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FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

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Design and Synthesis of Hybrid Compounds Based on Tacrine/Resveratrol Derivatives

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I would like to thank my supervisors, Prof. PharmDr. Martin Doležal, Ph.D., Prof. Maria Laura Bolognesi and PharmDr. Jan Korábečný, Ph.D., who lectured and oversaw my work all along. I am also very grateful to the research groups at the Department of Pharmaceutical Chemistry and the Faculty of Pharmaceutical Sciences, University of Bologna for a very pleasant working environment.

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I declare that this Diploma Thesis is an original author's copy, worked out by myself (under the supervision of Prof. PharmDr. Martin Doležal, Ph.D.). All literature and other sources used in this Thesis are quoted and listed in the literature section. This Diploma Thesis was not used to gain another or the same academic title.

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Supported by SVV 260 183 and by Erasmus program.

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ABSTRACT

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Title of Thesis: Design and Synthesis of Hybrid Compounds Based on Tacrine/Resveratrol Derivatives

Alzheimer‘s disease (AD) is a progressive neurodegenerative brain disorder, in which a progressive dementia appears. The cause of AD is currently unknown, however, scientific research has revealed several pathological hallmarks - β-amyloid plaques and neurofibrillary tangles. These changes cause gradual disintegration of nerve cells and they change the metabolism in the brain. The current drugs are not able to treat the cause of the disease, being able only to delay the onset of severe symptoms. The basic drugs for AD treatment are acetylcholinesterase (AChE, E.C. 3.1.1.7) inhibitors and, more recently approved, *N*-methyl-*D*-aspartate (NMDA) receptor antagonist memantine. These drugs are able to increase cholinergic activity or preventing glutamate excitotoxicity in the patient’s brain, thus improving cognitive functions and delaying severe stages of the disease. One of the emerging approaches in drug synthesis represents multi-target-directed ligands (MTDLs). Apart from the ability to inhibit AChE, they can also target more pathological processes at once. As such, they are able to bring an added value in a single molecule. In this work, we turned our attention to the preparation of hybrid compounds based on tacrine and resveratrol moieties. Tacrine scaffold act as cholinesterase inhibitor, whereas resveratrol is a strong antioxidant, naturally occurring in the vine. We assumed that coupling of these moieties could lead to the derivatives affecting multiple pathological targets of the disease and consequently represent new leads for AD therapy.

ABSTRAKT

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Název práce: Design and Synthesis of Hybrid Compounds Based on Tacrin/Resveratrol Derivatives

Alzheimerova choroba (AD) je progresivní neurodegenerativní onemocnění mozku, při kterém dochází k postupné demenci. Příčina vzniku AD je v současné době neznámá, nicméně vědecký výzkum odhalil několik patologických charakteristických znaků - β-amyloidní plaky a neurofibrilární klubka. Tyto změny způsobují postupný rozpad nervových buněk a mění metabolismus v mozku. Současné léky nejsou schopny léčit příčinu onemocnění, jsou pouze schopny oddálit nástup závažných příznaků. Základní léčiva pro léčbu AD jsou inhibitory acetylcholinesterasy (AChE, EC 3.1.1.7) a nedávno schválený antagonista N-methyl-D-aspartátových (NMDA) receptorů memantin. Tyto léky zvyšují cholinergní aktivitu nebo tlumí excitotoxicitu glutamátu v mozku pacienta, čímž dojde ke zlepšení kognitivních funkcí a oddálení závažných stádií onemocnění. Jedním z rozvíjených přístupů v syntéze nových léčiv je příprava multifunkčních léčiv (MTDLs). Kromě schopnosti inhibovat AChE, jsou schopny zacílit další patologické procesy probíhající v mozku pacienta. Tím přinášejí další přidanou hodnotu v jedné jediné molekule. V této práci jsme se zaměřili na přípravu hybridních látek na bázi takrinu a resveratrolu. Takrin zde působí jako inhibitor cholinesterasy, zatímco resveratrol je silný antioxidant, přirozeně se vyskytující ve vinné révě. Předpokládáme, že spojení těchto molekul by mohlo vést k přípravě derivátů, které ovlivňují více patologických cílů onemocnění a mohly by představovat vodítko v terapii AD.

1 ABBREVIATIONS

Aβ	amyloid β peptide
ACh	acetylcholine
AChE	acetylcholinesterase
AChEIs	acetylcholinesterase inhibitors
AD	Alzheimer‟s disease
ApoE ε4	Apolipoprotein E4
APP	amyloid precursor protein
BChE	butyrylcholinesterase
CAS	catalytic active site
ChAT	choline acetyltransferase
ChE	cholinesterase
ChEI	cholinesterase inhibitor
ER	endoplasmic reticulum
GSK-3	glycogen synthase kinase 3
InsP3R	inositol triphosphate
mAChR	muscarinic acetylcholine receptor
MMSE	Mini Mental State Examination
MMT	multiple medication therapy
MTDLs	multi-target-directed ligands
nAChR	nicotinic acetylcholine receptor
NMDA	*N*-methyl-*D*-aspartate
PAS	peripheral anionic site
PrPc	cellular prion protein
PSEN1	presenilin 1
ROS	reactive oxygen species
*Tc*AChE	*Torpedo californica* acetylcholinesterase

2 THEORETICAL PART

2.1 ALZHEIMER'S DISEASE

2.1.1 Clinical image

Alzheimer's disease (AD) is the most common cause of dementia, representing almost 60% of all cases of dementia. The main symptoms are progressive cognitive decline, emotional disorders, behavioural disorders and even the inability to perform daily activities in severe stages of the disease. AD has a slow start manifested by a set of nonspecific signs announcing the onset of the disease. This stage can be several years long. In the initial stage, failure of memory is the most obvious symptom of the disease, causing the patient troubles to remember recent events, forgetting to perform common tasks and having difficulties with learning new information. In the next stage, the memory is even more affected: the patient remembers deeply instilled memories, but forgets basic things such as the home address or the names of his or her relatives. Over time, it leads to further deterioration of thinking skills and other brain functions. Furthermore, depressions, anxieties and changes of personality and behaviour appear. Gradually, AD leads to an inability to perform activities of daily living (ADLs), such as hygiene, eating, dressing, using the toilet and the patient loses the ability of independence. In the severe stage, there is almost complete loss of memory, the patient is unable to perform ADLs and dependent on the institutional care. Death occurs on average 5-8 years after diagnosis. There are several tests used to evaluate the severity of the disease. The most common test is called Mini Mental State Examination (MMSE), which consists of 30 questions. Its output can determine the mild dementia (24 points), severe dementia starts at 9 points. This test can help physicians to determine what treatment to use.[5, 6]

2.1.2 Prevalence

AD is a worldwide problem. The number of individuals suffering from dementia in the age range 60-64 years is less than 1%, but it increases with age, almost exponentially. In the age group of 85+ years, almost 30% is affected. Currently, AD is one of the major health problems in the society.[7]

It is estimated that in 2001, 24,300,000 people suffered from dementia worldwide. It is expected that in 2020, this number will increase to 42.3 million and to 81.1 million in 2040, suggesting that the amount of new cases is almost doubled every 20 years. Countries and regions with the largest proportion of affected individuals are United States, China, Western Pacific and

Western Europe. In the Czech Republic, it is estimated that more than 120,000 patients suffer from this disease.[8, 9]

2.1.3 Risk factors

The prevalence of AD is closely related to age – the higher the age, the higher the incidence. It is estimated that from 65 years of age, every 8th person suffers from this serious illness. Women are more often affected by AD than men, especially in a very advanced age.[10]

Another important factors are the genetic determinants. Mutations occur to alleles of the amyloid precursor protein (APP), presenilins; however, the presence of the apolipoprotein E4 allele (ApoE ε4) is considered the most dangerous mutation. The risk of AD onset also promote cardiovascular diseases, hypertension, diabetes mellitus, dyslipidaemia, obesity, bad habits such as smoking or excessive alcohol consumption and brain traumas. On the other hand, the beneficial protective factors against AD onset seem to be a higher level of education, mental and physical activity. People with higher education and rich social and intellectual lives have a significantly lower risk of developing AD. Epidemiological studies suggest a correlation between the risk of developing AD and the use of certain types of substances such as antioxidants, vitamin C and E, non-steroidal anti-inflammatory agents, etc. However, the results of these studies are quite ambivalent. Some studies reject this correlation[11, 12] while other studies have found that statin and fruit intake for example were associated with a lower risk of AD mortality.[13]

2.1.4 Pathophysiology

An irreversible damage and neuronal death is the primary cause of AD type dementia. The affected areas are mainly temporal lobes, amygdala and hippocampus. On the other hand, motor and sensory nuclei seem to be intact. This is the reason for preserving the motor and sensory function in AD patients until the late stages of the disease. It also occurs to suppression of cholinergic functions. This fact is the principle for cholinergic theory.[2]

There are two biological hallmarks – β-amyloid plaques and neurofibrillary tangles. Microscopic changes are noticeable inside and outside of the affected cells. The helix-shaped fibrils form inside neurons, known as neurofibrillary tangles. The basic element of the neurofibrillary tangles is Tau protein that belongs to the family of proteins associated with microtubules. Normally, Tau is well soluble; in tangles, however, it occurs in an insoluble hyper phosphorylated form unable to perform its physiological function. The enzyme called Glycogen synthase kinase-3 (GSK-3β) is responsible for its synthesis. The phosphorylated form of the protein has a modified conformation

which is not capable of binding to microtubules. The integrity of cytoskeleton is significantly deteriorated, disrupting other cellular functions associated witch microtubules as well.[2, 14, 15]

Another biological hallmark is the presence of amyloid plaques that accumulate extracellularly. They are formed from APP by cut that is made by two secretases – β-secretase and γ-secretase. APP is found on the surface of many cell structures – the plasma membrane, Golgi apparatus, endoplasmic reticulum (ER), endosome, lysosome and mitochondria. Normally, α-secretase snips APP, releasing a soluble fragment of APP (sAPP). β-secretase and γ-secretase enzymes also snip APP to release slightly different fragments of APP, leading to the formation of short fragments of amyloid (Aβ) with a length of 38-43 amino acids. These fragments occur in low concentrations in the spinal fluid and also in the blood of healthy people without any cognitive symptoms. Nevertheless, the Aβ fragments can spontaneously aggregate to form soluble Aβ oligomers (diffuse plaques). As more fragments are added, these oligomers increase in size and become insoluble, eventually forming insoluble fibrillary forms. The whole process is finished by forming senile plaques.[2, 16, 17]

There is also the presence of activated microglial cells close around of amyloid deposits. In a healthy brain surface receptors of neurons and glial cells interact together. These interactions are essential to suppress the activation of glial cells leading to the development of pro-inflammatory cascade and subsequently to the damage of neurons. In the brain with amyloid structures it occurs to formation of antigens for microglia, which prompt their activation. An activated glial cell then produces free radicals and supports the pro-inflammatory and immune responses. It also occurs to damage of healthy neurons.

