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MALDI-TOF MS method based screening of inhibitors of amyloid beta fibrillization from a range of food colors.

Bachelor thesis

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Amüloid beeta fibrillisatsioooni inhibiitorite skriining toiduvärvide hulgast MALDI-TOF MS meetodiga.

Bakalaureuse lõputöö

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AUTORIDEKLARATSIOON

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ABBREVIATIONS USED

Aβ	amyloid beta peptide
ACN	acetonitrile
ApoE4	apolipoprotein E4
APP	amyloid precursor protein
AD	Alzheimer's disease
BACE	β-site of APP cleaving enzyme
BB41	basic blue 41
ESI	electrospray ionization
FDA	U.S. Food and Drug Administration
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MALDI	matrix-assisted laser desorption ionization
NSAID	non-steroidal anti-inflammatory drugs
TFA	trifluoroacetic acid

INTRODUCTION

Alzheimer's disease is an irreversible, progressive brain disease that slowly destroys memory and thinking skills, and eventually even the ability to carry out simplest tasks. This incurable, degenerative, and terminal disease was first described by German psychiatrist and neuropathologist Alois Alzheimer in 1906 and was consequently named after him (Berchtold and Cotman 1998). Most often, it is diagnosed in people over 65 years of age (Brookmeyer, Gray et al. 1998), although the less-prevalent early-onset Alzheimer's can occur much earlier. In 2006, there were 26.6 million sufferers worldwide. Alzheimer's disease currently affects nearly 2% of the population in industrialized countries and is predicted to affect 1 in 85 people globally by 2050 (Brookmeyer, Gray et al. 1998)(World population prospects: the 2006 revision, highlights). Finding an efficient drug that helps to stop the progression of AD is of utmost importance.

To achieve that goal one of the strategies is to inhibit the fibrillization of Abeta peptide. There are only a few suitable high throughput methods for identifying inhibitors of this pathological fibrillization of Abeta peptide.

At the proteomics laboratory in the Tallinn University of Technology at the Department of Gene Technology a high throughput MALDI-TOF MS method for quick and accurate screening of Abeta fibrillation inhibitors was developed. The aim of this work was to screen a library of nontoxic food colors with the formerly developed MALDI-TOF MS protocol. Different experiments for reproducibility using modified Met-Abeta42 were also performed. An inner standard, the intensity of insulin peak, was used to observe the change of A-beta monomer concentration in mass-spectrometer analysis.

This bachelor thesis will give a review of Alzheimer's disease and medical strategies to cure it. The thesis will mostly concentrate on the inhibition of Alzheimer A-beta peptide fibrillization.

LITERARY REVIEW

1.1ALZHEIMER'S DISEASE. HISTORY

In 1901, a 51-year-old woman, Auguste D, was admitted to the state asylum in Frankfurt. She was suffering from cognitive and language deficits, auditory hallucinations, delusions, paranoia and aggressive behavior, and was studied by Alois Alzheimer (1864–1915), a doctor at the hospital.

Alzheimer moved to the Munich medical school in 1903 to work with Emil Kraepelin – one of the foremost German psychiatrists of that era – and when Auguste D died in April 1906, her brain was sent to him for examination. In November of that year, Alzheimer presented Auguste's case at a psychiatry meeting, and he published his talk in 1907.

In 1910, Kraepelin coined the term 'Alzheimer's disease' – a term still used to refer to the most common cause of senile dementia.

Virtually until 1960s AD was considered a normal byprocess of aging when a link between A-beta plaques and tangles in the brain and impairment of cognitive abilities was found. These were the same plaques and tangles that Dr. Alzheimer had found on the autopsy of Auguste D. (Khachaturian 2006).

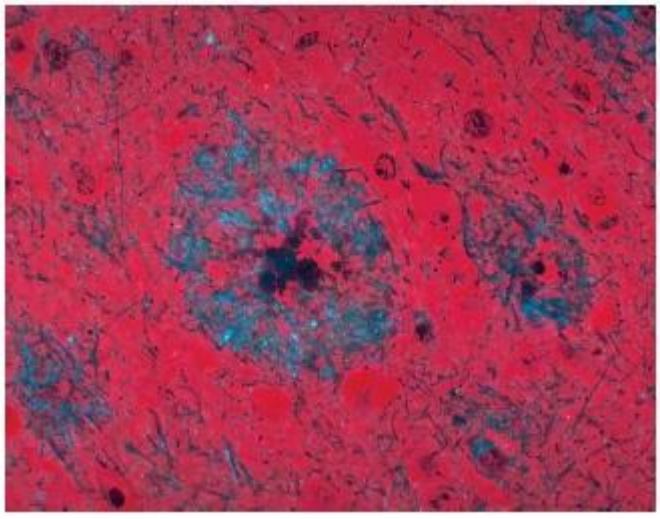


Figure1: Tissue cut from AD patient brain showing abnormal plaques. (picture from the public domain.)

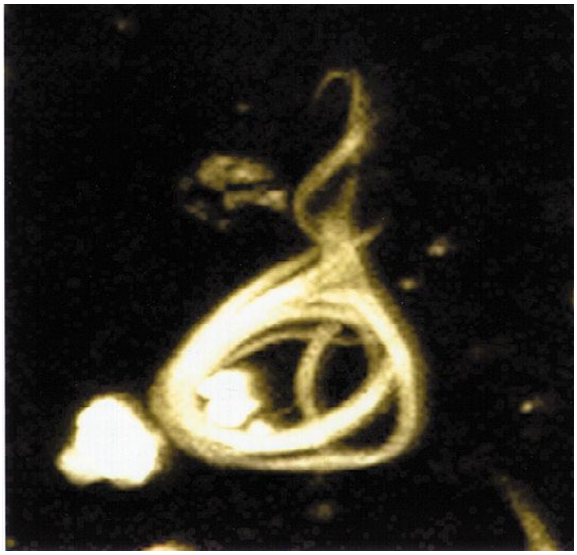


Figure 2: Microscopy image of a neurofibrillary tangle, conformed by hyper phosphorylated tau protein (National Institute on Aging*)*

These features are considered also today the prime molecular characteristics of AD pathology (Blennow, de Leon et al. 2006).

Plaques and tangles disrupt communication between neurons eventually leading to their death and thus to degeneration of memory, intellect and to behavioral and linguistic disorders.

1.2 PREVALENCE. RISK FACTORS. SYMPTOMS. DIAGNOSIS

In 2006, there were 26.6 million sufferers worldwide. Alzheimer's is predicted to affect 1 in 85 people globally by 2050 (Brookmeyer, Gray et al. 1998) .

Although the course of Alzheimer's disease is unique for every individual, there are many common symptoms. The earliest observable symptoms are often mistakenly thought to be 'age-related' concerns, or manifestations of stress (Waldemar, Dubois et al. 2007). In the early stages, the most common symptom is inability to acquire new memories, observed as difficulty in recalling recently observed events. When AD is suspected, the diagnosis is usually confirmed with behavioral assessments and cognitive tests, often followed by a brain scan if available.

As the disease advances, symptoms include confusion, irritability and aggression, mood swings, language breakdown, long-term memory loss, and the general withdrawal of the sufferer as their senses decline (Tabert, Liu et al. 2005; Waldemar, Dubois et al. 2007). Gradually bodily functions are lost, ultimately leading to death (National Institute of Aging 2007-10-26) Individual prognosis is difficult to assess, as the duration of the disease varies. AD develops for an indeterminate period of time before becoming fully apparent, and it can progress undiagnosed for years. The mean life expectancy following diagnosis is approximately seven years (Molsa, Marttila et al. 1986). Fewer than three percent of individuals live more than fourteen years after diagnosis (Molsa, Marttila et al. 1995).

The cause and progression of Alzheimer's disease are not well understood. And to

this moment all treatments are symptomatic and no cure has yet to be found. Because AD cannot be cured and is degenerative, management of patients is essential. The role of the main caregiver is often taken by the spouse or a close relative. Alzheimer's disease is known for placing a great burden on caregivers; the pressures can be wide-ranging, involving social, psychological, physical, and economic elements of the caregiver's life. In developed countries, AD is one of the most costly diseases to society (Meek, McKeithan et al. 1998).

The most important risk factor for AD is age. Genetic factors, sex, lifestyle, level of education and intelligence, head trauma, vascular diseases, heavy metals and radiation also play a role (Chen, Lin et al. 2009). For example mutations on chromosome 21 cause the formation of abnormal amyloid precursor protein (APP). A mutation on chromosome 14 causes abnormal presenilin 1 to be made, and a mutation on chromosome 1 leads to abnormal presenilin 2.

Even if only one of these mutated genes is inherited from a parent, the person will almost always develop early-onset AD. This inheritance pattern is referred to as “autosomal dominant” inheritance. In other words, offspring in the same generation have a 50/50 chance of developing FAD (familial AD) if one of their parents had it.

The vast majority of cases of AD are sporadic – they do not run in families. Nevertheless, molecular genetic analyses suggest that there are likely to be many more genes that influence one’s susceptibility to AD. The first such susceptibility gene identified was apolipoprotein E for which there are 3 alleles that encode 3 different isoforms of apolipoprotein E (E2, E3 and E4). Individuals that produce the E4 isoform are at increased risk of AD (Roses 1997).

1.3 PATHOLOGY OF THE ALZHEIMER'S DISEASE AND PATHWAYS TOWARDS IT.

1.3.1. GENETIC PROPENSITY AND SECRETASES.

The gene for ApoE4 isoform is located in chromosome 19 and it increases the risk of developing Alzheimer’s. ApoE4 raises the production of Aβ while lowering its clearance from the brain. Hereditary AD is an autosomal dominant disease, which is brought forth by mutations in three genes, all connected to the production of Aβ.

1.3.2. THE AMYLOID HYPOTHESIS OF ALZHEIMER'S DISEASE

Alzheimer’s disease is a complex and dynamic pathology with reasons for disease progression varying over different groups of patients. Nevertheless a hypothesis that amyloidal/Aβ plaques are the leading cause of AD pathogenesis has been gathering evidence.

A fundamental abnormality that plays a pivotal role in the dysfunction and death of neurons in AD is altered proteolytic processing of APP resulting in increased production and accumulation in the brain of neurotoxic forms of Aβ. In every case of autosomal dominant early-onset familial AD where the genetic abnormality has been

identified (mutations in the APP, PS1 and PS2 genes), the defective gene causes an increase in the production of the long 42 amino acid form of Aβ (Aβ42 as opposed to the normal Aβ40) in patients, in cultured cells and in transgenic mice (Scheuner, Eckman et al. 1996). Aβ42 has two extra terminal (Isoleucine and Alanine) residues which are hydrophobic and thus form more readily aggregates in aqueous solutions. It has also been shown that Aβ42 easily forms clusters of 5 or 6 monomers and stabilizes the so-called „paranucleus“. The paranuclei cluster forms even larger aggregates. Aβ40 by itself does not readily form clusters of more than 4 molecules (Chen and Glabe 2006).

The production of Aβ requires sequential cleavages of APP by β-secretase (BACE) at the N-terminus of Aβ and by γ-secretase at the C-terminus of Aβ. Alternatively, an enzyme called α-secretase cleaves APP within the Aβ sequence thereby precluding production of Aβ. Mutations in APP that cause familial AD result in one or two amino acid changes within or immediately adjacent to Aβ that enhance its cleavage by BACE and γ-secretase, while presenilin mutations alter γ-secretase activity (Hardy 1997) (Figure 3). The changes in APP metabolism may be genetic, age- or lifestyle related, due changes in energy metabolism and ion homeostasis.

