurosci.* 2:294-302.
- Spandl, R.J., Bender, A., Spring, D.R. (2008). Diversity-oriented synthesis; a spectrum of approaches and results. *Org. Biomol. Chem.* 6:1149-1158.
- Spuch, C., Antequera, D., Isabel Fernandez-Bachiller, M., Isabel Rodriguez-Franco, M., Carro, E. (2010). A new tacrine-melatonin hybrid reduces amyloid burden and behavioral deficits in a mouse model of Alzheimer's disease. *Neurotox Res.* 17:421-431.
- Stahura, F.L., Bajorath, J. (2004). Virtual screening methods that complement HTS. *Comb. Chem. High Throughput Screen.* 7:259-269.
- Steger, M., Hubschwerlen, C., Schmid, G. (2001). Solid- and solution-phase synthesis of highly-substituted-pyrrolidine libraries. *Bioorg. Med. Chem. Lett.* 11:2537-2540.
- Sterling, J., Herzig, Y., Goren, T., Finkelstein, N., Lerner, D., Goldenberg, W., Miskolczi, I., Molnar, S., Rantal, F., Tamas, T., Toth, G., Zagyva, A., Zekany, A., Finberg, J., Lavian, G., Gross, A., Friedman, R., Razin, M., Huang, W., Kraus, B., Chorev, M., Youdim, M.B., Weinstock, M. (2002). Novel dual inhibitors of AChE and MAO derived from hydroxy aminoindan and phenethylamine as potential treatment for Alzheimer's disease. *J. Med. Chem.* 45:5260-5279.

- Stojan, J., Zorko, M. (1997). Kinetic characterization of all steps of the interaction between acetylcholinesterase and eserine. *Biochim. Biophys. Acta.* 1337:75-84.
- Storch, A., Schrattenholz, A., Cooper, J.C., Abdel Ghani, E.M., Gutbrod, O., Weber, K.H., Reinhardt, S., Lobron, C., Hermsen, B., Soskic, V. (1995). Physostigmine, galanthamine and codeine act as 'noncompetitive nicotinic receptor agonists' on clonal rat pheochromocytoma cells. *Eur. J. Pharmacol.* 290:207-219.
- Sugimoto, H., Tsuchiya, Y., Sugumi, H., Higurashi, K., Karibe, N., Iimura, Y., Sasaki, A., Kawakami, Y., Nakamura, T., Araki, S. (1990). Novel piperidine derivatives. Synthesis and anti-acetylcholinesterase activity of 1-benzyl-4-[2-(N-benzoylamino)ethyl]piperidine derivatives. *J. Med. Chem.* 33:1880-1887.
- Suhonen, J., Pirttilä, T., Erkinjuntti, T., Koponen, H., Makkonen, M., Puurunen, M., Raivio, M., Rinne, J., Rosenvall, A., Strandberg, T., Vanninen, R., Vataja, R. (2010). Muistisairauksien diagnostiikka ja lääkehoito. *Duodecim* 126:2167-2168.
- Sui, Y., Wu, Z. (2007). Alternative statistical parameter for high-throughput screening assay quality assessment. *J. Biomol. Screen.* 12:229-234.
- Sukuru, S.C., Jenkins, J.L., Beckwith, R.E., Scheiber, J., Bender, A., Mikhailov, D., Davies, J.W., Glick, M. (2009). Plate-based diversity selection based on empirical HTS data to enhance the number of hits and their chemical diversity. *J. Biomol. Screen.* 14:690-699.
- Sulkava, R. (2010). Evidence-Based Medicine Guidelines: Alzheimer's disease. Publisher: 2010 Duodecim Medical Publications Ltd. DOI: Article ID: ebm00756 (036.054).
- Svetaz, L., Zuljan, F., Derita, M., Petenatti, E., Tamayo, G., Caceres, A., Cechinel Filho, V., Gimenez, A., Pinzon, R., Zacchino, S.A., Gupta, M. (2010). Value of the ethnomedical information for the discovery of plants with antifungal properties. A survey among seven Latin American countries. *J. Ethnopharmacol.* 127:137-158.