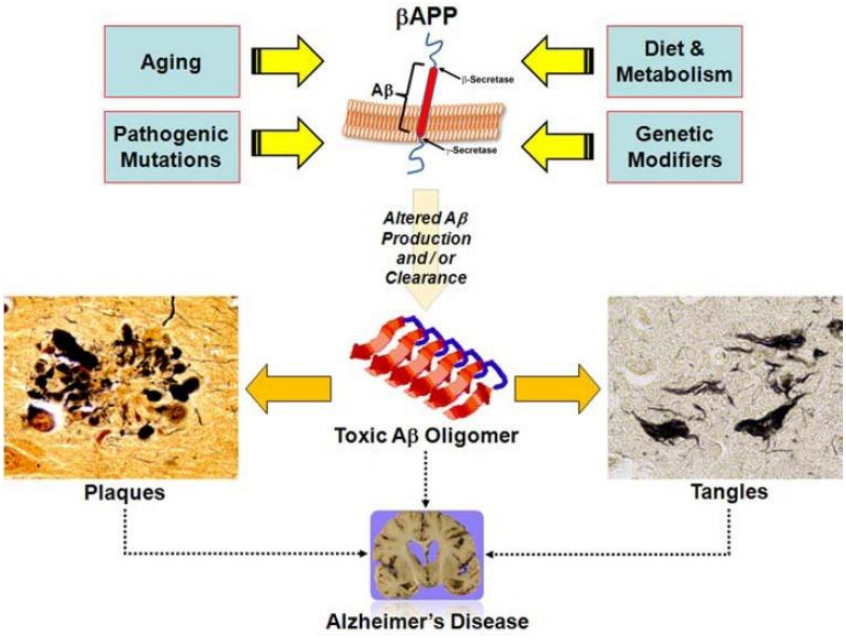


Figure 1. Factors Affecting Amyloid Accumulation and AD Pathology.[2]

An amyloid pathway also causes an increased influx of extracellular Ca^{2+} into the cell which is releasing from intracellular stores. Aβ affects receptors of cellular prion protein (PrPc) located outside of the cell membrane, thus affecting receptor channels, especially NMDA receptors. Moreover, Aβ can directly affect the influx of Ca^{2+} by forming transmembrane channels. A release of intracellular Ca^{2+} from the ER can be facilitated by several mechanisms. Mutated PSEN1 increases receptors' sensitivity to inositol triphosphate (InsP3R) and thereby provides an easier release of calcium from the ER. A certain role in the cellular Ca2 + homeostasis is played by an intracellular domain of APP resulting from cleavage of γ secretase. Modification of Ca^{2+} mediated signal negatively affects memory and learning, eventually induce cell death.[18, 19]

2.2 CHOLINERGIC HYPOTHESIS

2.2.1 Acetylcholine

Acetylcholine (ACh) is a small molecule composed of choline and acetyl, acting as a neurotransmitter and neuromodulator of autonomic nervous system and CNS. ACh is an agonist of two basic types of receptors:[20] Nicotinic acetylcholine receptors (nAChR, also known as "ionotropic" acetylcholine receptors) and Muscarinic acetylcholine receptors (mAChR, also known as "metabotropic" acetylcholine receptors). These two receptors are quite different, nAChR is linked to voltage-controlled ion channels and structurally divided into neuronal (NN) type, which is located in vegetative ganglia and in the CNS and muscular (NM) type that is located at the neuromuscular junction. The mAChRs are linked with signalling cascade and with a formation of other signalling molecules. Their response to stimulation is slower than the response of N receptors. There are five subtypes of M receptor (M1-5): M1,3,5 have excitatory response while M2,4 have inhibitory response.[20, 21]

ACh participate in a signal transmission of pre-ganglionic sympathetic and parasympathetic fibers, post-ganglionic parasympathetic fibers and neuromuscular transmission. It is also responsible for attention, motor skills, memory and learning ability in the CNS. ACh is synthesized in the cytosol of the presynaptic neurons from choline and acetyl-CoA by the enzyme choline acetyltransferase (ChAT, EC 2.3.1.6). If the action potential is changed, ACh is released into the synapse from the vesicles where the ACh is stored. The effect of ACh is rapidly terminated by cholinesterases which decompose ACh to choline and acetic acid. Choline is then scavenged and used for the synthesis of further ACh.[20, 22]

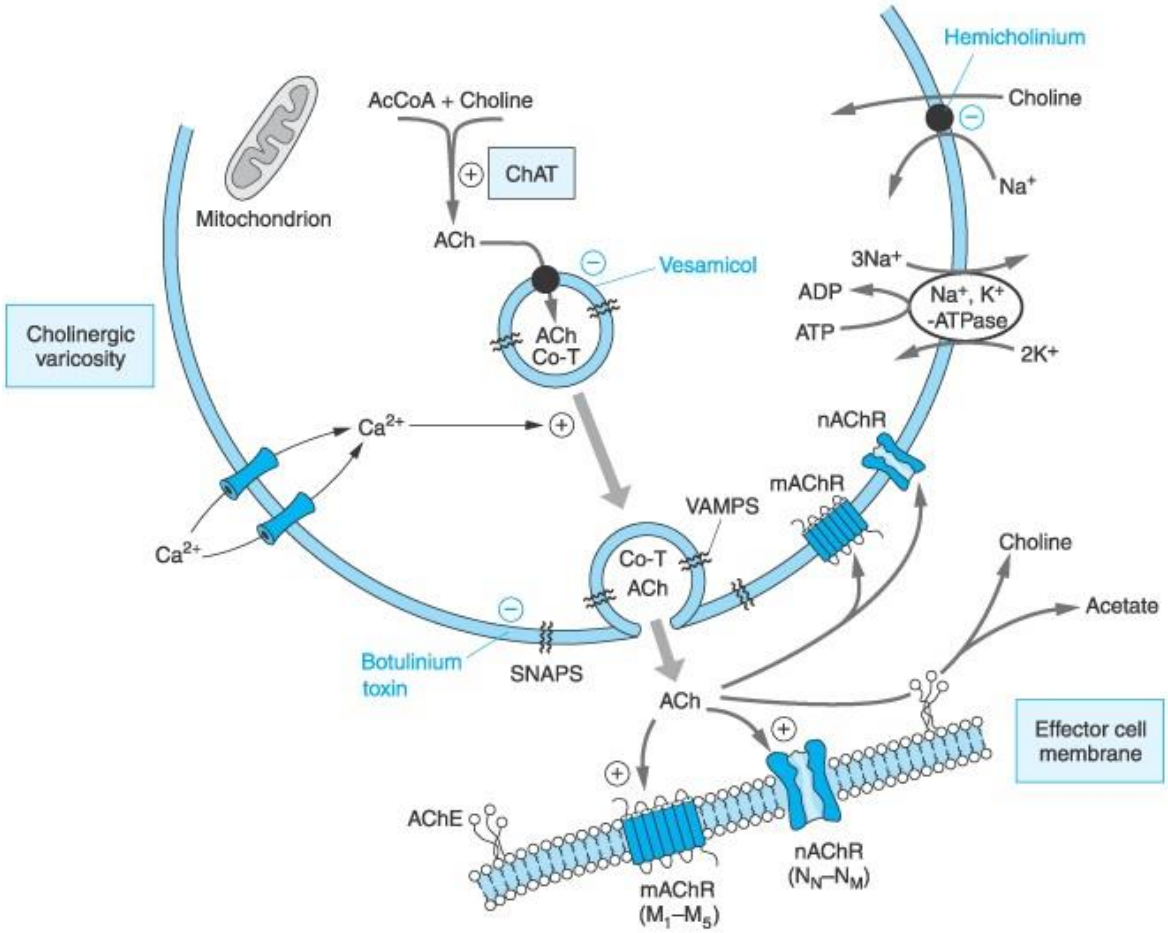


Figure 2. Synthesis, storage, and release of ACh and receptors on which ACh acts.[1]

2.2.2 Cholinergic hypothesis

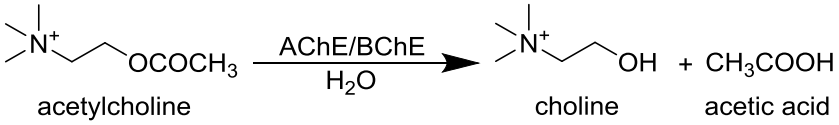
The cholinergic hypothesis was the first theory proposed to explain AD and led to the development of the only drugs currently approved to treat mild to moderate AD. The finding that a loss of cholinergic activity is present in the brains of AD patients is the base of this theory. Experimental studies in humans and non-human primates have suggested a role of ACh in learning and memory. By blocking cholinergic receptors with scopolamine, we can induce similar symptoms of memory impairment to the symptoms of patients with AD. The administration of cholinergic agent, Physostigmine, can reverse this process and the memory is improved. This finding supports ACh being a relevant factor in the learning and memory processes. Under further, more detailed examination of AD patients, a decline of activity of ChAT and decreased uptake of choline by presynaptic neurons was observed. [23]

Currently, this theory is still the basic theory in respect to AD treatment; an increase of cholinergic activity was the only AD treatment for a long time. For this purpose, the most important compounds are acetylcholinesterase inhibitors (AChEI). Clinical studies have confirmed their beneficial effects in AD treatment compared with placebo. Unfortunately, they are often short-term effects and the disease progresses. There are several approved drugs in AD treatment such as AChEIs which are used most often, but not all the patients respond to the treatment by this drugs.[24]

2.3 CHOLINESTERASES

2.3.1 Characteristic

Cholinesterases are the enzymes catalysing the conversion of choline esters to the corresponding acids and choline. By this mechanism, they are able to terminate impulse transmission at cholinergic synapses. The most important is the hydrolysis of ACh to choline and acetic acid in the synaptic cleft.



Scheme 1. Hydrolysis of ACh to choline and acetic acid.