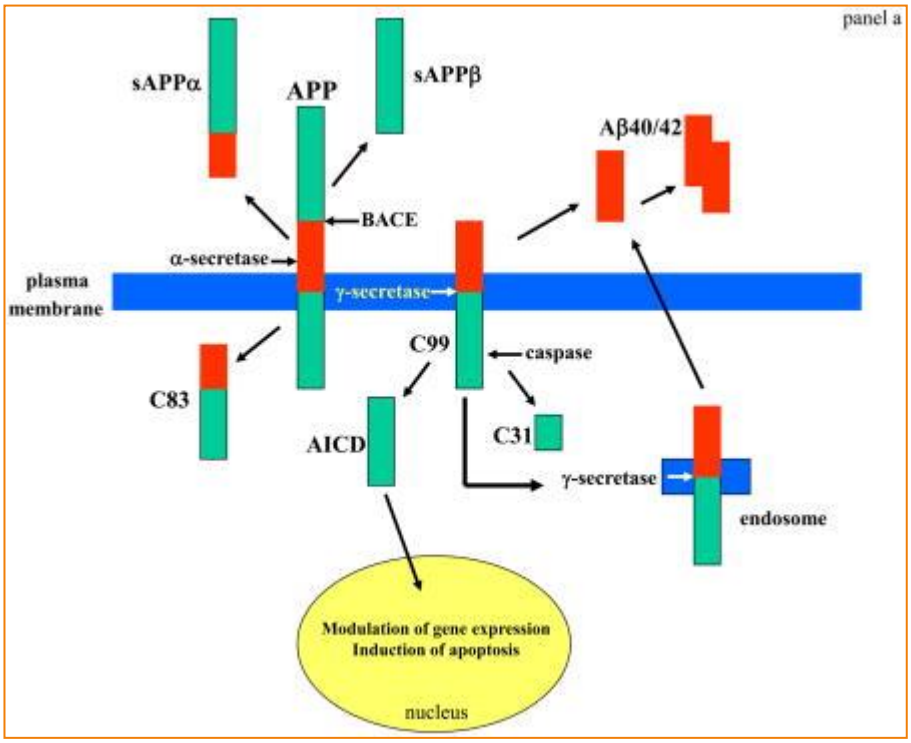


Figure 3: APP processing by different secretases and subsequent fate of the substrate. (Nature 2004 Mark P. Mattson)

1.4. THERAPEUTIC STRATEGIES

There are many treatment options for AD patients. Alas, many of them are symptomatic and aimed at giving temporary relief for the patients. Choline-esterase inhibitors aimed at the lack of acetylcholine and NMDA (n-methyl d-aspartate) receptor antagonist for the glutamate mediated cytotoxicity - to name a few (Klafki,

Staufenbiel et al. 2006). Although it is very important to improve the quality of life of AD patients and slow the development of the disease, symptomatic treatments will not be considered here. A score of mechanism-based treatment strategies will be examined below.

1.4.1. SUBSTANCES INHIBITING AB AGGREGATION

One of the most promising strategies is the inhibition of Aβ aggregation. Direct interaction with Aβ molecules and prevention of their accumulation blocks the key points of main pathological pathway for the development of AD. Neurochem Inc., a Canadian company, has completed a Phase II clinical trial of their glycosaminoglycan mimetic of Alzheimer's that has been designed to bind to Aβ peptides and thereby inhibits formation of Aβ aggregates. As of now it is in phase III clinical trials (Citron 2004). Tamiprosate which also bound directly to Aβ monomers unfortunately did not reach the market as drug exposed similar efficacy compared to placebo.

1.4.2. PASSIVE VACCINATION WITH ANTIBODIES

In a landmark paper in 1999 Dale Schenk and co-workers described that immunization with Aβ attenuates the Alzheimer's disease-like pathology in a transgenic mouse model of Alzheimer's disease (Schenk, Barbour et al. 1999). With amyloid plaque clearance Aβ immunization was shown to also reduce various aspects of the amyloid-associated pathology including neuritic dystrophy and synaptic degeneration as well as early tau accumulation (Lombardo, Stern et al. 2003; Oddo, Billings et al. 2004; Brendza, Bacskai et al. 2005; Buttini, Masliah et al. 2005). The treatment relies on activation of brain microglial cells and antibody Fc-part activated phagocytosis. That is probably the reason why the first clinical trials of Aβ immunotherapy, which used aggregated Aβ1–42 as antigen, had to be stopped in Phase II due to aseptic meningoencephalitis in 6% of the treated patients (Orgogozo, Gilman et al. 2003; Bayer, Bullock et al. 2005; Gilman, Koller et al. 2005; Nicoll, Barton et al. 2006). Another hypothesis suggests that by binding to Aβ peptides antibodies disrupt aggregation, thus acting like aggregation inhibitors. Supporting this hypothesis, antibodies can block and even reverse Aβ aggregation and toxicity *in vitro* (Solomon, Koppel et al. 1997; Frenkel, Solomon et al. 2000; McLaurin, Cecal et al. 2002; Du, Wei et al. 2003). Due to the fact that such macromolecules as antibodies have trouble passing the hemato-encephalic barrier it is evident that search for low molecular weight substances is needed.

A third hypothesis has been formulated suggesting that circulating antibodies were to sequester Aβ, shift the equilibrium towards the periphery and thereby reduce brain Aβ deposition (DeMattos, Bales et al. 2001). Consistent with this peripheral sink hypothesis an elevation of blood Aβ after immunization has been found (DeMattos, Bales et al. 2001; Pfeifer, Boncristiano et al. 2002; Lemere, Spooner et al. 2003; Gandy, DeMattos et al. 2004; Lemere, Beierschmitt et al. 2004; Wilcock, Munireddy et al. 2004) which reflected the brain amyloid burden (Dodart, Bales et al. 2002). Yet, this could also be explained by a simple stabilization of blood Aβ due to antibody binding.

1.4.3. B- AND Γ- SECRETASE INHIBITORS

An evident way for decreasing Aβ levels is to lower its production. β- and γ-secretases are responsible for cutting the precursor protein APP and releasing Aβ peptide. γ-secretase also cleaves the Notch receptor which is why dysfunction in it may increase toxicity. Taking into account the need for γ-secretase, modeling its action, as opposed to inhibition, is required (Wolfe 2008; Wolfe 2008; Wolfe 2008). As proof it has been found that certain non-steroidal anti-inflammatory drugs (NSAIDs) such as iboprofenum, indomezathin and sulindac sulfide can preferentially reduce the generation of the highly amyloidogenic Aβ42 species without affecting Notch cleavage (Weggen, Eriksen et al. 2001). A γ-secretase inhibiting compound (LY450139) by Eli Lilly has been tested in a 6-week Phase II trial. The compound was reported to reduce Aβ levels in plasma but not in CSF at concentrations that did not produce significant side effects (Siemers, Skinner et al. 2005). Orally administered γ-secretase inhibiting substance MRK-560-l has also been shown to be void of side effects (Best, Smith et al. 2007).

β-secretase gene (BACE) was discovered and cloned in 1999. BACE1 knock-out mice were reported to produce only very small amounts of Aβ thus confirming that BACE1 represents the primary β-secretase *in vivo*. The absence of severe pathological phenotypes in the knockout mice (Luo, Bolon et al. 2001; Roberds, Anderson et al. 2001), suggested that targeting β-secretase may be a particularly promising therapeutic approach, even though the identification of specific small molecule inhibitors suitable for drug development have proved to be elusive (Citron 2004).

1.4.4. THERAPEUTIC STRATEGIES TARGETING TAU

Beside Aβ fibrils neurofibrillary lesions made up of aggregated hyper phosphorylated microtubule-associated tau-protein is a second defining characteristic of Alzheimer's disease. Phosphorylation of tau regulates its ability to promote microtubule assembly from tubulin (Lindwall and Cole 1984) and abnormal hyper phosphorylation interferes with its normal biological function (Gustke, Steiner et al. 1992; Lichtenberg-Kraag, Mandelkow et al. 1992; Bramblett, Goedert et al. 1993; Alonso, Zaidi et al. 1994) by decreasing tau's ability to bind to and to stabilize microtubules. Under pathological conditions, aberrant hyper phosphorylation of tau results in its detachment from microtubules, breakdown of the microtubule network, disturbance of axonal transport and ultimately neurodegeneration (Mandelkow and Mandelkow 1998). Unable to bind microtubules tau aggregates to helical filaments further adding to the toxic effects. Drugs that aim to reduce tau-induced neuropathology comprise of molecule that inhibit tau multimerisation, lower tau phosphorylation through inhibition kinases and activation of phosphatases, intensifying degradation of tau or try to compensate for the lack of microtubule stabilization (Brunden, Trojanowski et al. 2009).

1.4.5. OTHER SUBSTANCES BLOCKING THE DEGENERATION OF NEURONS

According to the theoretical model for Aβ peptide channel formation least energy calculations position His13 and His14 residues around the entrance of the putative pore. This has been tested by trying to block the cytotoxic ion channels by binding the His rings with other compounds containing His diads. Blocking has also been

successful with known His coordination compounds such as Ni^{2+} and imidazole. Administering a custom-made peptide with a His-His diad can block the Aβ channels and avoid neural Ca^{2+} -impeded cytotoxicity and subsequent cell-death.

Ca^{2+} -mediated cytotoxicity is also brought forth by excessive activation of NMDA (N-methyl-D-aspartate) - receptor. This glutamate excitoxicity (NMDA-receptor being a glutamate-ligand gated ion channel) is believed to play a role in neuronal death observed in Alzheimer's and other major neurodegenerative conditions (Bleich, Romer et al. 2003; Hynd, Scott et al. 2004)

Thus NMDA receptor antagonists have surfaced as promising drug candidates if not for curing Alzheimer's then at least stabilizing the patient. There have been a score of potent NMDA-receptor antagonist substances which have not passed clinical trials for one or the other reason, for example MK-801 being one of them – left aside due to interference with the important role of glutamate in the brain. Others, such as Dimebon have not been sufficiently active when compared to placebo. But non-competitive NMDA-receptor antagonists on the other hand show greater promise. Memantine shows moderate affinity (Kornhuber, Bormann et al. 1989) and protects neurons while leaving physiological NMDA-receptor function unaffected (Sonkusare, Kaul et al. 2005).

1.5. INHIBITORS OF AB AGGREGATION

Aggregation of Aβ is one of the main ways in which AD finds its beginning and the reason for activation of many cytotoxic pathways which lead to death of neural cells and decline of cognitive abilities. Hence it is of no surprise that this aggregation is a therapeutical target for aggregation inhibitor substances. The idea is not so much about clearing old amyloid plaques but prevent the formation of newer ones which in this process are a cause for many pathological pathways. The goal is also to stop the formation of Aβ oligomers – the most cytotoxic Aβ species of them all. There are many substances that try to achieve these objectives one way or another. They can be divided into more-less distinct subclasses.

1.5.1. STRUCTURE AND SEQUENCE BASED SYNTHETIC PEPTIDES

When proteins aggregate, including Aβ, they form lower energy state structures such as β-sheets. So it follows that synthetic peptides try to disrupt such formations in their conception or strive to stop their progression into supra β-sheet structures such as amyloid plaques. There are three main paths to achieving this goal. First approach would be to use the self-associating property of proteins to poison fibril formation with short peptide segments (Tjernberg, Naslund et al. 1996; Findeis 2002; Sciarretta, Gordon et al. 2006). A second approach is based on screening for peptides that can disrupt fibril formation (Wiesehan, Buder et al. 2003; Larbig, Pickhardt et al. 2007). Lastly, a third option that has been shown applicable to fibril inhibition is structure-based design of non-natural peptides targeted to block the ends of fibrils (Sievers, Karanicolas et al. 2011). These peptides are designed against β-sheet stabilizing structures such as 'steric zippers'. And another similar method proposed inhibition amyloid aggregation by formation of helical assemblies: Aβ and synthetic peptides form stable coiled-coil fragments, which arrest further fibrillization and

have been shown to disassemble mature amyloid-like fibrils (Brandenburg, von Berlepsch et al. 2011).

