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**Investigating the biochemical and structural
alterations in tau mutants caused by VQIVYK-
targeting tau inhibitors**

BACHELOR'S THESIS

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Prehlasujem, že som bakalársku prácu vypracovala samostatne pod vedením Viswanatha Dasa M.Sc., Ph.D., s vyznačením všetkých použitých prameňov a spoluautorstiev. Súhlasím so zverejnením bakalárskej práce podľa zákona č. 111/1998 Sb., o vysokých školách, v znení neskorších predpisov. Bola som zoznámená s tým, že sa na moju prácu vzťahujú práva a povinnosti vyplývajúce zo zákona č. 121/2000 Sb., autorský zákon, v znení neskorších predpisov.

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Alica Chudáčková

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Abstract

Tauopathies represent a group of neurodegenerative diseases characterized by intracellular accumulation of abnormal tau protein. These diseases, such as Alzheimer's disease and frontotemporal dementia, are caused by excessive phosphorylation of tau protein, inducing the formation of fibrillar clusters of tau protein. The VQIVYK motif, located in the third repeat region (R3) of tau, is associated with its aggregation upon phosphorylation, thus contributing to the progression of these diseases. This thesis aims to investigate the effects of selected inhibitors targeting the VQIVYK motif of tau protein on the biochemical and biophysical properties of wild-type tau and tau mutant S285R/R2. Fluorescence and dot blot analyses revealed that several compounds, including entacapone, purpurin and palmatine chloride, were able to reduce tau aggregation and fibril formation in both wild-type and S285R mutant peptides. The S285R mutation consistently showed higher aggregation, confirming its utility as an experimental model of pathological tau. results suggest that inhibitors targeting aggregation-prone motifs may represent promising therapeutic strategies against tauopathies.

Keywords	tau protein, frontotemporal dementia, tauopathy, VQIVYK motif, inhibitors
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Abstrakt

Tauopatie predstavujú skupinu neurodegeneratívnych ochorení charakterizovaných vnútrobunkovou akumuláciou abnormálneho tau proteínu. Tieto ochorenia, ako napríklad Alzheimerova choroba a frontotemporálna demencia, sú spôsobené nadmernou fosforyláciou tau proteínu, ktorá vyvoláva tvorbu fibrilárnych zhlukov tau proteínu. Motív VQIVYK, ktorý sa nachádza v tretej opakovanej oblasti (R3) tau, je spojený s jeho agregáciou pri fosforylácii, čím prispieva k progresii týchto ochorení. Cieľom tejto práce je preskúmať účinky vybraných inhibítorov zameraných na motív VQIVYK proteínu tau na biochemické a biofyzikálne vlastnosti tau divokého typu a tau mutantu S285R/R2. Fluorescenčné a dot blot analýzy odhalili, že niekoľko zlúčenín vrátane entakapónu, purpurínu a palmatínchloridu dokázalo znížiť agregáciu a tvorbu fibríl tau v peptidoch divokého typu aj mutantu S285R. Mutácia S285R dôsledne vykazovala vyššiu agregáciu, čo potvrdzuje jej využiteľnosť ako experimentálneho modelu patologického tau. výsledky naznačujú, že inhibítory zamerané na motívy náchylné na agregáciu môžu predstavovať sľubné terapeutické stratégie proti tauopatiám.

Kľúčové slová	tau proteín, frontotemporálna demencia, tauopatia, VQIVYK motív, inhibítory
Počet strán	41
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Aims and Objectives

Frontotemporal dementia is a group of neurodegenerative tauopathies characterized by different tau mutations. Mutations, particularly around the P301 residue such as P301L and P301S, disrupt the protective mechanism of the flanking sequence, exposing the VQIVYK motif in repeat 3 of the tau microtubule-binding domain, resulting in increased aggregation of tau in frontotemporal dementia.

The primary aim of this thesis is to test the effect of selected tau VQIVYK-targeting inhibitors in altering the biochemical and biophysical properties of wild-type tau in comparison to tau mutants - S320F and S285R. The theoretical objectives focus on understanding how these inhibitors influence tau aggregation, fibrillization, and structural properties, providing insights into their potential therapeutic implications.