There are two types of cholinesterases: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8). Their names are based on natural substrates which are able to decompose. Substrate for BChE hasn't been known yet. This enzyme is often referred to as

plasma cholinesterase or pseudocholinesterase. Despite their similarity, these enzymes' primary functions are different: AChE terminates cholinergic neurotransmission and is indispensable for life, while the exact physiological function of BChE is not fully known and its deficiency or defect does not lead to pathological manifestations. It can play a role of reserve cholinesterase in case of the inhibition of AChE.[25]

2.3.2 Structure

The structure of the AChE has been studied intensively since the 90s. The first AChE was acquired from *Torpedo californica* in a crystalline form designated as *Tc*AChE. *Tc*AChE is α/β hydrolase composed of 537 amino acids. It consists of β folded sheets and catalytic triad Ser-His-Glu surrounded by α helix. The most noticeable part of AChE is the 20 amino acid long straight cavity situated in the middle of the enzyme. Its walls are formed by aromatic amino acids and there is a active site of the enzyme (CAS) at the bottom. The active site is composed of two main parts – the acyl site and the anionic site. The acyl site of *Tc*AChE contains catalytic triad – Ser 200, His 440, Glu 327 –, while the anionic site, being close to the acyl site, is composed of amino acids – Trp 84, Tyr 330, Phe 331. The acyl site hydrolyzes the ester bond while the anionic site enables a correct orientation of ACh for interaction with quaternary nitrogen and carbonyl oxygen by forming a hydrogen bond. Aromatic walls of the cavity ensure substrate selectivity. BChE is slightly different and lacks the peripheral anionic site which allows binding of larger molecules.[26]

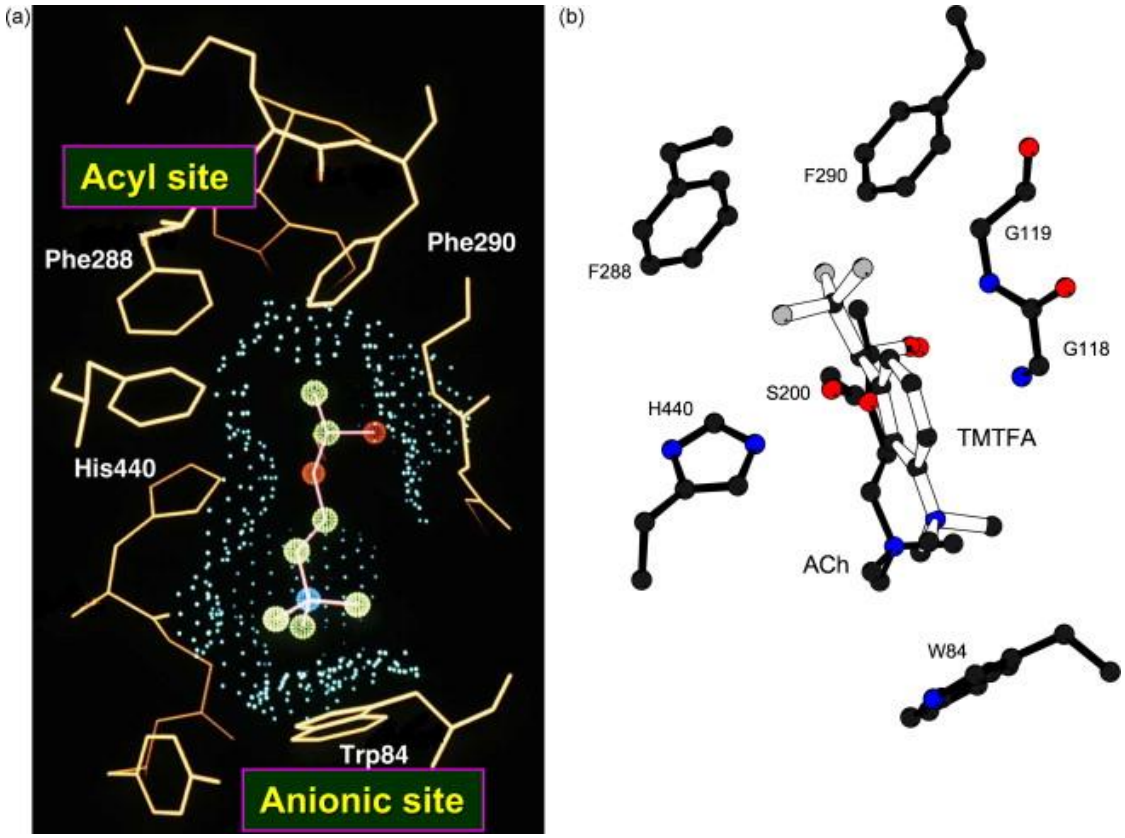


Figure 3. The active site of AChE. (a) Model of ACh bound in the active site of *Tc*AChE; (b) close-up of the active site of the TMTFA-*Tc*AChE complex, showing the experimentally determined TMTFA moiety (open-face lines) together with a superimposed model of ACh docked in the active site (solid lines). Several key residues in the binding pocket are indicated.[3]

Another indispensable part for the functionality of AChE is the peripheral anionic site (PAS) located at the entrance of CAS and composed of five amino acids – Tyr 70, Asp 72, Tyr 121, Trp 279 and Tyr 334. This site has the ability to allosterically modify CAS and prevent access to the cavity of the substrate. Its function is the uptake of the substrates on the way to the active site and thus increasing the catalytic efficiency of the enzyme – this is the main difference between AChE and BChE. In the case of AChE, a high substrate concentration can inhibit the enzyme by substrate inhibition. BChE, on the other hand, does not have the peripheral anionic site so there is no substrate inhibition. Peripheral anionic site has the ability to bind many different molecules, even Aβ, and thus induce its aggregation. Targeting of PAS can even affect pathogenesis of AD.[27, 28]

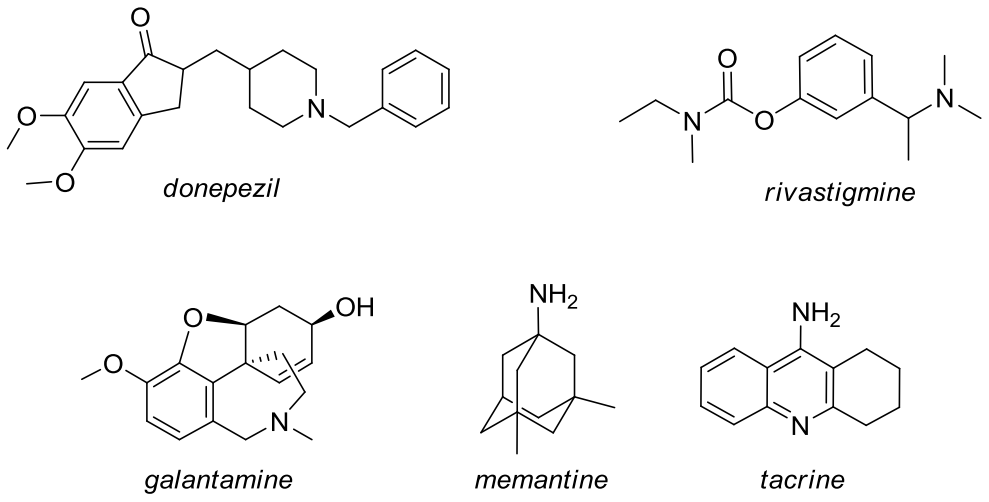
2.4 CHOLINESTERASE INHIBITORS

As described above, the major cause of memory impairment and cognitive function deterioration is the degeneration of cholinergic neurons and the associated disorder of cholinergic neurotransmission. One of the possibilities to improve cholinergic neurotransmission is increasing the levels of ACh at synapses. The inhibition of AChE is the most preferred method for increasing the ACh levels.[2]

ChEIs are classified according to their selectivity and the type of inhibition into selective/non-selective and reversible/irreversible inhibitors respectively. Selective inhibitors have a higher affinity for either AChE or BChE, whereas non-selective inhibitors are equally effective in inhibiting both of the enzymes, AChE and BChE. Reversible inhibitors have the ability to form a complex with the enzyme; however, the complex disintegrates over time and the enzyme activity is restored. Irreversible inhibitors bind to cholinesterase irreversibly. It is necessary to use AChE reactivators for the restoration of the activity of the enzyme or a new enzyme must be synthesized. Reversible inhibitors include for example non-selective tacrine or selective donepezil. Irreversible inhibitors are mainly organophosphates, e.g., Sarin and Tabun which are used as chemical weapons.[29]

Adverse effects manifested in AChEI therapy are most often associated with exaggerated peripheral cholinergic activity including gastrointestinal disturbances such as nausea, vomiting and diarrhoea; occasionally also muscle spasms and bradycardia.[29]

2.4.1 AChE inhibitors used in therapy of Alzheimer's disease



Scheme 2. FDA - approved drugs + tacrine

2.4.1.1 Tacrine

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) was synthesized in 1945 and pharmacologically described in 1949. It was observed that tacrine has the effect upon AChE and BChE in 1960. In 1986, almost 30 years later, the beneficial effects of tacrine in AD were reported.[29]

In 1993, tacrine was approved by FDA for treatment of mild to moderate stages of the disease under the brand name Cognex. Unfortunately, it was shortly found that tacrine has quite serious side effects, especially hepatotoxicity. Approximately half of the patients who had received tacrine during clinical trials had increased levels of liver enzyme alanine aminotransferase (ALT; EC 2.6.1.2). One quarter of the patients showed an increased ALT levels up to three times and 8% even ten times higher than the highest physiological levels of this enzyme in blood. In some cases, liver biopsies showed lobular hepatitis. Currently, tacrine is no longer registered in the USA and is almost unused due to its hepatotoxicity.[29]

2.4.1.2 Galantamine

Galantamine is a tertiary alkaloid which was approved in 2001 by the FDA and is commonly marketed. It was isolated in 1952 from snowdrop (Galanthus nivalis - Amaryllidaceae) and used in the AD treatment for the first time in 1987. [29]

The meta-analysis examined the effects of galantamine on cognitive and global functions using a dosage range of 8-36 mg/day. Generally, galantamine treatment produced a significant improvement on the ADAS-cog scores in six trials that used this scale. These effects were significant at 3 months and even more pronounced at 6 months.[30]

The action's mechanism is the inhibition of AChE by binding galantamine into the active site of the enzyme and the allosteric modulation of nicotinic receptors. A therapy is initiated at a dose of 8 mg/day and gradually increases to 24 mg/day. Galantamine is taken twice a day, in the morning and evening hours.[29]

2.4.1.3 Donepezil

In 1996, FDA approved donepezil (2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1-one) for treatment of all AD stages under the brand name Aricept.[29]

The meta-analysis found that donepezil showed improvements on the ADAS-cog and Mini-Mental State Examination (MMSE) in a dosage range of 5-10 mg/day. There are some evidences indicating that donepezil protects cortical neurons against glutamate toxicity, prevents apoptotic cell

death, increases expression of nicotinic receptors and decreases Aß production and Aß-induced toxicity.[30]

Donepezil has selectivity for AChE resulting in lower incidence of cholinergic side effects in the periphery. A therapy is initiated at a dose of 5 mg/day and gradually increases to 10 mg/day. Donepezil is taken once a day.[29]

2.4.1.4 Rivastigmine

In 2000, FDA approved Rivastigmine [3-[(1*S*)-1-(dimethylamino)ethyl]fenyl]-*N*-ethyl-*N*-methylcarbamate for treatment of all stages of the disease under the brand name Exelon.[29]

"The meta-analysis found that active treatment had a modest benefit in measures of cognition (ADAS-cog), function (PDS, NOSGER) and global assessment of change (CIBIC-Plus). Results on behaviour were not found to be significant."[30]

Rivastigmine is a pseudo-irreversible non-competitive inhibitor. Therapy is initiated at a dose of 1-2 mg/day and gradually increases to 12 mg/day. Rivastigmine is administered twice a day in the morning and evening.[29]

2.4.2 Multi-target-directed ligands

As described above, AD is a multifactorial disease connected with many pathological processes. In this context, ChEIs's mechanism of action represents an outdated drug design strategy which means that one molecule is prepared for single target. In many cases the "one-molecule-one-target" strategy fulfils its purpose, but drugs that affect a single target may not be clinically effective when dealing with multifactorial diseases like AD.[31]

There are three main strategies designed to target more pathological processes at once. The first strategy is a multiple medication therapy (MMT). MMT generally combines two or more different drugs with different therapeutic mechanisms, bringing great problems with compliance, financial efficiency and logistics. The second strategy is a multiple-compound medication (MCM). MCM incorporates different drugs into one pill, providing an increased patient compliance and other efficiency benefits. The third strategy is called a multi-target-directed ligands (MTDLs). A single compound of MTDL can target more pathological processes at once. As such, they are able to bring an added value in a single molecule. Most often, MTDLs are prepared by combining distinct molecules, resulting in acquiring new hybrid molecules. In fact, each moiety can retain its ability to aim at its own specific target and thus the whole molecule can produce multiple pharmacological responses with a positive impact on neurodegenerative processes. Some other

advantages include better ADME profile and a much lower risk of drug-drug interactions due to the simplification of the medication.[4]