A rather more straightforward way towards this aim is to use N-methylated peptides to target N-terminal ends of aggregating proteins (Cruz, Tusell et al. 2004; Amijee, Madine et al. 2009). N-methylated peptides or "meptides" bind to the fibril through β-sheet outer NH-group and by sterically inhibiting the further formation of stabilizing hydrogen bonds inhibit further amyloid formation. A single N-Me-Alanine coupled to a truncated Aβ (31-42) has been found to be necessary and sufficient for inhibiting further fibril formation (Kokkoni, Stott et al. 2006; Li, Zemel et al. 2012).

1.5.2. INHIBITORS BASED ON AMYLOID DYES

Dyes used to stain amyloid fibrils in higher concentrations often inhibit their formation. When common features as obligatory phenyl groups that bind to Aβ are taken into account, it is evident why amyloid dyes have been of interest in the search for the Aβ aggregation inhibitors. Sulphonated dyes such as Congo red do not pass the BBB and cannot be used (Lorenzo and Yankner 1994; Frid, Anisimov et al. 2007). ThioflavinT is toxic to humans. Some evidence supporting ThioflavinS has also been found (Sharp, Crabb et al. 2009). On the other hand dye derivatives are more promising due to the ability to reduce their toxicity and improve Aβ specificity. Active research of curcumin analogues have been conducted with outcome of substances capable of Aβ fibril disruption, chelating metal ions suspected in assisting oxidative damage and with good antioxidative characteristics to boast (Chen, Chen et al. 2011). (Reinke and Gestwicki 2007) have described three main attributes to consider when designing new molecules. Firstly, there has to be several phenyl groups to accommodate for proper binding with Aβ. Secondly terminal phenyl group has to have its hydroxyl moieties substituted. Thirdly the compound has to include a rigid 8Å-16Å length linker.

1.5.3. POLYPHENOLS

Most of cytotoxicity manifests though oxidative damage and the activation of caspases and kinases. Plant secondary metabolites polyphenols have many known antioxidative qualities to counter changes in the brain struck by Alzheimer's disease. Green tea has many such polyphenol catechins, most prominent of them being EGCG. Primarily researched for its anticancerogenic properties (Lin and Liang 2000; Lin 2002; Moyers and Kumar 2004) new evidence suggests that it also modulates α-secretase activity and thus the production of Aβ itself (Rezai-Zadeh, Shytle et al. 2005). EGCG benefits cell survival due effect on protein kinase C (Levites, Amit et al. 2002) and nuclear factor kappaB (NF-κB) (Levites, Youdim et al. 2002). Resveratol found in wine is also implicated in the action on NF-κB (Chen, Fischle et al. 2001). Other useful properties include the ability to diffuse reactive oxygen species and the ability to stop apoptosis (Jang and Surh 2003). Gingko biloba extract EGb 761 which contains 24% of flavonoids and 6% of terpenes is a scavenger of peroxyl radicals and restores membrane fluidity (Ramassamy 2006). Studies have shown that when these polyphenols are taken together a synergistic effect is observed and their impact is increased (Singh, Arseneault et al. 2008). That is why it is

important to have a well-diversified diet. It is to be noted that most of polyphenolic compounds do not inhibit Aβ plaque formation directly, but ameliorate its symptoms.

1.5.4. METAL CHELATORS

Aβ can bind transition metal ions which in their turn facilitate oxidative damage. Cu(II) and Zn(II) ions catalyze Aβ aggregation and Cu and Fe ions are primarily responsible for the neurotoxic oxidation (Atwood, Robinson et al. 2002; Tougu, Karafin et al. 2009; Tougu, Tiiman et al. 2011). Metal chelators offer a way, such as clioquinol, to bind metals and to reduce their redoxreactivity and toxicity (Cherny, Atwood et al. 2001; Gnjec, Fonte et al. 2002; Rauk 2009). So far metal chelator treatment in AD has only been demonstrated in animal models.

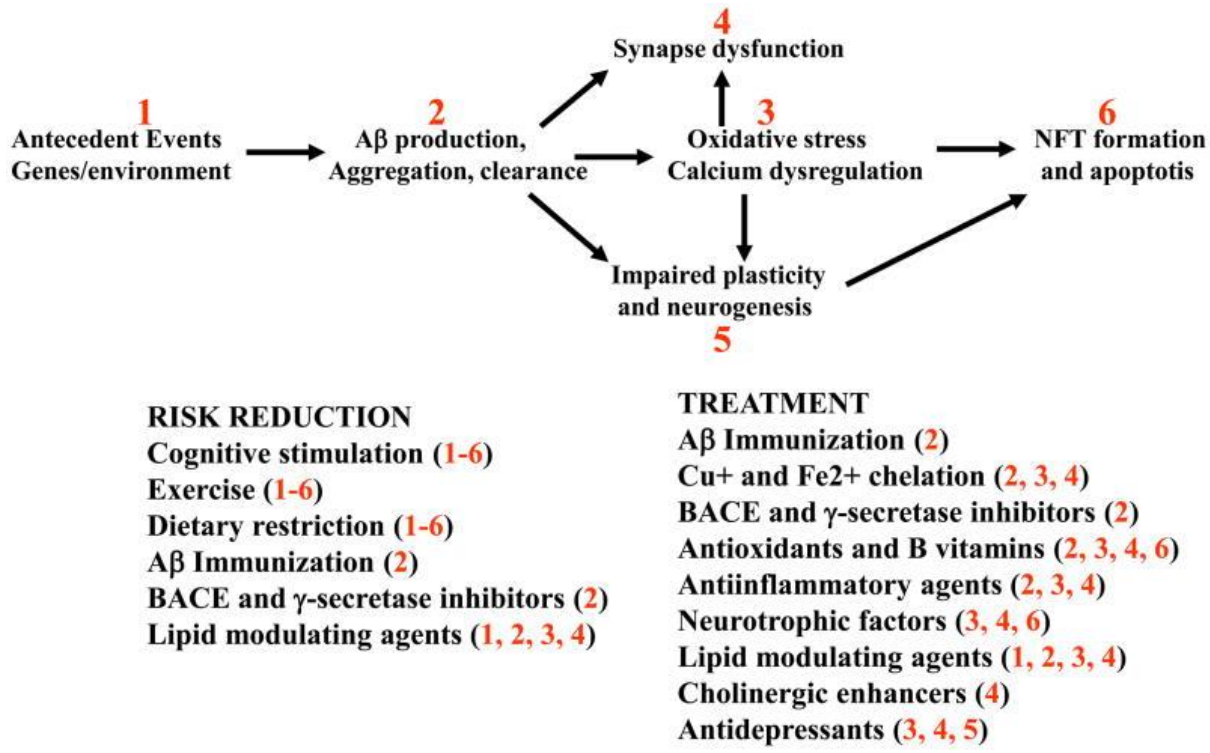


Figure 5: Strategies and targets for the prevention and treatment of AD (Nature. 2004 August 5; 430(7000): 631–639)

Approaches that are being tested in clinical and/or primary prevention trials include Aβ immunization, Cu+/Fe2+ chelation, cholesterol-lowering drugs (statins), anti-inflammatory agents, antioxidants and folic acid. Epidemiological and animal studies suggest the potential benefits of cognitive stimulation, regular physical exercise and dietary restriction, but these remain to be critically examined in controlled prospective studies or clinical trials.

1.6. METHODS FOR DETECTION OF AGGREGATION

Due to etiology of Alzheimer's disease inhibition of Aβ aggregation is proven to be an effective approach towards finding a cure (Estrada and Soto 2007). Many

different ways of measuring aggregation and detecting its inhibition have been developed.

1.6.1. FLUORIMETRIC

Amyloid fibrils share common characteristics, such as unbranched fibrillar morphology, cross-β-sheet structure, and binding of the amyloid-specific dyes Congo red and thioflavin T (ThT) (Dolphin, Ouberai et al. 2007). This has been successfully used to detect formation of Aβ fibrils by (Blanchard, Chen et al. 2004; Kim, Kim et al. 2006; Bolder, Sagis et al. 2007; Brunden, Trojanowski et al. 2009) and others. Binding of ThT and Aβ can easily be detected with fluorescence spectroscopy by measuring the rise of fluorescence intensity. Traditionally these tests have taken days to perform, but using different conditions, this time can shortened considerably. For instance using fibril model $(A\beta_{16-37}Y_{20}K_{22}K_{24})_4$, which is a covalent assembly of four Aβ fragments and adding some HPO_4^{2-} to the solute, the experiment can be completed in about an hour (Dolphin, Ouberai et al. 2007). Another way to approach the problem of time is to stir the incubated solute with a magnetic stirrer at 200 rpm (Tougu, Karafin et al. 2009). Main drawback of these methods is that potential inhibitors can compete with the fluorescent ligands (ThT in these cases) and by dislodging them from the fibrils or by binding too close to them extinguish fluorescence thereby produce false positive results (Hudson, Ecroyd et al. 2009). Evidence for false positives was also found in MALDI MS high-throughput screening assay developed by (Zovo, Helk et al. 2010).

1.6.2. IMMUNOLOGICAL METHODS

Immunological methods are specific, precise and so far most clinically advanced (Necula, Kayed et al. 2007). Using Aβ antibodies it is possible to detect monomers, oligomers and fibrils. Oligomers, being the most toxic form of Aβ aggregates, have received most attention (Walsh, Klyubin et al. 2002). Still, a major problem is method's relatively high cost, making it unsuitable for use in screening of large libraries of substances. Presently, immunological methods are best suited to assay Aβ oligomers in vivo, be it in human plasma or in tissue of laboratory animals (Necula, Breydo et al. 2007; Xia, Yang et al. 2009).

1.6.3. CELL-BASED EXPERIMENTS

Many cell-based systems have been developed to study Abeta aggregation in vivo. Often fused or truncated proteins or deletions in regulating genes are used (Goto and Tanzi 2002; von der Haar, Josse et al. 2007). Some of the methods circumvent Aβ aggregation altogether and focus on its cytotoxicity by stopping the cell-death cascade (Seyb, Schuman et al. 2008). Cell-based systems offer a useful view of how Aβ peptide oligomers and fibrils interact with cell organelles and cause oxidative damage in situ. This is also their main drawback, because these altered proteins might not give a true view of the aggregation and often to not pass through intracellular membranes. It is also hard to judge the efficacy of potential inhibitors when they have to interact with a myriad of cytoplasmic molecules. On the other hand studying extracellular Aβ fibril formation and its inhibition by measuring cell's response is more promising (Liu and Piasecki 2001). (Hong, Maezawa et al. 2007) have combined rapid MTT formazan exocytosis assay and the MC65 protection assay to screen for small molecule inhibitors of Abeta oligomer-induced cytotoxicity.

1.6.4. MASS SPECTROMETRY

From 1958 when amino acids and peptides where successfully first analyzed by MS (mass spectrometry) in its many forms has become the basis of modern proteomics and analytical biology. It enables quickly, cheaply and with high sensitivity to analyze large libraries of substances. At the same time being versatile and allowing to be coupled in tandem with an array of other analytical methods (Aebersold and Mann 2003; Glish and Vachet 2003; Marvin, Roberts et al. 2003; Payne and Glish 2005).