The experimental aims focused on examining β -sheet-rich structural changes induced by the inhibitors by Thioflavin T (ThT) binding kinetics assay. ThT enables real-time tracking of the fibrillization process by measuring fluorescence intensity over time. Furthermore, this study evaluates the effects of VQIVYK inhibitors on hydrophobicity changes in wild-type and mutant tau using the 8-anilinonaphthalene-1-sulfonic acid (ANS) assay. Additionally, the Immuno-dot blot assay, performed after the pelleting assay, is used to analyse the distribution of tau between soluble and insoluble fractions, providing insights into the extent of tau aggregation and the effects of VQIVYK inhibitors on fibril formation.

1 INTRODUCTION

Tauopathies represent a clinically and pathologically diverse group of neurodegenerative disorders (NDD) characterized by the intracellular accumulation of abnormal tau protein.

Tau protein is found mostly in human neuronal cells. Tau is affected by post-translational modifications, with phosphorylation being the most prevalent and functionally significant. Abnormal hyperphosphorylation induces the dissociation of tau from microtubules, leading to microtubule disassembly and the formation of fibrillary tangles accumulating in the brain, contributing to neuropathological changes associated with tauopathies like Alzheimer's disease (AD) and frontotemporal dementia (FTD).

VQIVYK motif is a six-amino-acid sequence located in the third repeat (R3) region of the tau protein. Phosphorylation of tau can expose or enhance the aggregation-prone nature of the VQIVYK region, thereby accelerating pathological tau aggregation and contributing to disease progression.

The inhibitors used in this study are categorized into three groups based on their origin. Catechols, naturally occurring benzene derivatives, are commonly employed in the treatment of Parkinson's disease due to their neuroprotective properties. Natural inhibitors include: Purpurin, a trihydroxyanthraquinone derived from the anthracene family, shows potential as a lead molecule for AD treatment; Oleocanthal, an anti-inflammatory compound, exhibits properties akin to the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen; Rosmarinic acid is known for its antioxidant and anti-inflammatory effects and Curcumin, a beta-diketone, demonstrates anti-inflammatory, antineoplastic and neuroprotective properties. The other inhibitor, palmatine chloride, is an alkaloid exhibiting antioxidant and anti-inflammatory effects.

2 CURRENT STATE OF THE PROBLEM

2.1 Tauopathies

2.1.2 Pathogenesis and diagnosis

Tauopathies are neurodegenerative and fatal diseases defined by abnormal tau protein disassembling and forming aggregates in the brain. As phosphorylated tau detaches from microtubules, it undergoes self-assembly into neurotoxic oligomers and fibrillar aggregates, driving disease progression, such as FTD, AD, amyotrophic lateral sclerosis, and Pick's disease, among many others (Annadurai, 2023).

Tauopathies are classified into two major groups according to their underlying causes. Primary tauopathies are distinguished by the accumulation of abnormal tau protein within cells, whereas secondary tauopathies arise from a different pathological process, with tau protein accumulation occurring as a secondary effect (Dugger & Dickson, 2017). In primary tauopathies, which include progressive supranuclear palsy, corticobasal degeneration, and Pick's disease, tau aggregates show distinct morphological and compositional characteristics compared to those observed in AD. Notably, while tau pathology in AD is predominantly limited to neurons, primary tauopathies frequently demonstrate tau deposition in non-neuronal cell types, including astrocytes and oligodendrocytes (Lanero-Rodriguez et al., 2024).