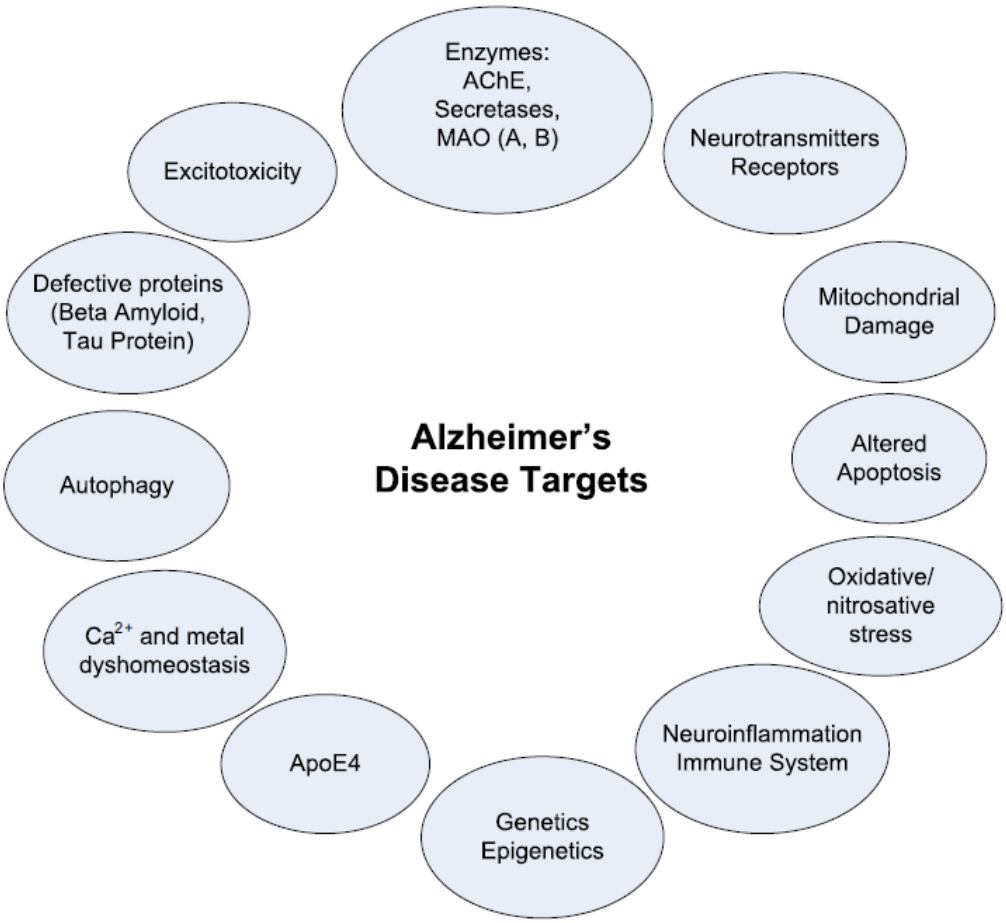


Figure 4. Possible therapeutic targets for designing of new MTDLs in AD treatment.[4]

2.5 ANTIOXIDANTS IN AD TREATMENT

2.5.1 AD and Oxidative stress

As was mentioned previously, AD has a very complex pathogenesis. An increasing amount of evidence suggests a connection between free radical formation and destruction of neurons involved in AD pathogenesis. This thesis is supported by the concept of free radical hypothesis of aging where accumulation of free radicals results in damaged cell components. The age is the key risk factor which connects AD even with free radical hypothesis. There are many specific markers including lipid, DNA and protein oxidation which are definitely involved in the AD pathogenesis. Moreover, epidemiologic studies suggest that the intake of antioxidants present in diet reduces the

risk of AD. Often, the antioxidants also have the ability to inhibit amyloid β deposition. For these reasons, an antioxidant therapy is regarded as one of the more promising approaches in AD therapy.[32]

2.5.1.1 Lipid Peroxidation

Lipid peroxidation plays a role in oxidative stress by attacking the side chain of lipids, which are located in the brain, by reactive oxygen species (ROS). The action's mechanism includes removing of a hydrogen atom from the methylene carbon. The higher the number of double bonds in the molecule, the more vulnerable the molecule is. Therefore, polyunsaturated fatty acids (PUFA) are particularly vulnerable to its peroxidation.

Several clinical studies suggest an increased lipid peroxidation compared with healthy brains. The products of this oxidation are elevated in certain brain regions such as frontal/temporal cortex and hippocampus.[32]

2.5.1.2 Protein Oxidation

Protein oxidation involves a reaction between ROS and backbone and the side chain of the protein. Attack of ROS to side-chains of the proteins leads to formation of multitude of products which can attack again to amino acids to produce carbonyl functions.

Products of this oxidation, carbonyl moieties among others, are elevated in the frontal and parietal lobe as well as in hippocampus.[32]

2.5.1.3 DNA/RNA Oxidation

Nucleic acids can be also attacked by ROS. The resulting defects are very different in kind, including DNA–protein cross-links, base release, strand breaks and a direct modification of purine and pyridine bases. The most popular biomarker linked with the damaged nucleic acids represents hydroxylated guanine.

Mecocci *et al.* reported increased levels of this biomarker in nuclear and mitochondrial DNA obtained from brain samples of AD patients.[32]

2.5.1.4 Glyco-oxidation

Proteins can by damaged even by posttranslational oxidative modifications. Modified glycation produces pathological proteins which are formed when the amino group of proteins reacts

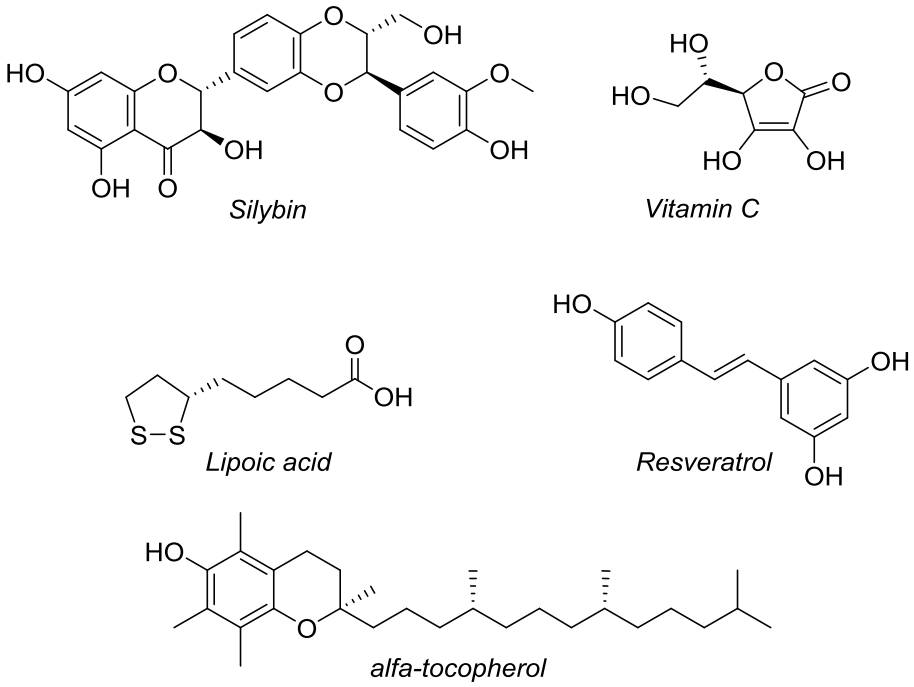
non-enzymatically with monosaccharides. These products are linked with Aβ aggregation and increased levels of this biomarker are widely found in AD brains.[32]

2.5.2 Overview of antioxidants

Generally, antioxidants are molecules with the ability to eliminate ROS or RNS that naturally occur in the body and brain. Most often, ROS and RNS occur in places where an intense aerobic activity takes place, for example in the brain. There are many antioxidants located in nature including vitamins with antioxidant capacity such as vitamins E and C or β-carotene. These compounds can protect cells against oxidation. Being fat soluble, thus protecting lipid membrane or different lipoproteins against damage, vitamin E (α-tocopherol) is considered to be the most important vitamin. Several clinical studies have proved that vitamin E suppresses toxic effect of Aβ and even improves cognitive function of AD patients. Vitamin C (ascorbic acid) is a water soluble vitamin and as such, it is found mainly in blood or blood plasma.[33]

Some plant antioxidants have also a strong antioxidant capacity, showing potential for AD therapy. It could be silybin located in the thistle (*Silybum marianum - Asteraceae*) or resveratrol naturally occurring in the vine. Animal studies have demonstrated that silybin delays memory loss and protects cells from oxidative stress caused by Aβ. Resveratrol is the stilbene derivative and the reason why the vine consumption is linked with health benefits. This theory is supported by the fact that Mediterranean cultures have a lower incidence of coronary heart diseases, heart attacks and a higher life expectancy in general.[33, 34]

Mitochondrial antioxidants form another group of antioxidants. Oxidative stress can damage mitochondria causes its fragmentation and influence many physiological functions, which is very common for AD. Coenzyme Q10 is a typical antioxidant able of reaching not only the cell, but even mitochondria. It has many physiological functions such as participating in electron transport in mitochondria, sustaining of membrane potential and protection of neurons against intracellular Aβ deposition. Generally, coenzyme Q10 has very strong antioxidant capacity and has the ability to regenerate α-tocopherol in the redox cycle. Lipoic acid is also a strong antioxidant with the ability to reach mitochondria, to assist in coenzyme activity and to recycle some other antioxidants such as vitamins C or E.[33]



Scheme 3. Structures of common antioxidants

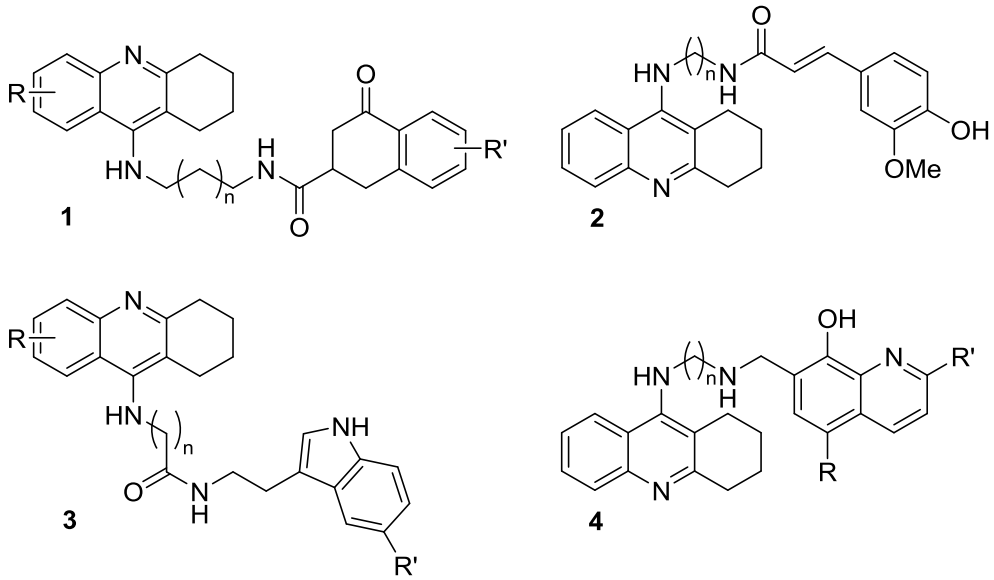
2.5.3 Tacrine/antioxidant hybrid molecules

Tacrine derivatives endowed with additional antioxidant properties might have beneficial effects for reducing toxicity and considerable benefits in the suppression of oxidative stress according to the MTDL strategy. Recently, this strategy has led to the preparation of several anti-AD drug candidates, for example tacrine–ferulic acid hybrids, tacrine-melatonin hybrids, tacrine-8-hydroxyquinoline hybrids and tacrine-4-oxo-4H-chromene hybrids.