The name 'mass spectrometry' is a misnomer of sorts. The mass is not what is measured; instead, mass spectrometry determines the mass-to-charge (*m/z*) ratio or a property related to *m/z*. A mass spectrum is a plot of ion abundance versus *m/z*. This means that in order to measure a compound, it needs to be given a charge, *i.e.* protonated or deprotonated. Donor of the proton is the solute substances are diffused in. Different MS methods use different ways of ionization, for brevity an overview of the two most common ones will be given: ESI and MALDI. In Electrospray ionization, or ESI for short, ions are generated at atmospheric pressure by passing a sample based in solution through a small capillary (internal diameter < 250 μm) that is at a potential difference relative to a counter electrode at voltages between +500 and +4,500 V. Electrostatic spraying of a sample solution initially generates an aerosol of charged droplets. Sometimes a concentric flow of gas, such as N_2, is used to facilitate further nebulization process. ESI has some very impressive attributes that allow it to be used for a wide variety of biological problems (Glish and Vachet 2003). There seems to be no inherent limit to the size of molecules that can be ionized, as observed by the ionization of nominal weight of megaDaltons of DNA (Fuerstenau and Benner 1995). In addition, ESI is a very 'soft' technique that allows non-covalent biomacromolecular complexes to be ionized intactly. Despite the power of ESI, it does have two notable shortcomings. First, the flowing nature of ESI means that a sample is constantly being consumed. Unfortunately, however, no mass spectrometer constantly analyses ions, which means that some of the sample is wasted. In addition, when complex mixtures of compounds are present, the higher-concentration analytes can suppress ion formation by lower-concentration analytes.

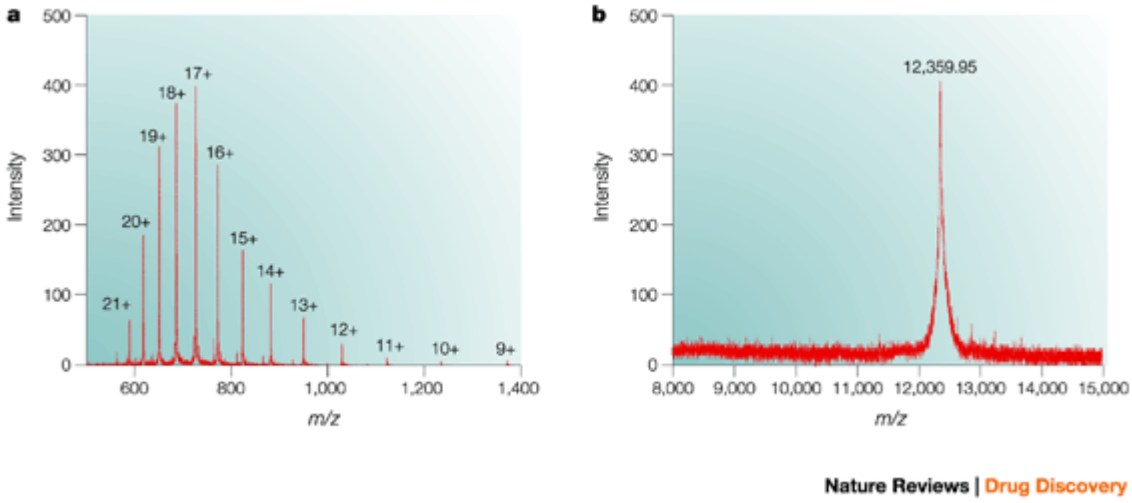


Figure 6: **a |** Electrospray ionization (ESI) mass spectrum of cytochrome *c*: multiple peaks are observed due to the different charge states that arise from varying degrees of protonation. **b |** Matrix-assisted laser

desorption/ionization (MALDI) mass spectrum of cytochrome *c*: only a single peak is observed for the analyte because ionization in MALDI generally occurs by the addition of a single proton. Note the different mass-to-charge (*m/z*) scales.(Glish and Vachet 2003)

MATRIX-ASSISTED LASER DESORPTION/IONIZATION

Unlike ESI, in which analyte ions are produced continuously, ions in matrix-assisted laser desorption/ionization (MALDI) are produced by pulsed-laser irradiation of a sample. The sample is co-crystallized with a solid matrix, a Brønsted-acid able to donate a proton to the analyte and that can absorb the wavelength of light emitted by the laser. Usually the sample is mixed with an appropriate matrix on a probe that is then inserted into the vacuum system. After the laser irradiation of the probe the gas-phase ions that are formed are directed toward oppositely charged acceleration electrode and then "fired" in the direction of mass analyzer (M. Karas 1987).

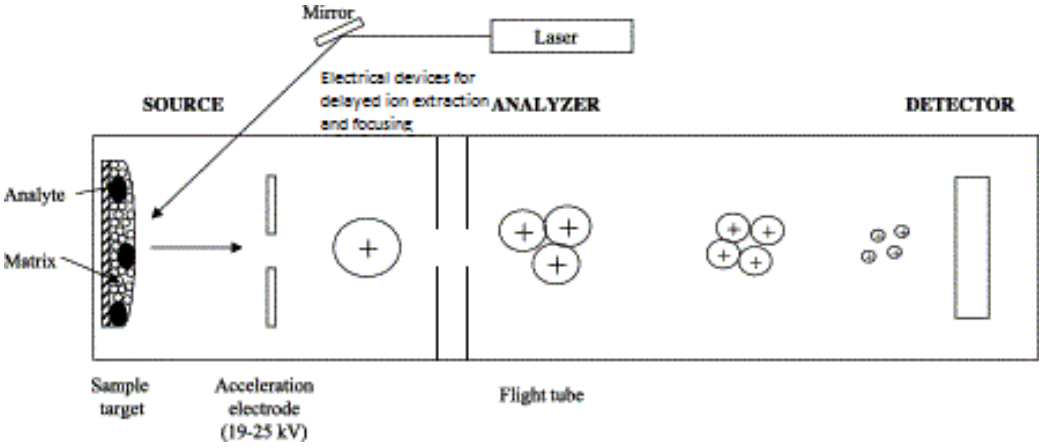


Figure 7: Principle of matrix-assisted laser desorption/ionization mass spectrometry. The analyte mixed with a saturated matrix solution forms crystals. The irradiation of this mixture by the laser induces the ionization of the matrix, desorption, transfer of protons from photo-excited matrix to analyte to form a protonated molecule. *Modified from a version of (Marvin, Roberts et al. 2003)*

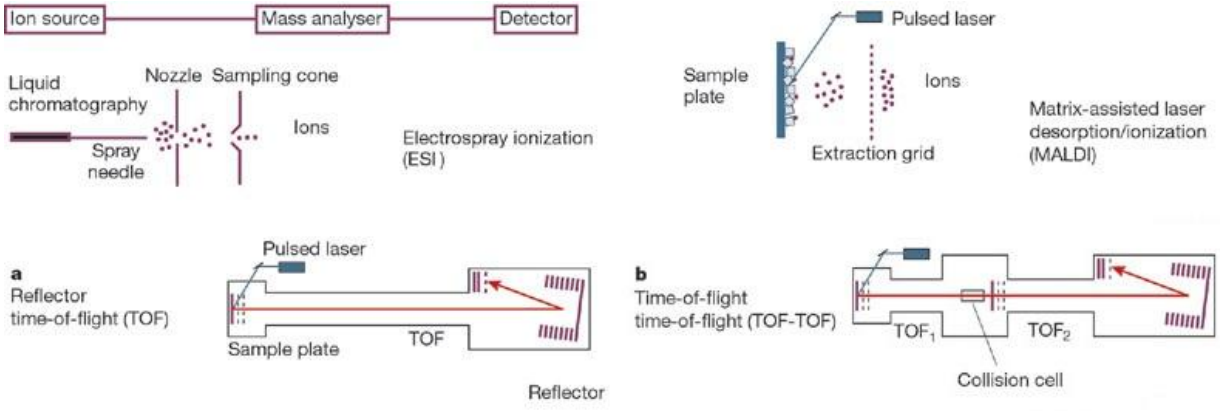


Figure 8: Uppermost panels depict the ionization and sample introduction process in electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). **a**, In reflector time-of-flight (TOF) instruments, the ions are accelerated to high kinetic energy and are separated along a flight tube as a result of their different velocities. The ions are turned around in a reflector, which compensates for slight differences in kinetic energy, and then impinge on a detector that amplifies and counts arriving ions. **b**, The TOF-TOF instrument incorporates a collision cell between two TOF sections. Ions of one mass-to-charge (*m*/*z*) ratio are selected in the first TOF section, fragmented in the

collision cell, and the masses of the fragments are separated in the second TOF section. *Cut down version from (Aebersold and Mann 2003)*

Further on, only MALDI MS-TOF (time of flight) will be discussed as it is most proper and relevant to current work. In TOF spectroscopy time of flight is measured to determine the mass of the molecule. As all molecules should in theory receive the same kinetic energy, then the smaller ones will be first to reach the detector and *vice versa*. A reflector extends ions' time of flight at the same time focusing them enabling analyzer to reach a higher resolution.

MALDI MS-TOF is a well-tested and –used method and it has been well documented that it can be used for Alzheimer's amyloid beta protein identification (Mori, Takio et al. 1992; Naslund, Karlstrom et al. 1996; Skribanek, Balaspiri et al. 2001; Zovo, Helk et al. 2010) Other technical reasons to prefer this method to others, such as ESI MS, were presented beforehand.

1.7. STATEMENT OF PURPOSE

Proteomics laboratory of the Tallinn University of Technology has developed a MALDI-TOF high-throughput method for screening of inhibitors of Aβ aggregation. Following that K.Zovo and E.Helk have tested the method with a screen of library consisting of 80 substances to find inhibitors of Aβ fibrillization. The aim of this thesis is to further test the applicability of current method and continue the research conducted by Helk & Zovo. In the focus of interest are a range of food colors, because there is reason to suspect that this group of substances has potential to inhibit Aβ fibrillization. The fact that food color safety is heavily researched and is beyond any reasonable doubt also plays a role in the decision. Most of the substances screened readily pass the blood-brain-barrier and would not have any considerable delivery issues. In short, if any of the colors would yield a sufficient rate of inhibition, a cheap, safe and efficient drug for targeting AD etiology and also for prophylactic treatment would be available without further delay.

2. MATERIALS AND METHODS

2.1. MATERIALS

Lyophilized amyloid beta peptide 1-42 (rPeptide, Bogart, GA, USA)

Lyophilized amyloid beta peptide 1-43 (Met-$A\beta_{42}$ prepared by Andra Noormägi)

Bovine insulin (Sigma-Aldrich, St. Louis, MI, USA)

α-Cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich, St. Louis, MI, USA)

Trifluoroacetic acid (TFA) (Riedel de-Haen, Seelze, Germany)

Acetonitrile (AcN) (Rathburn, Walkerburn, UK)

Ethanol

HEPES buffer (USB Corporation, Cleveland, Ohio, USA)

NH_3xH_2O (Sigma-Aldrich, St. Louis, MI, USA)

2.2. METHODS

2.2.1. PREPARATIONS OF SOLUTIONS

All of the substances examined were weighed using a microscale with an accuracy of 0,1μg. They were dissolved in 20% ethanol and stored in 2ml eppendorf tubes wrapped in aluminium foil to protect from light. Substances screened were stored at concentration of 26μM. When mixed into the inhibition sample the end concentration was 5μM.

α-Cyano-4-hydroxycinnamic acid is suitable for analyzing proteins under 10-kDa and was thus used as a matrix. The matrix was dissolved in 0,3%TFA; 60% ACN/H_2O in concentration of 10mg/ml. Matrix has to be dissolved in a volatile solvent to equalize the analyte-matrix crystallization. This is necessary to reduce the difference between spectrums acquired from a single sample (Duncan, Roder et al. 2008). Following dissolution matrix was mixed on Vortex for 1 minute, incubated for 10 minutes, vortexed for 1 minute and centrifuged for 3 minutes at 6000 rpm. Supernatant was transferred to folium-coated vial to protect matrix from light-induced degradation.

Bovine insulin was used for an internal standard and it was mixed with the 0,3%TFA; 60% ACN/H_2O just before mixing it with the matrix. The end concentration of insulin in the matrix was 0,3μM.

All of the demonstrated results are obtained from the experiments with a native Aβ-42 peptide. When calibrating the method Met- Aβ-42 / Aβ-43 was also used and Aβ-40 was tested as a seed for fibrillization, but these substances did not yield consistent results and did not behave as expected and were not applied to the method.