- Taft, C.A., Da Silva, V.B., Da Silva, C.H. (2008). Current topics in computer-aided drug design. *J. Pharm. Sci.* 97:1089-1098.
- Takahashi, J., Hijikuro, I., Kihara, T., Muruges, M.G., Fuse, S., Kunimoto, R., Tsumura, Y., Akaike, A., Niidome, T., Okuno, Y., Takahashi, T., Sugimoto, H. (2010a). Design, synthesis, evaluation and QSAR analysis of N(1)-substituted norcymserine derivatives as selective butyrylcholinesterase inhibitors. *Bioorg. Med. Chem. Lett.* 20:1718-1720.
- Takahashi, J., Hijikuro, I., Kihara, T., Muruges, M.G., Fuse, S., Tsumura, Y., Akaike, A., Niidome, T., Takahashi, T., Sugimoto, H. (2010b). Design, synthesis and evaluation of carbamate-modified (-)-N(1)-phenethylnorphysostigmine derivatives as selective butyrylcholinesterase inhibitors. *Bioorg. Med. Chem. Lett.* 20:1721-1723.
- Tang, H., Ning, F.X., Wei, Y.B., Huang, S.L., Huang, Z.S., Chan, A.S., Gu, L.Q. (2007). Derivatives of oxoisoaporphine alkaloids: a novel class of selective acetylcholinesterase inhibitors. *Bioorg. Med. Chem. Lett.* 17:3765-3768.
- Tang, H., Wei, Y.B., Zhang, C., Ning, F.X., Qiao, W., Huang, S.L., Ma, L., Huang, Z.S., Gu, L.Q. (2009). Synthesis, biological evaluation and molecular modeling of oxoisoaporphine and oxoaporphine derivatives as new dual inhibitors of acetylcholinesterase/butyrylcholinesterase. *Eur. J. Med. Chem.* 44:2523-2532.

- Thorne, N., Auld, D.S., Inglese, J. (2010). Apparent activity in high-throughput screening: origins of compound-dependent assay interference. *Curr. Opin. Chem. Biol.* 14:315-324.
- Toiber, D., Berson, A., Greenberg, D., Melamed-Book, N., Diamant, S., Soreq, H. (2008). N-acetylcholinesterase-induced apoptosis in Alzheimer's disease. *PLoS One.* 3:e3108. DOI: 10.1371/journal.pone.0003108.
- van Marum, R.J. (2008). Current and future therapy in Alzheimer's disease. *Fundam. Clin. Pharmacol.* 22:265-274.
- Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M.A., Biere, A.L., Curran, E., Burgess, T., Louis, J.C., Collins, F., Treanor, J., Rogers, G., Citron, M. (1999). β -secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 286:735-741.
- Vellom, D.C., Radic, Z., Li, Y., Pickering, N.A., Camp, S., Taylor, P. (1993). Amino acid residues controlling acetylcholinesterase and butyrylcholinesterase specificity. *Biochemistry* 32:12-17.
- Vilar, S., Chakrabarti, M., Costanzi, S. (2010). Prediction of passive blood-brain partitioning: straightforward and effective classification models based on *in silico* derived physicochemical descriptors. *J. Mol. Graph. Model.* 28:899-903.
- Villalobos, A., Blake, J.F., Biggers, C.K., Butler, T.W., Chapin, D.S., Chen, Y.L., Ives, J.L., Jones, S.B., Liston, D.R., Nagel, A.A. (1994). Novel benzisoxazole derivatives as potent and selective inhibitors of acetylcholinesterase. *J. Med. Chem.* 37:2721-2734.
- Vinutha, B., Prashanth, D., Salma, K., Sreeja, S.L., Pratiti, D., Padmaja, R., Radhika, S., Amit, A., Venkateshwarlu, K., Deepak, M. (2007). Screening of selected Indian medicinal plants for acetylcholinesterase inhibitory activity. *J. Ethnopharmacol.* 109:359-363.
- Wagenaar, M.M. (2008). Pre-fractionated microbial samples--the second generation natural products library at Wyeth. *Molecules* 13:1406-1426.