Ferulic acid, which belongs to natural phenolic acids, is one of the most common antioxidants in nature. It is contained in cash crops such as wheat (*Triticum aestivum*) or eucalyptus (Eucalyptus globulus). Studies suggest that a long-term administration of this antioxidant induces resistance against Aβ toxicity in the brain. Therefore, a connection of ferulic acid with a tacrine moiety via an alkylene diamine side chain could lead to a preparation of a novel class of target molecules, which might have a preserved ability to inhibit AChE originating from the tacrine template and the antioxidant capacity originating from the ferulic acid moiety.[35, 36]

The new tacrine-melatonin derivatives have the ability to inhibit cholinesterases at low nanomolar levels, even lower levels than tacrine itself. Compounds prepared from 6-chlorotacrine and 6,8-dichlorotacrine combine high potency and selectivity toward AChE. These new compounds should be able to activate mostly the central cholinergic transmission and improve mental abilities without side effects due to its selectivity.[37]

The new tacrine-4-oxo-4 H-chromene hybrids manifested some interesting invitro biological activities for a potential AD treatment, including the inhibition of human ChEs as well as the radical scavenger activity and reducing the formation of senile plaques. IC_{50} values were in the nano- and picomolar ranges and their antioxidant properties were most often more potent than trolox or vitamin E analogue. Generally, these tacrine−flavonoid hybrids did not show a clear selectivity toward human ChEs, but some derivatives showed a strong selectivity toward h-AChE or h-BChE.[38]

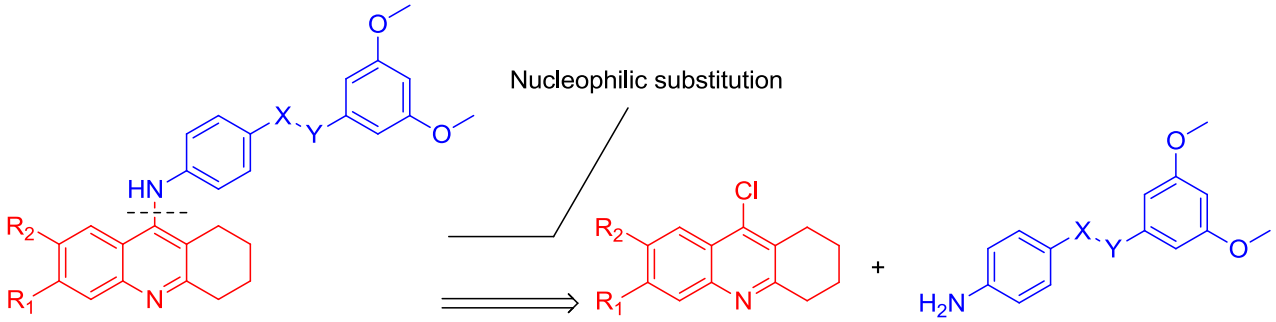


Scheme 4. Structures of Tacrine/antioxidant hybrid molecules. **1:** Tacrine-4-Oxo-4H-chromene Hybrids, **2:** tacrine-ferulic acid hybrids, **3:** Tacrine-Melatonin Hybrids, **4:** Tacrine-8-Hydroxyquinoline Hybrids.

3 EXPERIMENTAL PART

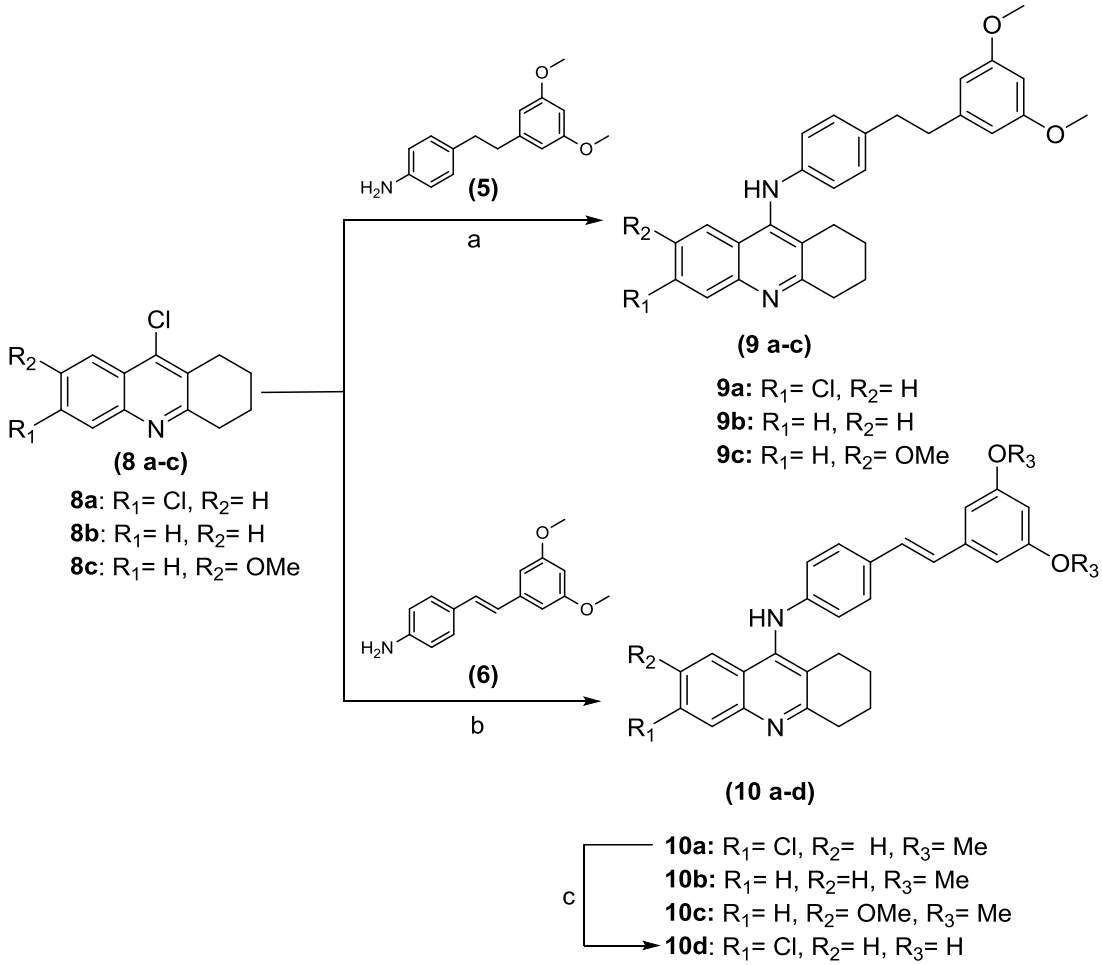
3.1 SYNTHETIC PART

3.1.1 General synthetic part



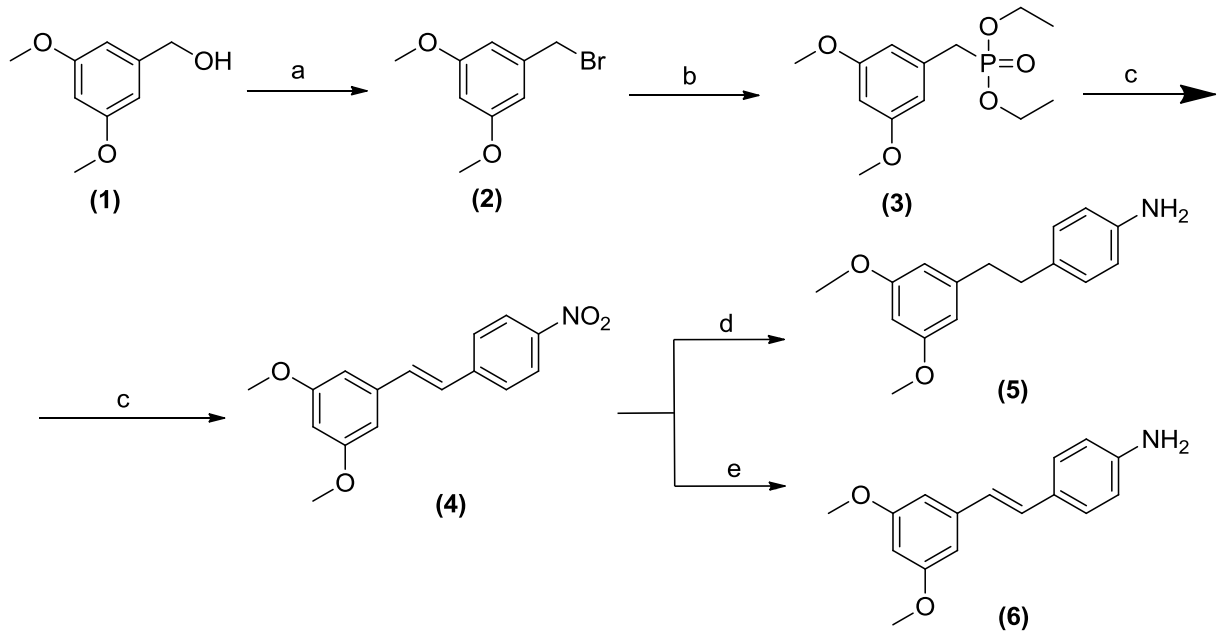
R_1= Cl, R_1= H,
R_2=H, R_2=OMe
X-Y= CH-CH, CH=CH

Scheme 1: General synthesis of the final hybrid derivatives **9a-c**, **10a-d**



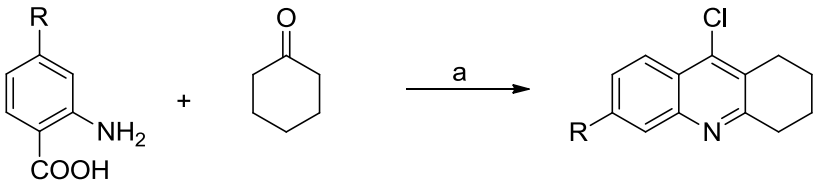
Reagents and conditions: (a) phenol, MW, 150W, 120 °C, 60min; (b) phenol, MW, 150W, 120 °C, 120min; (c) anhydrous DCM, BBr3 (1M in DCM), -78 °C, 20h.