Native Aβ-42 peptide was dissolved in 0,02% NH_3xH_2O yielding a concentration of 10μM. Final concentration in the testing sample was 5μM.

When zero timepoint was pipetted on a probe and analyzed by MALDI MS then the concentration of Aβ-42 vs. insulin was 5μM vs. 0,3μM. This yielded a calibrated m/z ratio of 3:2 on the MS spectrum.

2.2.2. PERFORMING THE AGGREGATION

Aggregation was performed on microplates or in separate eppendorf tubes in series of six: a control sample without seed and potential inhibitors; a control sample with a seed to start the aggregation, but without inhibitors; and 4 samples each with different testing substance and same seed concentration. Each sample's volume was 26μl.

Fibrillization was kick-started by adding and aggregation nucleus – a seed. These seeds where made from mature Aβ fibrils of at least 1 month of age. Aβ fibrils in concentration of 5μM were subjected to sonication at 130W; 20 kHz; amplitude of 50%; 5s:10s (on-off cycle) pulse; for 1,5 minutes.

Experiments with different starting concentration of seeds were performed: 3%, 5% and 10% of the sample Aβ concentration. It was determined that the concentration of 5% was most suitable, giving enough precision in an acceptable 2 – 2,5 hour timeframe of aggregation.

2.2.3. MEASUREMENTS WITH MALDI TOF MS

Samples were pipetted on MALDI MS plate manually while first columns of the plate were left empty. Each spot had a parallel spot from the same sample to minimize statistical, quantitative and random errors. Ratio of matrix vs. sample on a probe was 3:1. MALDI MS plate was covered from light and left to dry and crystallize for 20 minutes.

MALDI MS spectrums were acquired in linear mode using *Voyager -DE™ STR Biochemistry Workstation (Applied Biosystems)* automated program. The program was set for: accelerating voltage -25 000V; grid voltage – 93%; delay time – 485 ns; pulses per sector – 40; mass range – 1500 - 10 000Da; laser intensity – 1900V; number of spectra collected – 10.

To analyze MALDI TOF MS spectrums *Data Explorer™* software was used.

3. RESULTS AND DISCUSSION

3.1. OBSERVING FIBRILLIZATION OF AB_{42}

Effect of test substances on $A\beta_{42}$ fibrillization were measured by comparing relative intensity of insulin and $A\beta_{42}$ peaks in MS. Molecular weight of insulin (5734 Da) is

close to that of $A\beta_{42}$ (4514 Da) making possible easy monitoring of both intensities. The fact that intensity does not change during the experiment makes it an ideal internal standard. It is easy to see that if test substances did not inhibit $A\beta_{42}$ aggregation, then the $A\beta_{42}$ monomers disappeared from the sample and the relative intensity of the MS peak became lower. On the other hand when test substances inhibited $A\beta_{42}$ aggregation, then the amount of monomer in the solute did not change or change only slightly and the intensity of amyloid peak remained constant.

Every series of experiment also had its control sample to ensure seed potency and correct zero timepoint starting concentrations. Control samples did not have any added seed and/or (two separate control) test substances added. Acceptable aggregation for seedless control sample was 10%-15%. Control sample with seed but without test substances was expected to fibrillize completely. Some known Aβ fibrillization inhibitors, such as BB41 (Basic Blue 41) and tannic acid were used to judge the potency of detected inhibitors.

Dynamics of the $A\beta_{42}$ peak in MALDI MS spectra:

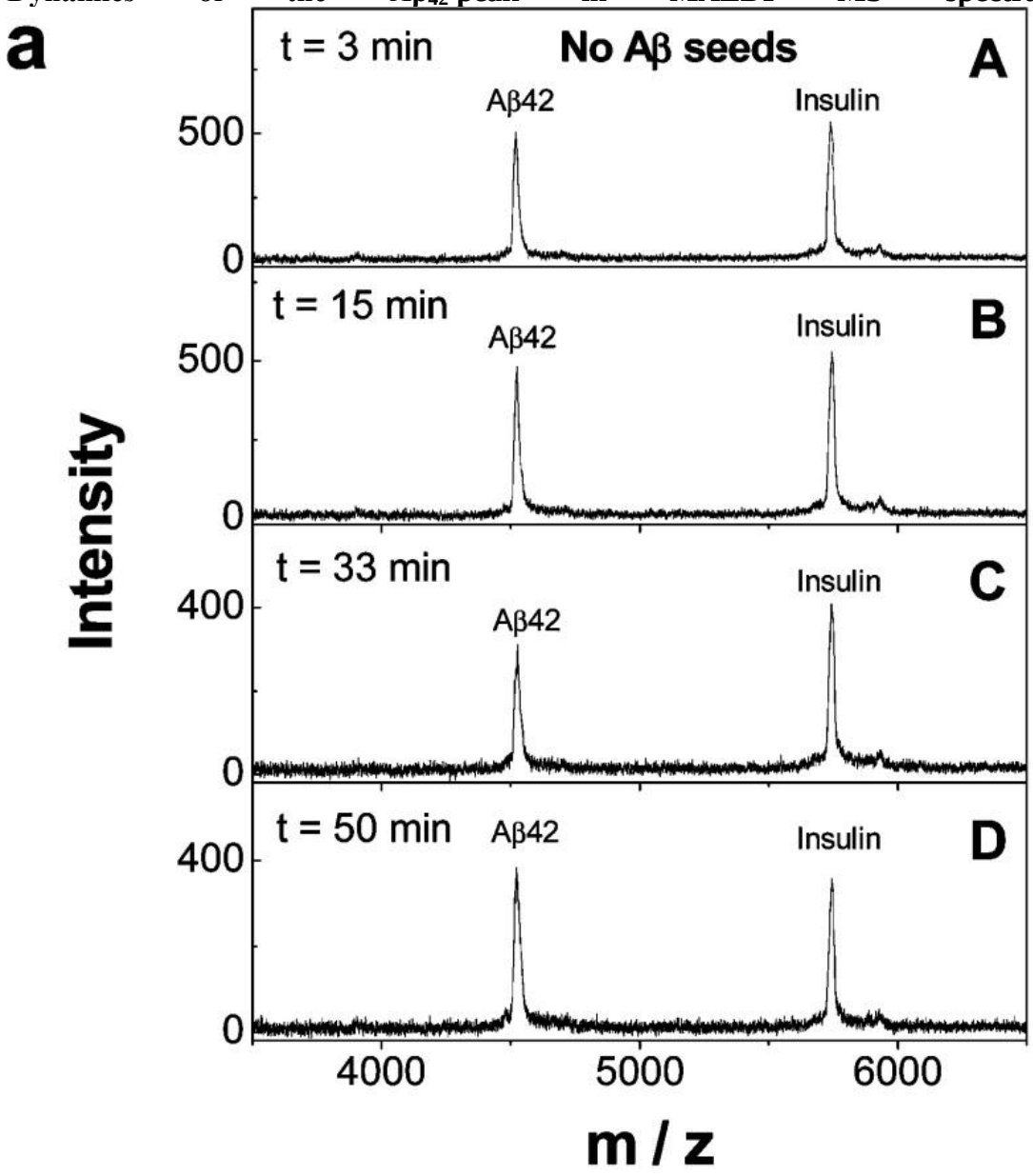


Figure 9: **(a)** averaged spectra of 5 μM $A\beta_{42}$ registered at different incubation times in the presence of 0,3 μM insulin as an internal standard (Zovo, Helk et al. 2010) – results reproduced in current work.

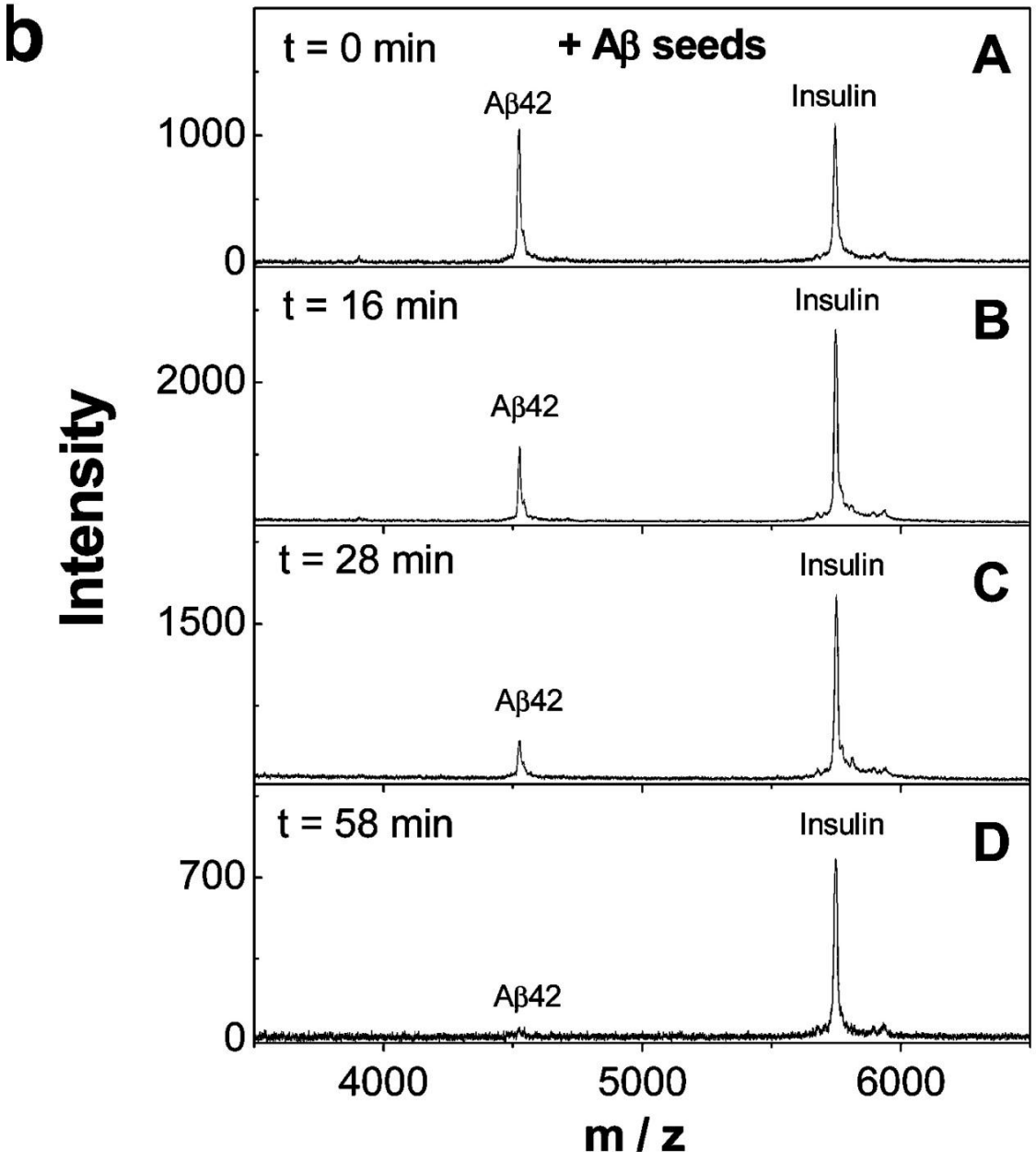


Figure 10: **(b)** averaged spectra of 5 μM $A\beta_{42}$ after addition of 0.25 μM $A\beta_{42}$ fibrillar seeds registered in the presence of 0.3 μM insulin as an internal standard. Incubation conditions: 20 mM HEPES, pH 7.3, 25 °C.(Zovo, Helk et al. 2010) – results reproduced in current work.