- Wang, H., Zhou, G., Gao, X., Wang, Y., Yao, W. (2007). Acetylcholinesterase inhibitory-active components of *Rhodiola rosea* L. *Food Chem.* 105:24-27.
- Waring, M. (2010). Lipophilicity in drug discovery. *Expert Opin. Drug Discover.* 5:235-248.
- Waring, M.J. (2009). Defining optimum lipophilicity and molecular weight ranges for drug candidates-Molecular weight dependent lower logD limits based on permeability. *Bioorg. Med. Chem. Lett.* 19:2844-2851.
- Wen, H., Lin, C., Que, L., Ge, H., Ma, L., Cao, R., Wan, Y., Peng, W., Wang, Z., Song, H. (2008). Synthesis and biological evaluation of helacid analogues as novel acetylcholinesterase inhibitors. *Eur. J. Med. Chem.* 43:166-173.
- Wennberg, T., Kreander, K., Lähdevuori, M., Vuorel, H., Vuorela, P. (2004). Primary Screening of Natural products using micro fractionation combined with a bioassay. *J Liq Chromatogr R. T.* 27:2573-2592.
- Wiesner, J., Kriz, Z., Kuca, K., Jun, D., Koca, J. (2007). Acetylcholinesterases--the structural similarities and differences. *J. Enzyme Inhib. Med. Chem.* 22:417-424.

- Williams, M.M., Xiong, C., Morris, J.C., Galvin, J.E. (2006). Survival and mortality differences between dementia with Lewy bodies vs Alzheimer disease. *Neurology* 67:1935-1941.
- Wolters, A.M., Jayawickrama, D.A., Webb, A.G., Sweedler, J.V. (2002). NMR detection with multiple solenoidal microcoils for continuous-flow capillary electrophoresis. *Anal. Chem.* 74:5550-5555.
- Young, K.J., Bennett, J.P. (2010). The mitochondrial secret(ase) of Alzheimer's disease. *J. Alzheimers Dis.* 20 Suppl 2:S381-400.
- Yu, Q., Holloway, H.W., Flippen-Anderson, J.L., Hoffman, B., Brossi, A., Greig, N.H. (2001). Methyl analogues of the experimental Alzheimer drug phenserine: synthesis and structure/activity relationships for acetyl- and butyrylcholinesterase inhibitory action. *J. Med. Chem.* 44:4062-4071.
- Zaheer-ul-Haq, Halim, S.A., Uddin, R., Madura, J.D. (2010). Benchmarking docking and scoring protocol for the identification of potential acetylcholinesterase inhibitors. *J. Mol. Graph. Model.* 28:870-882.
- Zhan, Z.J., Bian, H.L., Wang, J.W., Shan, W.G. (2010). Synthesis of physostigmine analogues and evaluation of their anticholinesterase activities. *Bioorg. Med. Chem. Lett.* 20:1532-1534.
- Zhang, H.Y., Tang, X.C. (2006). Neuroprotective effects of huperzine A: new therapeutic targets for neurodegenerative disease. *Trends Pharmacol. Sci.* 27:619-625.
- Zhang, J.H., Chung, T.D., Oldenburg, K.R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* 4:67-73.
- Zhao, B., Moochhala, S.M., Tham, S.Y. (2004). Biologically active components of *Physostigma venenosum*. *J. Chromatogr. B. Analyt Technol. Biomed. Life Sci.* 812:183-192.
- Zhou, X., Wang, X.B., Wang, T., Kong, L.Y. (2008). Design, synthesis, and acetylcholinesterase inhibitory activity of novel coumarin analogues. *Bioorg. Med. Chem.* 16:8011-8021.
- Zhu, Y., Xiao, K., Ma, L., Xiong, B., Fu, Y., Yu, H., Wang, W., Wang, X., Hu, D., Peng, H., Li, J., Gong, Q., Chai, Q., Tang, X., Zhang, H., Li, J., Shen, J. (2009). Design, synthesis and biological evaluation of novel dual inhibitors of acetylcholinesterase and beta-secretase. *Bioorg. Med. Chem.* 17:1600-1613.