Scheme 2: synthesis of stilbenes **5-6**



Reagents and conditions: (a) anhydrous DCM, PBr_3 (1M in DCM), reflux, 4h (b) $PO(OEt)_3$, 140 °C, 3h (c) anhydrous DCM, CH_3ONa, 18-Crown-6 ether, 4-nitrobenzaldehyde, 120 °C, 5h (d) EtOAc, Pd/H_2, r.t. (e) EtOAc, $SnCl_2.2H_2O$, reflux, 12h.

Scheme 3: synthesis of tacrines **8a-b.**



(7 a-b)

7a: R= Cl
7b: R= H

(8 a-b)

8a: R= Cl
8b: R= H

Reagents and conditions: (a) $POCl_3$, 130 °C, 3h.

General Chemical Methods

Reaction progress was monitored by TLC on pre-coated silica gel plates (Kieselgel 60 F_{254}, Merck) and visualized by UV254 light. Flash column chromatography was performed on silica gel (particle size 40-63 μM, Merck). If required, solvents were distilled prior to use. All reagents were obtained from commercial sources and used without further purification. When stated, reactions were carried out under an inert atmosphere. Reactions involving microwave irradiation were performed using a microwave synthesis system (CEM Discover® SP, 2.45 GHz, maximum power 300 W), equipped with infrared temperature measurement. Catalytic hydrogenation was performed

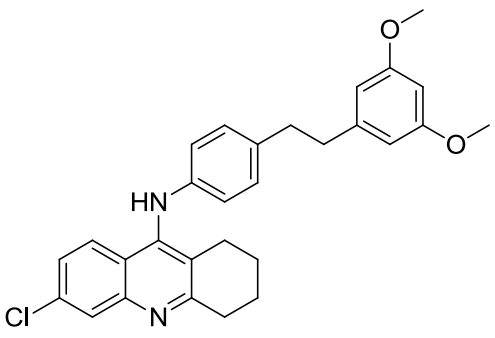
on H-CubeTM Continuous-flow Hydrogenation Reactor (H-Cube, ThalesNano Nanotechnology, Budapest, Hungary). Compounds were named relying on the naming algorithm developed by CambridgeSoft Corporation and used in Chem-BioDraw Ultra 12.0. Unless state otherwise, ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Varian Gemini at 400 MHz and 100 MHz respectively. Chemical shifts (δ_H) are reported relative to TMS as internal standard. Mass spectrum was recorded on a V.G. 7070E spectrometer or on a Waters ZQ 4000 apparatus operating in electrospray (ES) mode.

General procedure for coupling reactions (9a, 9b, 9c, 10a, 10b, 10c)

Tacrine derivatives **8a-c** (1 equiv), the appropriate amine **5-6** (1-1.5 equiv) and phenol (4-9 equiv) were charged in a pressure tight microwave tube containing a stirring bar. The reaction mixture was submitted to microwave irradiation at 120 °C with an irradiation power of 150W for 1h. The mixture was dissolved in DCM and washed with sodium hydroxide 10%, brine and water, dried over sodium sulfate and evaporated. Crude product was purified by flash chromatography = (petroleum ether/ EtOAc, elution gradient 90:10 to 80:20).

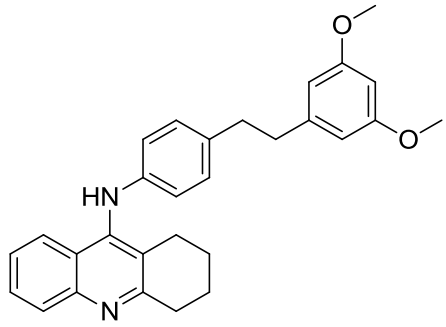
3.1.2 Characterization of the derivatives

6-chloro-N-(4-(3,5-dimethoxyphenethyl)phenyl)-1,2,3,4-tetrahydroacridin-9-amine (9a)



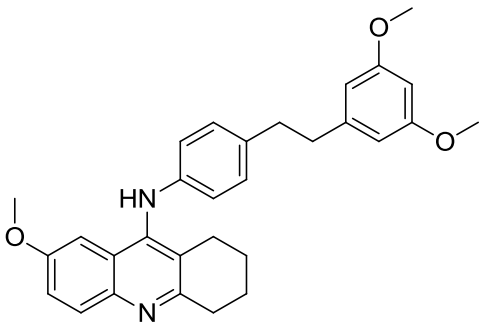
Coupling of compound **5** (0.103 g, 0.40 mmol) and **8a** (0.100 g, 0.40 mmol) with phenol (0.338 g, 9 equiv) was performed according to the general procedure described above. Brown solid, 0.064 g, 0.14 mmol, 34% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.84-1.88 (m, 2H), 1.91-1.95 (m, 2H), 2.7 (t, J=6.2Hz, 2H), 2.82 (s, 4H), 3.11 (t, J= 6.6Hz, 2H), 3.75 (s, 6H), 5.93 (br, 1H, -NH-), 6.29-6.32 (m, 3H, aromatic), 6.63 (d, J= 8Hz, 2H, aromatic), 7.01 (d, J=8Hz, 2H, aromatic), 7.21 (dd, J=2Hz, 8.8Hz, 1H, aromatic), 7.64 (d, J=8.8Hz, 1H, aromatic), 7.95 (d, J=1.6Hz, 1H, aromatic), ^{13}C-NMR (chloroform-*d*, 100 MHz) δ: 22.8, 22.9, 25.3, 34.2, 37.0, 38.4, 55.4, 98.0, 106.7, 106.8, 115.4, 117.5, 121.0, 122.5, 125.1, 125.7, 127.8, 129.36, 129.44, 134.5, 134.9, 142.4, 144.0, 144.3, 148.0, 160.9, 161.2.

N-(4-(3,5-dimethoxyphenethyl)phenyl)-1,2,3,4-tetrahydroacridin-9-amine (9b)



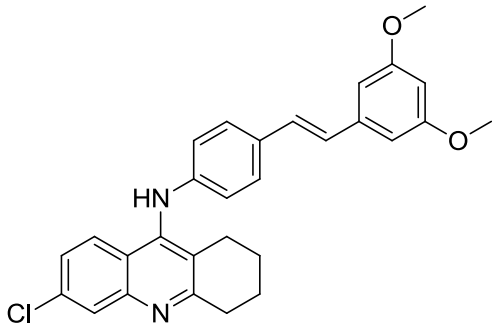
Coupling of compound **5** (0.170 g, 0.66 mmol) and **8b** (0.144 g, 0.66 mmol) with phenol (0.560 g, 9 equiv) was performed according to the general procedure described above. Yellow solid, 0.15 g, 0.34 mmol, 52% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.85-1.88 (m, 2H), 1.93-1.99 (m, 2H), 2.73 (t, J=6.2Hz, 2H), 2.83 (s, 4H); 3.15 (t, J= 6.4Hz, 2H), 3.77 (s, 6H), 5.82 (br, 1H, -NH-), 6.31-6.34 (m, 3H, aromatic), 6.64 (d, J=8.4Hz, 2H, aromatic), 7.02 (d, J=8Hz, 2H, aromatic), 7.32 (t, J=7.6Hz, 1H, aromatic), 7.59 (t, J= 7.6Hz, 1H, aromatic), 7.76 (d, J= 8.4Hz, 1H, aromatic), 7.98 (d, J=8Hz, 1H, aromatic), ^{13}C-NMR (chloroform-*d*, 100 MHz): 22.6, 23.0, 25.5, 34.3, 37.0, 38.5, 55.4, 98.0, 106.7, 117.1, 122.9, 123.0, 123.3, 124.9, 128.7, 129.0, 129.3, 134.3, 142.7, 143.6, 144.4, 147.6, 160.0, 160.9.

N-(4-(3,5-dimethoxyphenethyl)phenyl)-7-methoxy-1,2,3,4-tetrahydroacridin-9-amine (9c)



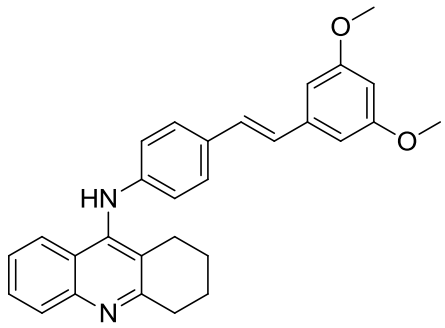
Coupling of compound **5** (0.100 g, 0.39 mmol) and **8c** (0.096 g, 0.39 mmol) with phenol (0.328 g, 9 equiv) was performed according to the general procedure described above. Brown solid, 0.096 g, 0.13 mmol, 33.4% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.84-1.88 (m, 2H), 1.92-1.96 (m, 2H), 2.74 (t, J= 6.4Hz, 2H), 2.83 (s, 4H), 3.11 (t, J= 6.4Hz, 2H), 3.66 (s, 3H), 3.77 (s, 6H), 5.70 (br, 1H, -NH-), 6.30-6.34 (m, 3H, aromatic), 6.63 (d, J= 8.4Hz, 2H, aromatic), 6.98 (d, J= 2.8Hz, 1H, aromatic), 7.03 (d, J= 8Hz, 2H, aromatic), 7.24 (m, 1H, aromatic), 8.89 (d, J= 9.2Hz, 1H, aromatic). ^{13}C-NMR (chloroform-*d*, 100 MHz) δ: 22.9, 23.1, 25.5, 33.9, 37.0, 38.5, 55.38, 55.44, 98.0, 101.8, 106.7, 117.0, 121.3, 123.4, 123.8, 129.3, 130.4, 134.1, 142.6, 143.6, 144.3, 156.8, 157.2, 160.9.

N-(4-(3,5-dimethoxyphenethyl)phenyl)-6-methoxy-1,2,3,4-tetrahydroacridin-9-amine (10a)



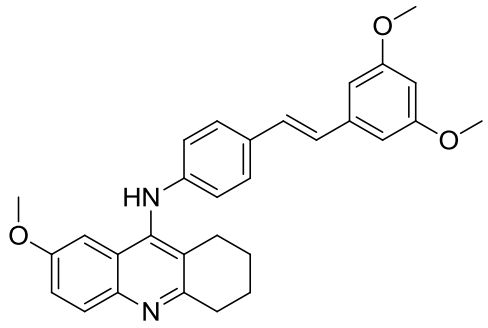
Coupling of compound **6** (0.255 g, 0.10 mmol) and **8a** (0.251 g, 0.10 mmol) with phenol (0.375 g, 4 equiv) was performed according to the general procedure described above. Yellow solid, 0.153 g, 0.33 mmol, 33% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.86-1.90 (m, 2H), 1.94-1.98 (m, 2H), 2.75 (t, J= 6.2Hz, 2H), 3.14 (t, J=6.4Hz, 2H), 3.83 (s, 6H), 5.87 (br, 1H, -NH-), 6.38 (t, J= 2Hz, 1H, aromatic), 6.64-6.69 (m, 4H, aromatic), 6.89 (d, J= 16Hz, 1H, aromatic), 7.01 (d, J=16.4Hz, 1H, aromatic), 7.25-7.28 (m, 3H, aromatic), 7.37 (d, J= 8Hz, 2H, aromatic), 7.70 (d, J= 8.8Hz, 1H, aromatic), 7.99 (d, J= 2Hz, 1H, aromatic). ^{13}C-NMR (chloroform-*d*, 100 MHz) δ: 22.7, 22.8, 25.4, 34.2, 55.5, 99.8, 104.6, 117.0, 121.5, 123.8, 125.0, 126.1, 126.8, 127.9, 128.7, 130.4, 134.8, 139.8, 143.4, 144.0, 148.1, 161.15, 161.22.