3.2 TEST SUBSTANCES

1. E131 – Patent Blue V; Mw 579.71 g mol^{-1}
2. E141 – Chlorophyllin; Mw 724.15 g mol^{-1}
3. E120 – Carmine; Mw 492,386 g mol^{-1}
4. E171 – Titanium dioxide; Mw 79.866 g mol^{-1}
5. E172 – Iron oxide; Mw 71,844 g mol^{-1}
6. E174 – Silver; Mw 107,868 g mol^{-1}
7. Unidentified green chocolate color
8. Unidentified blue chocolate color
9. Unidentified red chocolate color
10. E100 with E1450 - Starch sodium octenyl succinate
11. E104 - Quinoline Yellow WS; Mw 477.38 g/mol
12. E110 - Sunset Yellow FCF; Mw 452.37 g mol^{-1}
13. E124 – Ponceau 4R; Mw 604.47 g mol^{-1}
14. E102 – Tartrazine; Mw 534.3 g mol^{-1}
15. E132 – Indigo Carmine (Patent Blue); Mw 466.36 g mol^{-1}
16. Tannic acid; Mw 1701.2 g mol−1
17. Thioflavin T; Mw 318.86 g mol^{-1}
18. Piracetam; 142,156 g mol^{-1}

Same chemicals in different mixtures (as used in processed foods) were tested and will be shown where results were demonstrated.

3.3 OVERVIEW OF INHIBITION QUALITIES

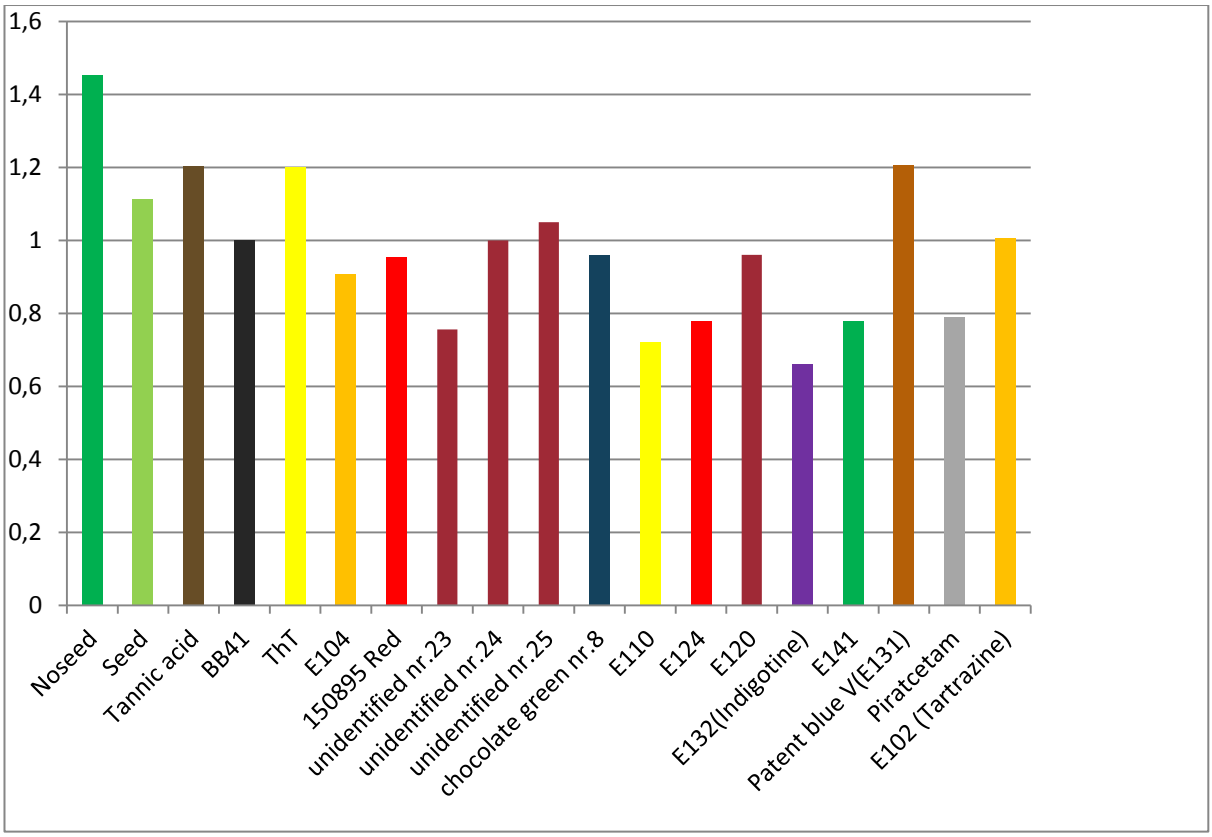


Figure 11: Ratio of $A\beta_{42}$:insulin (m/z) on MALDI MS spectrum. Timepoint 0 minutes. 5µM concentration of test substances.

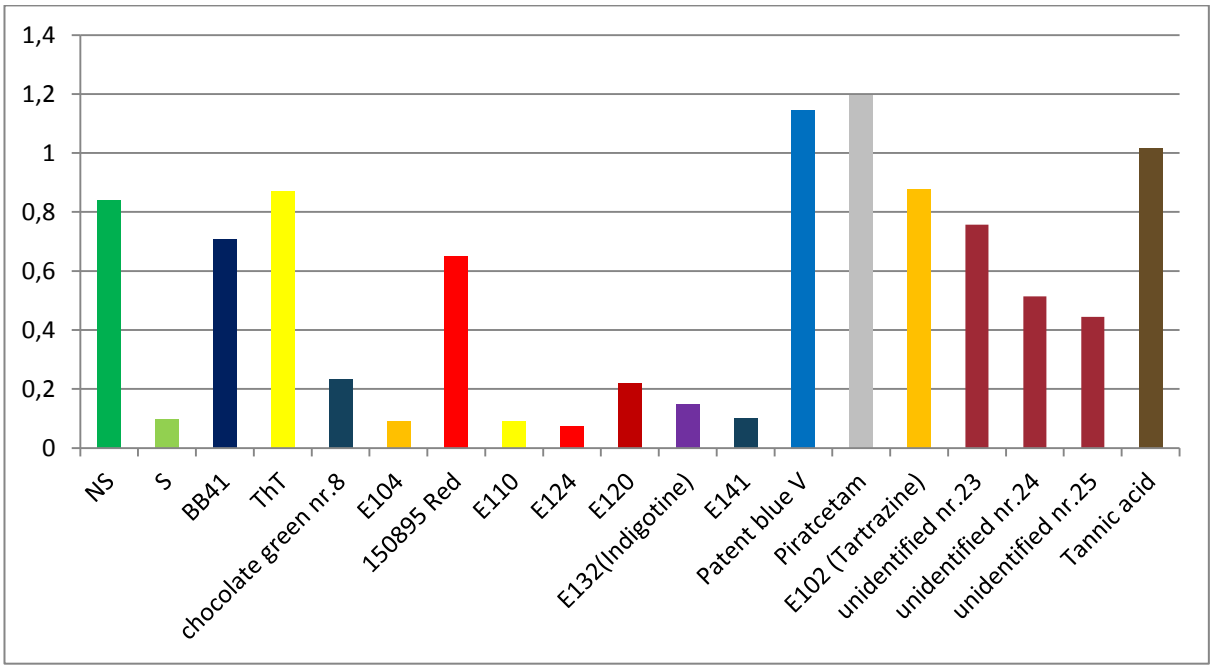


Figure 12: Ratio of $A\beta_{42}$:insulin (m/z) on MALDI MS spectrum. Timepoint 120 min. 5µM concentration of test substances.

3.4 INHIBITION KINETICS.

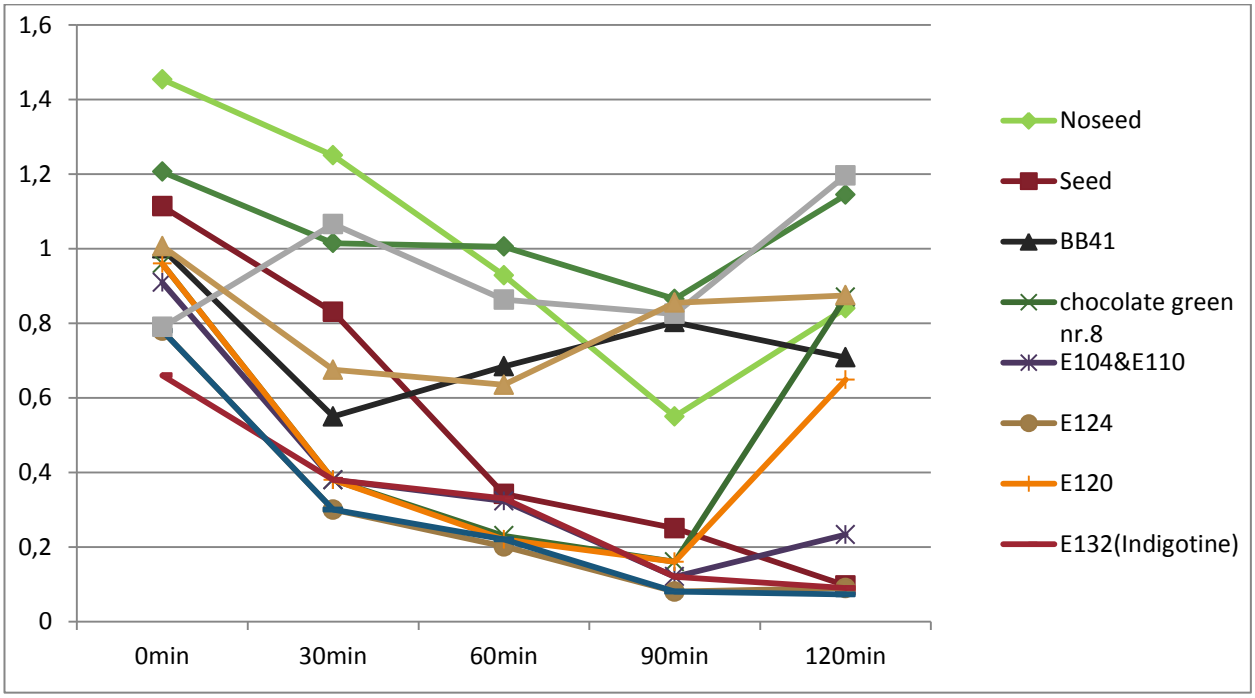


Figure 13: Inhibition kinetics for a set of substances. The line showing aggregation is not linear due to natural fluctuations in the reaction and measuring of thereof. Following figures give a view of the degree of aggregation over several timepoints, represented in column form. Test substances in concentration of 5µM.

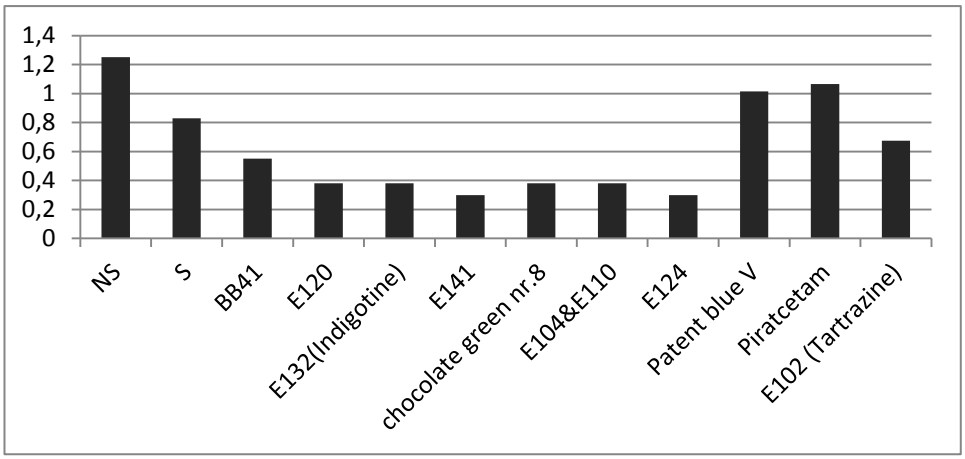


Figure 14: 30 minutes. Test substances in concentration of 5µM.