(E)-N-(4-(3,5-dimethoxystyryl)phenyl)-1,2,3,4-tetrahydroacridin-9-amine (10b)



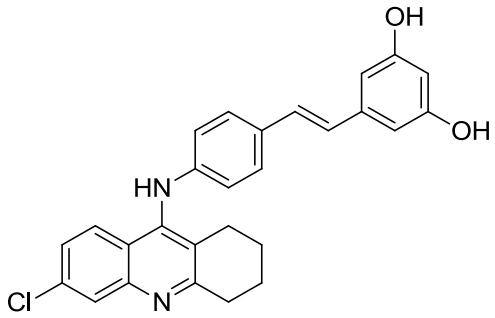
Coupling of compound **6** (0.176 g, 0.69 mmol) and **8b** (0.100 g, 0.46 mmol) with phenol (0.390 g, 9 equiv) was performed according to the general procedure described above. Orange solid, 0.093 g, 0.21 mmol, 46% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.86-1.90 (m, 2H), 1.95-1.99 (m, 2H), 2.76 (t, J=6.2Hz, 2H), 3.17 (t, J= 6.6Hz, 2H), 3.82 (s, 6H), 5.93 (br, 1H, -NH-), 6.37 (s, 1H, aromatic), 6.63-6.70 (m, 4H, aromatic), 6.89 (d, J=16.4Hz, 1H, aromatic), 7.01 (d, J= 16Hz, 1H, aromatic), 7.33-7.38 (m, 3H, aromatic), 7.61 (t, J= 7.6Hz, 1H, aromatic), 7.79 (d, J=8.8Hz, 1H, aromatic), 8.02 (d, J= 8Hz, 1H, aromatic). ^{13}C-NMR (chloroform-*d*, 100 MHz) δ: 22.8, 23.0, 25.6, 31.0, 34.1, 55.5, 99.8, 104.5, 110.2, 116.8, 123.2, 125.3, 126.5, 127.8, 128.9, 129.0, 130.1, 139.9, 144.3, 144.5, 160.2, 161.2.

(E)-N-(4-(3,5-dimethoxystyryl)phenyl)-7-methoxy-1,2,3,4-tetrahydroacridin-9-amine (10c)



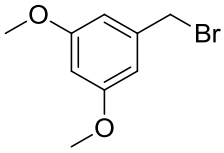
Coupling of compound **6** (0.268 g, 0.69 mmol) and **8c** (0.174 g, 1.05 mmol) with phenol (0.590 g, 9 equiv) was performed according to the general procedure described above. Brown solid, 0.106 g, 0.23 mmol, 32% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.85 (m, 2H), 1.83-1.87 (m, 2H), 1.91-1.95 (m, 2H), 2.75 (t, J= 6.2Hz, 2H), 3.11 (t, J= 6.6 Hz, 2H), 3.68 (s, 3H), 3.80 (s, 6H), 5.77 (br, 1H, -NH-), 6.35 (t, J= 2.2Hz, 1H, aromatic), 6.62-6.65 (m, 4H, aromatic), 6.86 (d, J= 16.4Hz, 1H, aromatic), 6.98 (d, J= 2.8Hz, 1H, aromatic), 6.99 (d, J= 16.4Hz, 1H, aromatic), 7.24-7.27 (m,1H, aromatic), 7.34 (d, J= 8.4Hz, 2H, aromatic), 7.89 (d, J= 9.6Hz, 1H, aromatic). ^{13}C-NMR (chloroform-*d*, 100 MHz) δ: 15.4, 22.9, 23.0, 25.5, 33.8, 55.50, 55.53, 66.0, 99.8, 101.7, 104.5, 106.9, 116.6, 121.5, 124.1, 126.3, 127.8, 128.9, 129.7, 130.25, 130.32, 139.9, 144.3, 157.1, 161.2 .

(E)-5-(4-((6-chloro-1,2,3,4-tetrahydroacridin-9-yl)amino)styryl)benzene-1,3-diol (10d)



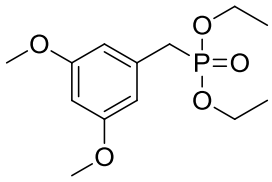
Compound **10a** (0.246 g, 0.52 mmol) was dissolved in anhydrous DCM (10 mL). After the mixture was cooled to -78 °C, BBr_3 (1.04 mL 1M in DCM, 1.04 mmol) was added dropwise. The resulting reaction mixture was allowed to warm up to room temperature and stirred for 20h, than cooled at 0 °C and treated as follows. Aqueous NaOH 10% was added and mixture was separated with EtOAc (3 x 20 mL). Combined organic phases were dried over sodium sulfate and evaporated. Crude product was purified by flash chromatography (petroleum ether/EtOAc 50:50). Yellow solid, 0.072 g, 0.16 mmol, 31% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.82-1.85 (m, 2H), 1.94-1.97 (m, 2H), 2.71 (t, J= 6.4Hz, 2H), 3.08 (t, J=6.4Hz, 2H), 6.17 (t, J= 2.2Hz, 1H, aromatic), 6.44 (d, J= 2.4Hz, 2H, aromatic), 6.70 (d, J= 8.8Hz, 2H, aromatic), 6.82 (d, J= 16.4Hz, 1H, aromatic), 6.95 (d, J= 6.4Hz, 1H, aromatic), 7.30-7.37 (m, 3H, aromatic), 7.85-7.87 (m, 2H, aromatic). ^{13}C-NMR (chloroform-*d*, 100 MHz) δ: 23.6, 26.7, 34.3, 102.8, 105.9, 118.4, 122.8, 124.8, 126.6, 126.8, 126.9, 127.6, 128.5, 128.6, 129.2, 131.6, 141.2, 145.4, 146.7, 148.2, 159.7, 162.3. (Substance is not completely pure).

3,5-Dimethoxybenzyl bromide (2)[39]



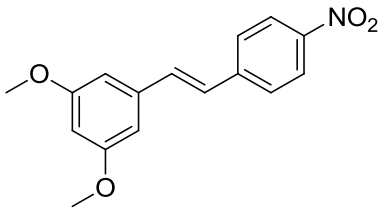
(3,5-dimethoxyphenyl)methanol **1** (1 g, 5.90 mmol) was dissolved in anhydrous DCM (20 mL). Then tribromophosphine 1M in DCM (6 mL, 5.90 mmol) was added. The reaction was refluxed under nitrogen for 4h, then cooled to room temperature. Crushed ice was added and mixture was extracted with DCM (3 x 10 mL). The organic phases were collected, washed with aqueous sodium carbonate and water, dried over sodium sulfate and evaporated to gain an oil. Oil was dried over high vacuum to gain a white solid. The compound was used in the next reaction without any further purification. 1.23 g, 5.32 mmol, 90% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 3.79 (s, 6H), 4.42 (s, 2H), 6.39 (s, 1H, aromatic), 6.54 (d, J = 4.4Hz, 2H, aromatic).

Diethyl 3,5-dimethoxybenzylphosphonate (3)[39]



3,5-Dimethoxybenzyl bromide **2** (1g, 4.30 mmol) was heated with excess of triethyl phosphite (3 mL, 17.50 mmol) at 140 °C for 3h. The remaining triethyl phosphite was removed by distillation at 140 °C under high vacuum to obtain the whitish oil. The compound was used in the next reaction without any further purification. 1.23 g, 4.27 mmol, 99% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.25 (t, J= 14,8 Hz, 6H), 3.07 (d, $J_{H,P}$=43.2 Hz, 2H), 3.76 (s, 6H), 3.94-4.09 (m, 4H), 6.33 (d, J= 4.4 Hz, 1H, aromatic), 6.44 (t, J=4.3 Hz, 2H, aromatic).

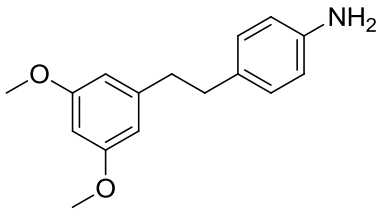
(E)-1,3-dimethoxy-5-(4-nitrostyryl)benzene (4)[39]



Diethyl 3,5-dimethoxybenzylphosphonate **3** (0.72 g, 2.50 mmol) was dissolved in anhydrous DCM (5 mL). Sodium methoxide (0.27 g, 5 mmol) and 18-Crown-6 ether (0.13 g, 0.50 mmol) were added and the mixture was stirred at room temperature for 5 minutes. Then, 4-nitrobenzaldehyde (0.57 g, 3.75 mmol) dissolved in anhydrous DCM (2.5 mL) was added dropwise at 0 °C and the mixture was stirred at room temperature for 1h, followed by heating to 120 °C for 5h. Water (5mL) was added and the mixture was extracted with diethyl ether (3x10 mL). The organic phases were collected, dried over sodium sulfate and evaporated. Crude product (0.56 g) was redissolved in DCM (5 mL). Girard's reagent (1.48 g, 8.83 mmol) and concentrated acetic acid (5.1 mL, 88.59 mmol) were added and the resulting mixture was stirred at room temperature

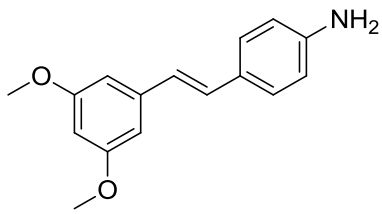
for 2h. Water (5 mL) was added, organic layers were collected, washed with aqueous sodium carbonate and brine, dried over sodium sulfate and evaporated to gain a yellow solid. Crude product was purified by flash chromatography (petroleum ether/EtOAc 90/10). 0.42 g, 1.47 mmol, 59% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 3.85 (s, 6H), 6.46 (s, 1H, aromatic), 6.70 (d, J= 2Hz, 2H, aromatic), 7.11 (d, J= 15.6 Hz, 1H, aromatic), 7.20 (d, J= 16.4Hz, 1H, aromatic), 7.63 (d, J=8.8Hz, 2H, aromatic), 8.22 (d, J=8.8Hz, 2H, aromatic).

4-(3,5-dimethoxyphenethyl)aniline (5)



A solution of (E)-1,3-dimethoxy-5-(4-nitrostyryl)benzene **4** (0.2 g, 0.70 mmol) in EtOAc (20 mL) was reduced on an H-Cube™ flow hydrogenator using a Palladium catalyst cartridge (T= r.t.; P($H_{2)=}$ 1 bar; flow rate: 1.0 mL/min). Volatile components were evaporated *in vacuum* to give the crude 4-(3,5-dimethoxyphenethyl)aniline as brown oil. **5** used in the following step without further purification. 0.17 g, 0.65 mmol, 93% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 2.81 (s, 4H), 3.77 (s, 6H), 6.31 (t, J= 2.4Hz, 1H, aromatic), 6.35 (d, J= 2.4Hz, 2H, aromatic), 6.63 (d, J=8Hz, 2H, aromatic), 6.99 (d, J=8Hz, 2H, aromatic).

(E)-4-(3,5-dimethoxystyryl)aniline (6)[40]

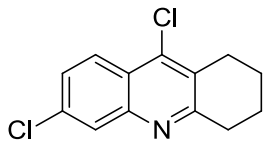


(E)-1,3-dimethoxy-5-(4-nitrostyryl)benzene **4** (0.2 g, 0.70 mmol) was dissolved in EtOAc (30 mL). 6 equivalents of Tin (II) chloride dehydrate (0.95g, 4.20 mmol) were added and mixture was refluxed for 7h. Than other 2 equivalents of Tin (II) chloride dehydrate (0.32g, 1.42 mmol) were added and mixture was refluxed for further 5h. Saturated aqueous sodium bicarbonate was added to achieve alkalinity. Precipitate was formed. The mixture was filtered and the filtrate was extracted with EtOAc (3x 10mL), than evaporated to obtain the product as brown solid. The compound was used in the next reaction without any further purification. 0.17 g, 0.65 mmol, 93% yield. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 3.83 (s, 6H), 6.36 (t, J= 2.4Hz, 1H, aromatic), 6.64 (d, J= 2.4Hz, 2H, aromatic), 6.68 (dd, J=2.0Hz, 6.4Hz, 2H, aromatic), 6.85 (d, J=16.4Hz, 1H, aromatic), 7.00 (d, J= 16.4, 1H, aromatic), 7.33 (dd, J=2.0Hz, 6.8Hz, 2H, aromatic).

General procedure for tacrines synthesis (8a, 8b)

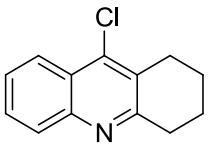
To a mixture of 4-chloranthranilic acid **7a** (1 equiv) or anthranilic acid (1 equiv) **7b** and cyklohexanone (1 equiv), $POCl_3$ was added in an ice bath. The mixture was refluxed for 3h. The excessive $POCl_3$ was removed by distillation to give a black residue. The residue was dissolved in DCM (15 mL). This mixture was washed with crashed ice and aqueous sodium carbonate, brine, water, dried over sodium sulfate and evaporated. The product was crystallized with acetone.

6,9-dichloro-1,2,3,4-tetrahydroacridine (8a)[41]



Reaction of 4-chloranthranilic acid **7a** (2,14 g, 12.50 mmol) and cyclohexanone (1.3 mL, 12.50 mmol) with $POCl_3$ (10mL) was performed according to the general procedure above. 4.89 g, 19.50 mmol, 78% yield as white solid. ^{1}H-NMR (chloroform-*d*, 400 MHz) δ: 1.92-1.95 (m, 4H), 2.99 (t, J= 6.4Hz, 2H), 3.09 (t, J=6.4Hz, 2H), 7.47 (dd, J=2.4Hz, 9.2Hz, 1H, aromatic), 7.96 (d, J= 2Hz, 1H, aromatic), 8.09 (d, J= 9.2Hz, 1H, aromatic).

9-chloro-1,2,3,4-tetrahydroacridine (8b)[41]



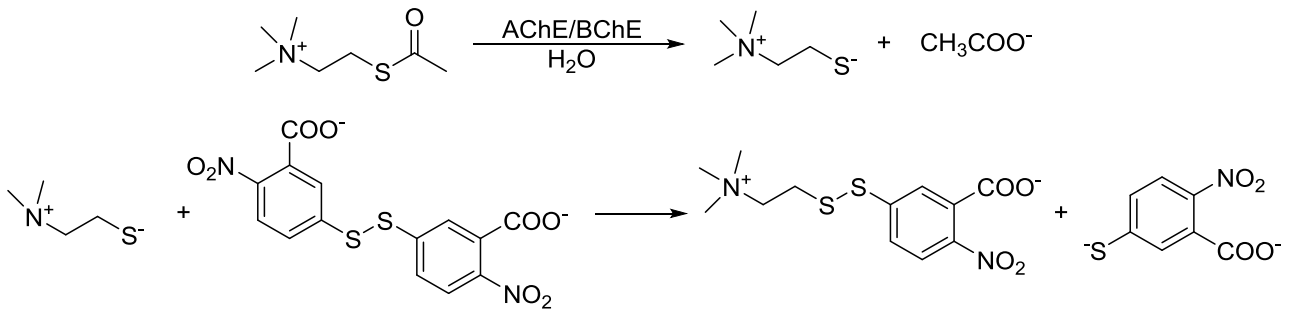
Reaction of Anthranilic acid **7b** (1.0 g, 7.29 mmol) and cyclohexanone (0.76 mL, 7.29 mmol) with $POCl_3$ (6 mL) was performed according to the general procedure above. 0.39 g, 1.77 mmol, 24% yield as white solid. ^{1}H-NMR (chloroform-*d*, 200 MHz) δ: 1.84 (d, J=2.6Hz, 4H), 2.87 (s, 2H), 3.04 (s, 2H), 7.50 (dt, J= 7.6Hz, 30Hz, 2H, aromatic), 7.97 (dd, J=8.4Hz, 25.4Hz, 2H, aromatic).

3.2 BIOLOGICAL SCREENING

3.2.1 Principle and procedure

Inhibitory parameters of tacrine/resveratrols were determined in vitro by Ellman method for human recombinant AChE (hAChE; EC 3.1.1.7) and human plasma BChE (hBChE; EC 3.1.1.8). This is a very sensitive spectrophotometric method applicable even for very small amounts of the substance. The principle of the method is based on the hydrolysis of acetylthiocholine by AChE or BChE to form thiocholine and acetic acid. Thiocholine subsequently reacts with 5,5'-dithiobis (2-

nitrobenzoic acid) to form a yellow-colored ion of 5-thio-2-nitrobenzoic acid. This ion is detected spectrophotometrically.[42]



Scheme 5. Hydrolysis of acetylthiocholine by cholinesterases to form thiocholine and its subsequent reaction with 5,5'-dithiobis-2-nitrobenzoic acid for release 5-thio-2-nitrobenzoic acid.

For measuring cholinesterase activity, a multichannel spectrophotometer Multi-mode microplate reader Synergy 2 (Vermont, USA) was used. Polystyrene plates with 96 holes (ThermoFisher Scientific, USA) were used as cuvettes. The entire assay was performed using 0,1 M buffer KH_2PO_4/H_2HPO_4 – pH 7,4. Cholinesterases were diluted in this buffer to the activity of 2.0 U / ml. To this enzyme solution (10 µl), solutions of 0.01 M DTNB (20 µl), 0.01 M acetylthiocholine chloride (20µl), 0.1M phosphate buffer - pH 7.4 (40 µl) and the corresponding inhibitor (10 µl) were added. Absorbance was measured at 412 nm and 37 °C after 5 min of incubation while shaking in an automatic cuvette. Three independent measurements for each compound were carried out and an evaluation was performed using GraphPad Prism 5 software (San Diego, USA).

3.2.2 Results

There were three independent measurements of inhibition activity performed for each compound. Results are expressed as their median and standard error of the mean (SEM).

Inhibitor	**IC_{50} *h*AChE±SEM (μM)**	**IC_{50} *h*BChE±SEM (μM)**	**SI (*h*BChE/*h*AChE)**
Tacrine	0.5 ± 0.1	0.02 ± 0.003	0.05
7-MEOTA	10.50 ± 2.000	21.00 ± 3.000	2.00
6-Cl-Tacrine	0.01 ± 0.001	0.85 ± 0.031	117.95
9a	0.825 ± 0.0501	n.d.	d.a.
9b	14.18 ± 2.966	n.d.	d.a.
9c	n.d.	n.d.	-
10a	n.d.	n.d.	-
10b	n.d.	n.d.	-
10c	n.d.	n.d.	-
10d	8.818 ± 0.3594	n.d.	d.a.

Table 1. Results of inhibitory parameters of hybrid compounds based on tacrine/resveratrol derivatives. n.d. = no enzyme inhibition at compound's concentration of 10 μM. d.a. = definite affinity for *h*AChE.

4 DISCUSSION

Within the thesis, seven novel hybrid compounds were prepared based on tacrine/resveratrol derivatives. Combining these two moieties, we assumed affecting more pathological processes

according to the MTDL strategy. Tacrine acts as a well-known AChE inhibitor which was studied for a long time at the Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences in the Czech Republic and resveratrol which acts as a strong antioxidant.

Each derivative exerted a great difference in its biological activity suggesting high structural requirements. Only three derivatives proved an inhibitory effect toward AChE represented by IC_{50} values in micromolar to submicromolar range; other derivatives did not have the ability to inhibit AChE at tested concentration scale. None of the derivatives had the capability to inhibit BChE suggesting high enzyme selectivity. All active compounds have definite affinity for AChE. Based on the results listed in Table 1, it is possible to determine the structure-activity relationship (SAR).

Generally, synthetized derivatives possess relatively weak inhibitions of AChE. Inhibitory ability of 6-chlorotacrine has not been overcome by none of the derivatives, 7-MEOTA activity has been exceeded by two derivatives (**9a**, **10d**). Insertion of a chloride atom leads AChE activity enhancement reaching low-micromolar IC_{50} values. The saturation of the double bond between aromatic rings and resveratrol also led to improvement of inhibitory parameters. This can be explained by allowing the free rotation around a simple sigma bond of the ligand which ultimately leads to a better ligand accommodation into the AChE active site.

The best inhibitory activity toward AChE displayed the derivative **9a** containing chloride atom in position 6 of tacrine moiety and saturated double bond in resveratrol scaffold. Comparing hybrids **9b** and **10d**, we can conclude that the chloride atom in position 6 is more important for the activity of the derivative than the saturation of double bond in resveratrol moiety.

The crucial step in preparation (*E*)-1,3-dimethoxy-5-(4-nitrostyryl)benzene represents Wittig reaction. Due to the contamination of the product by the starting material (4-nitrobenzaldehyde), which has a very similar R_f value, the purification of the product by flash-chromatography was very difficult. An improvement of the purification efficiency was achieved through the use of Girard's reagent; nevertheless, the flash-chromatography had to be applied for several times. The yield of the coupling was improved over time by microwave synthesis system (CEM Discover® SP) optimization and modifying the purification method. This enabled yield increase up-to 30%.

5 CONCLUSION

The aim of this diploma thesis has been to prepare six new hybrid derivatives. Using Ellman method, in vitro testing was performed in order to compare the inhibitory activity toward hAChE and hBChE, the results of which were then compared with tacrine, 7-MEOTA and 6-Cl-tacrine. Despite relatively weak inhibitions of the prepared molecules and their definite affinity for AChE, we assume some additional interesting properties, such as antioxidant capacity or a beneficial effect

on Aβ aggregation/toxicity, may be assigned to the molecules in question. These substances will be subjected to further testing, including tests to determine free radical scavenging activity using DPPH method, molecular orientation to active sites of AChE using X-ray crystallography or molecular docking method, and Aβ inhibition. The tests will be carried out in the near future.

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