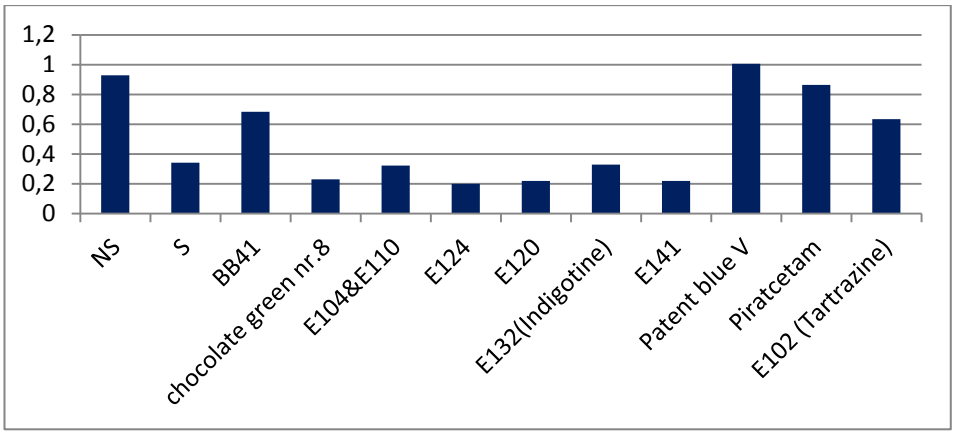


Figure 15: 60 minutes. Test substances in concentration of 5µM.

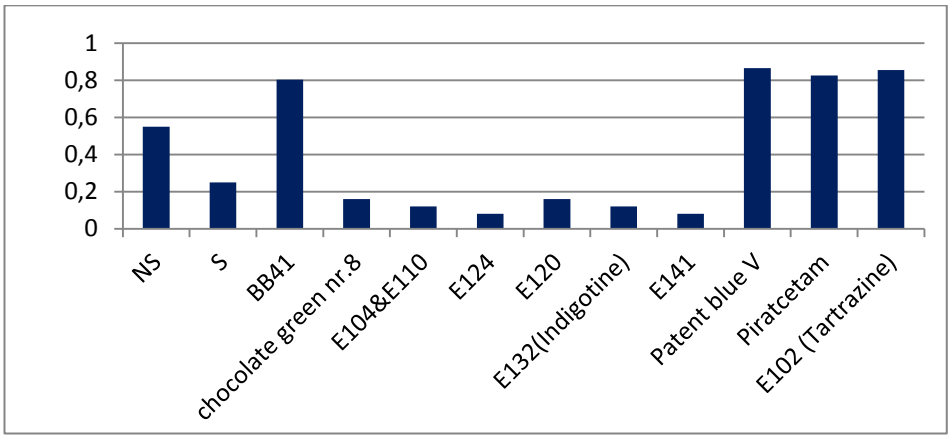


Figure 16: 90 minutes. Test substances in concentration of 5µM.

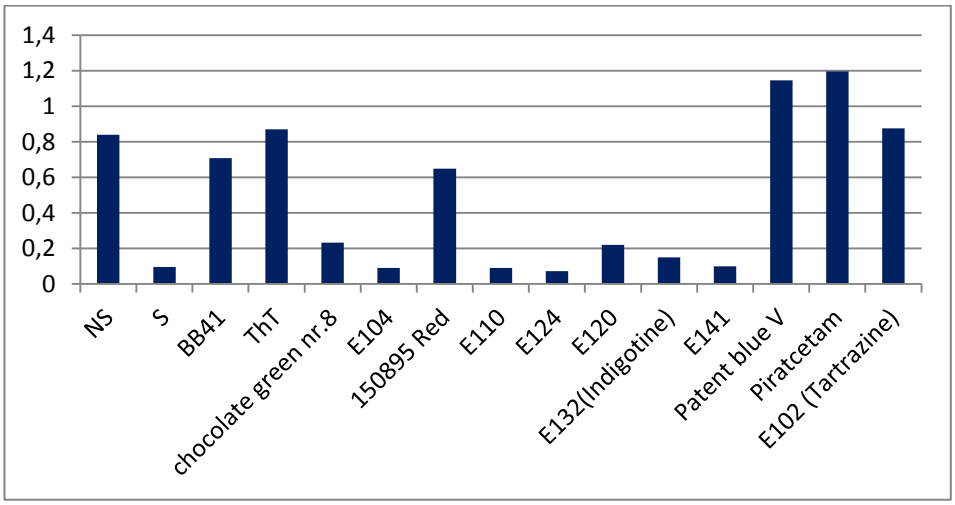
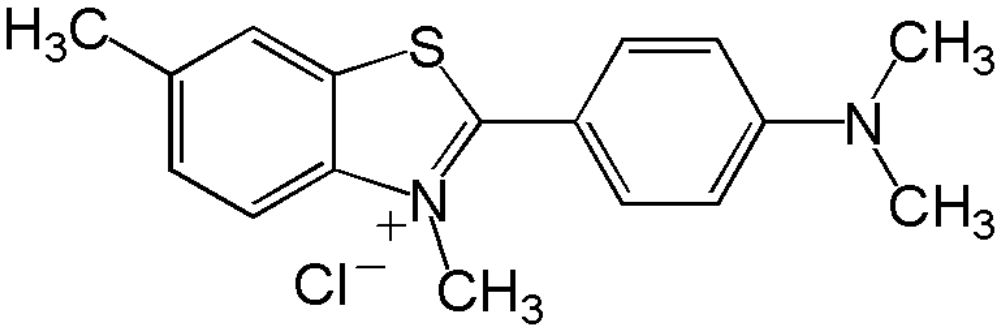


Figure 17: 120 minutes. Test substances in concentration of 5µM.

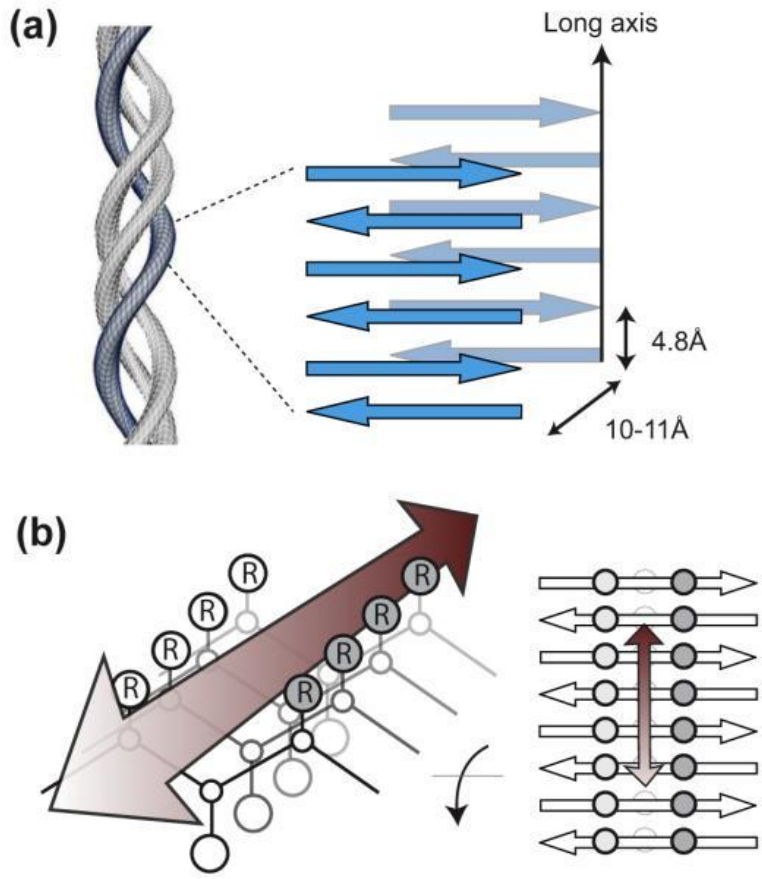
SUBSTANCES FOUND TO INHIBIT AB FIBRILLIZATION

Basic Blue (BB41) and tannic acid were tested to keep accordance with earlier works conducted on this subject. It was found that they substantially inhibit fibrillization of Aβ, this being in unison with earlier literature (Ono, Hasegawa et al. 2004; Taniguchi, Suzuki et al. 2005; Necula, Breydo et al. 2007).

Other substances found to inhibit Aβ fibrillization were Thioflavin T, Patent Blue V, Piracetam and Tartrazine. A slightly lower inhibition was detected in compounds 150895 Red and unidentified color nr.23. Therein ThT was tested due to its interference with Aβ fibril formation in florimetric experiments when in higher than normal concentration. Though a potent inhibitor of Aβ aggregation with the apparent mechanism of competing with Aβ monomers for fibril space, it probably cannot be used for humans due to its toxicity in such active concentrations. There is still a hope that a modified molecule will have use.



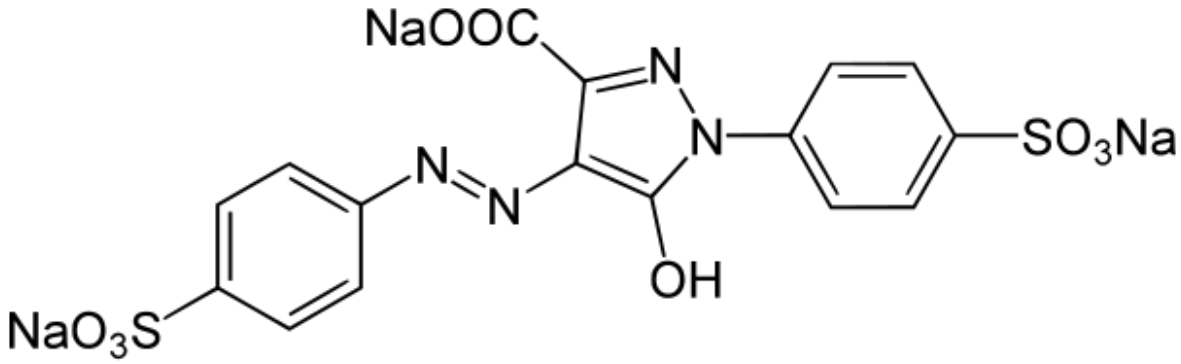
Thioflavin T



The common structure of fibrils and a structural rationale for fibril-ThT interactions (Biancalana and Koide 2010).

(**a**) Cross-β structure of amyloid fibrils, formed from layers of laminated β-sheets.
(**b**) "Channel" model of ThT binding to fibril-like β-sheets. ThT is proposed to bind along surface side-chain grooves running parallel to the long axis of the β-sheet.

TARTRAZINE



Tartrazine (otherwise known as E number E102, C.I. 19140, or FD&C Yellow 5) is a water soluble synthetic lemon yellow azo dye used for food coloring.

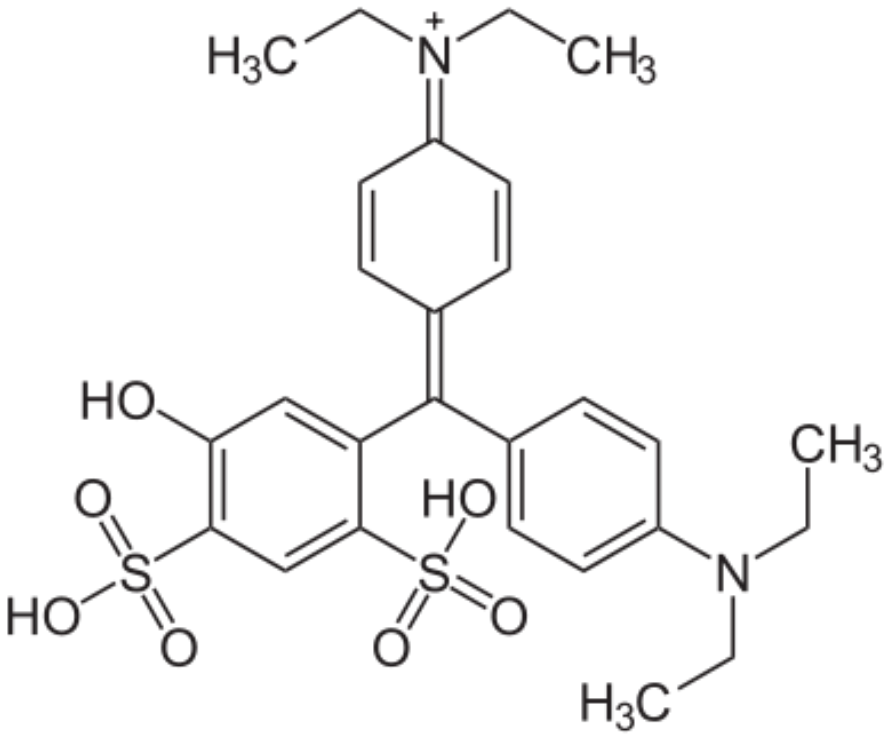
It is a very widely used food coloring agent, although nowadays there is a trend to substitute for tartrazine. This is due to the fact that Tartrazine appears to cause the most allergic and intolerance reactions of all the azo dyes. Symptoms from tartrazine sensitivity can occur by either ingestion or cutaneous exposure to a substance

containing tartrazine. A variety of immunologic responses have been attributed to tartrazine ingestion, including anxiety, migraine (Alvarez Cuesta, Alcover Sanchez et al. 1981), clinical depression, blurred vision, itching, general weakness, heatwaves, feeling of suffocation, purple skin patches, and sleep disturbance and has been suspected in causing hyperactivity (Rowe and Rowe 1994). Direct studies in mice have been conducted with noticeable behavioral effect on young mice and several generations of offspring (Tanaka, Takahashi et al. 2008; Gao, Li et al. 2011).

According these studies tartrazine seems to exert most unfavorable physiological effects on young individuals. In context of Alzheimer's disease being age-related pathology where patients are past the age of having children then many of the listed effects of tartrazine use become irrelevant. On the other hand there are cytogenetic effects of tartrazine to consider. Tartrazine is capable to strongly bind to linear dsDNA causing its degradation and has shown cytotoxicity at 1 and 2 mM concentration (Mpountoukas, Pantazaki et al. 2010). The study also speculated toxic potential on human lymphocytes.

Another study found that tartrazine and sunset yellow can behave as xenoestrogen (Axon, May et al. 2012). Although a small double-blind investigation with 26 atopic patients yielded no evidence that tartrazine contact results in allergic response (Pestana, Moreira et al. 2010) and even in the case that subsequent testing will verify tartrazine inhibition of Aβ fibrillization it would not be advisable to use as a therapeutical agent due to the many concerns already mentioned.

PATENT BLUE V



Patent Blue V, also called Food Blue 5 or Sulphan Blue, is a dark bluish synthetic dye used as a food coloring. As a food additive, it has E number E131. It has the appearance of a violet powder. In aqueous solution, its color will vary depending on the pH value of the medium: it is deep blue in a basic medium, yellowish-green in an acidic medium.

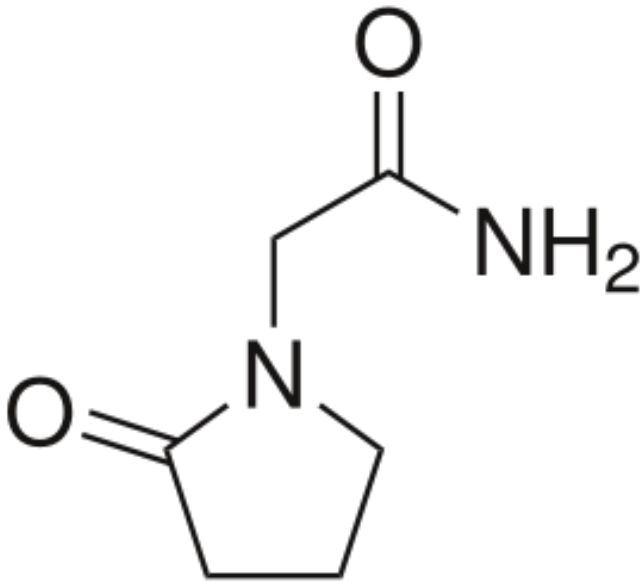
An advantage of E131 is the very deep color it produces even at low concentration. This trait has led to its application in medicine where Patent Blue V is used in lymphangiography and sentinel node biopsy as a dye to color lymph vessels. It is also used in dental disclosing tablets as a stain to show dental plaque on teeth.

In course of these treatments an anaphylactic effect has been registered (Rogler, Rohm et al. 2010; Howard, Moo et al. 2011) . It has been estimated that such allergy occurs in 0,9% of cases. As anaphylaxis can be very dangerous even leading to anaphylactic shock it is important that before treatment patients are tested for Patent Blue V sensitivity. The measurement of serum mast-cell tryptase (MCT) and skin prick test (SPT) are used in the investigation of suspected anaphylaxis because positive results are supportive of type-1 mediated hypersensitivity (Manson, Juneja et al. 2012). Similarly to tartrazine, evidence for its genotoxicity has been found (Sarikaya, Selvi et al. 2012)

Patent Blue V is banned as a food dye in Australia and USA, because health officials in these countries suspect that it may cause allergic reactions, with symptoms ranging from itching and nettle rash to nausea, hypotension, and in rare cases anaphylactic shock.

Still, as its use is well documented and practiced in medicine and as there are far less drawbacks of using Patent Blue than from tartrazine, it would be recommendable to use it in vivo inhibition of Aβ fibrillization.

PIRACETAM



Piracetam is considered a nootropic drug and is a cyclic derivative of GABA. It is one of the group of racetams. With its structure it does not follow the rules for amyloid-dye based inhibitors by (Reinke and Gestwicki 2007), nevertheless its utility is long-proven and it is possible that inhibitory activity is constituted through some kind of supramolecular structure or interaction. Piracetam is prescribed mainly in the case of myoclonus, but is also used for treatment of alcoholism, blood clotting, coagulation and vasospastic disorders, depression and anxiety, stroke, ischemia, dyspraxia and dysgraphia, schizophrenia, closed craniocerebral trauma, Alzheimer's, senile dementia and other age related neurological deteriorations (Winblad 2005). The latter part of the list is what prompted to include this substance in current study. Research on establishing safe dosage has concluded that piracetam, as with other racetams, is safe even in larger quantities and has very low toxicity (Gouliaev and Senning 1994).

Till now beneficial qualities of piracetam have been attributed to improvement of blood circulation, acceleration of neuronal metabolism, action on ion channels and channel-gated-receptors, activation of microglial cells and all-around neuroprotective properties. Newer evidence suggests that piracetam ameliorates the impairment of mitochondrial function and neurite outgrowth damaged by beta-amyloid peptide (Kurz, Ungerer et al. 2010). In another work it has been found that piracetam inhibits the lipid-destabilizing effect of the amyloid peptide Aβ C-terminal fragment. Piracetam, when preincubated with lipids, coats the phospholipid headgroups. Calculations suggest that this prevents appearance of the Aβ peptide-induced negative curvature that destabilizes membranes and gives way to Aβ cross-membrane channel formation. In addition, insertion of molecules with an inverted cone shape, like piracetam, into the outer membrane leaflet should make the formation of such structures energetically less favorable and therefore decrease the likelihood of membrane fusion (Mingeot-Leclercq, Lins et al. 2003). If subsequent testing will validate that piracetam does indeed inhibit the formation of amyloid-fibrils *in vitro* and *in vivo*, then a very valuable attribute would be added to already large arsenal that will be helpful in treating and stabilizing Alzheimer patients.

SUMMARY

From 1906 when Alois Alzheimer first described this disease, regardless of world-wide research, there has not been found a cure completely stopping or reversing the course f Alzheimer's disease (AD). Current work describes etiology of AD and gives an overview of different targets and treatment strategies. Next it proposes safe and active substances directed against the main cause of AD, against the formation of amyloid plaques and Aβ (amyloid beta) peptide oligomers. These substances would help to prevent the fibrillization of Aβ with to avoid all resulting pathological effects up to the Alzheimer's disease itself.

A more comprehensive research towards these molecules will be required, but it is clear that big pros are universal application and profylactic potential. On the other hand, when treatment strategies targeting α- and γ-secretases have more narrow, genetically linked application.Profylactic potential comes from the fact, that these aggregation inhibitors, mostly comprising of approved for consumption food colours, do not intervene with cell's natural metabolism and homeostasis. Inhibitors of Aβ fibrillizations here proposed have been identified using a high-throughput method of MALDI TOF mass spectometry developed in the Proteomics Laboratory in the Tallinn University of Technology. Method uses insulin as an inner standard to determine the change of soluble Aβ concentration in time. Aβ fibrills where used as 'seeds' to catalyze peptide's aggregation and the formation of other fibrills. Florimeter was used to make the fibrils in the presence of ThT. These fibrils were later sonicated to form the 'seeds'. Test were performed with peptides of different length: Aβ-40, Aβ-42 and Aβ-43. The most appropriate combination was determined to be native Aβ-42 fibril as 'seed' and native Aβ-42 monomer as aggregation substrate.

Tartrazine, Patent Blue V, piracetam and thioflavin T(ThT) were found to have considerable inhibitory properties. Previously reported inhibitors of Basic Blue (BB41) and tannic acid were confirmed. Two slightly weaker inhibitors 150985 Red and an unidentified sample nr.23. Due to established use of Patent Blue V and piracetam in medicine (and toxicity concerns of other colors) these two molecules are suggested for use as Aβ fibrillization inhibitors. MALDI TOF magnetospectography based method for screening those substances has proven reliable and further research with this method is recommended.

KOKKUVÕTE

Aastast 1906, mil Alois Alzheimer seda haigust kirjeldas pole hoolimata laialdasest ülemaailmsest teadustööst leitud seda neurodegenaratiivset haigust täielikult peatavat ja haiguskulgu pööravat ravi. Praegune uurimustöö kirjeldab Alzheimeri tõve etioloogiat, annab ülevaate erinevatest ravi strateegiatest ning pakub välja aktiivseid ja ohutuid aineid, mis on suunatud Alzheimeri haiguse peamise põhjustaja - amüloidsete naastude vastu. Need ained aitavad ära hoida amüloid beeta valgu agregeerumise ning seega vältida sellest tulenevaid kahjulikke mõjusid kuni Alzheimeri haiguse enda tekkeni välja. Pakutud amüloid beeta fibrillisatsiooni inhibiitorid on tuvastatud kasutades Tallinna Tehnika Ülikooli Proteoomika labori poolt välja arendatud unikaalse MALDI TOF magnetspektrograafial põhineva kõrge läbilaskvusvõimega skriinimismeetodit. Enamus leitud inhibiitoreid kuuluvad toiduvärvide hulka, mis tähendab, et nende ohutus inimesele on eelnevalt põhjalikult testitud ning on väljaspool mõistlikku kahtlust. Tartrasiinil, Patent Sinisel V, thioflafiin T leiti tugev inhibiitorne toime. Veel täheldati kahte nõrgemat inhibiitorit: 150985 Punane ja identifitseerimata proovi nr. 23. Lisaks on leitud tõendeid lisamaks piratsetaami kasulike omaduste hulka veel amüloidsete naastude vastase toime. Näidatud sai MALDI TOF MS meetodi sobivust madalamolekulaarsete ainete skriinimisel ning tulemused annavad põhjust edasiseks uurimustööks.

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