At the microscopic level, tauopathies can be further categorized into six tau isoforms present in affected cells based on the alternative splicing of tau protein's exons 2, 3, and 10 (Dugger & Dickson, 2017). The isoforms differ in the number of repeated amino-acid sequences in the microtubule-binding domain and the presence of N-terminal insertions. Alternative splicing of exons 2 and 3 generate isoforms with 0, 1, or 2 N-terminal insertions (0N, 1N, 2N), whilst alternative splicing of exon 10 yields three or four microtubule-binding repeats in the microtubule-binding domain (3R, 4R) (Strang et al., 2019). Thus, tauopathies can be categorized into 3R-tauopathies, 4R-tauopathies or mixed 3R/4R-tauopathies (Lanero-Rodriguez et al., 2024). In secondary tauopathy AD, tau aggregates consist of all isoforms (Buchholz & Zempel, 2024).

The diagnosis of tauopathies remains a clinical and research challenge due to their heterogeneity, involvement of multiple proteins in the pathological processes and overlapping symptoms with other NDDs (Orr et al., 2017; Zhang et al., 2022). Currently,

the clinical diagnosis depends on the patient's medical history, focusing on symptom presentation and progression. For example, AD can manifest itself in impaired memory and hippocampal atrophy, while supranuclear palsy is characterized by frequent falls due to subcortical degeneration (Orr et al., 2017). FTD represents 2,6 % of all dementia cases, and although it is the most prevalent form of presenile dementia, FTD remains highly underdiagnosed (Zhang et al., 2022). Additionally, dementia risk factors like hearing loss, hypertension, traumatic brain injury, poor education or lack of physical activity are also considered in the diagnostic process (Das et al., 2024).

Tauopathies are diagnosed with several methods, including neuroimaging techniques such as positron emerging tomography, which assesses brain function, and with magnetic resonance imaging, which detects structural changes in grey and white matter (Orr et al., 2017). However, these diagnostic imaging patterns are often unreliable in accurately categorizing specific NDDs due to the limited sensitivity of the method (Riley et al., 2022).

In addition to neuroimaging, the identification of biomarkers in cerebrospinal fluid (CSF) and blood has shown potential in improving diagnostic accuracy for tauopathies. By detecting molecular abnormalities years before clinical symptoms appear, early diagnosis becomes possible, allowing for the implementation of targeted therapies. These therapies include small molecule drugs, antibodies, and cell-based treatments. Understanding the molecular mechanisms underlying neurodegenerative diseases also facilitates a biomarker-driven classification, which aids in the development of more precise imaging techniques and targeted therapeutic strategies (Riley et al., 2022).

Presently, there are no approved disease-modifying agents for tauopathies capable of halting disease progression, nor any that offer a cure. Drugs employed in tauopathy treatment primarily target cognitive and behavioural symptoms, which may lead to reduced long-term efficacy or contribute to further health decline over time (Islam et al., 2022a).

The WHO identifies dementia as a major contributor to age-related cognitive decline, affecting up to 55 million people globally, with an estimated 7 million new cases each year – a number expected to double by 2050 (Das et al., 2024; Zhang et al., 2022). While significant progress has been made, early and differential diagnosis of tauopathies remains an evolving field. Ongoing research into tau structure, including aggregation

motifs like VQIVYK, holds promise for more precise and personalized diagnostic tools (Orr et al., 2017).

2.1.2 Frontotemporal dementia

Frontotemporal lobar degeneration refers to a group of neurodegenerative conditions characterized by neuropathological changes in the frontal and temporal lobes of the brain. These changes are responsible for various clinical syndromes, which often overlap. Frontotemporal dementia (FTD) is the most common of these, marked by progressive cognitive decline that primarily affects behaviour, language, and executive functions (Antonioni et al., 2023). FTD is further divided into different variants based on the symptoms, which include behavioural variant (bvFTD), semantic and nonfluent variants of primary progressive aphasia (svPPA and nfvPPA) and logopenic variant with language dysfunction and variable behavioural change (Olszewska et al., 2016).

The epidemiology of FTD has evolved significantly in recent years, with an increased recognition of the condition. Together with Alzheimer's disease, FTD is a leading cause of dementia that manifests in midlife or earlier. While FTD is typically diagnosed in the sixth decade of life, there are cases where individuals are affected much earlier, where it can be mistaken for psychiatric disorders like schizophrenia, bipolar disorder, and major depression (Onyike & Diehl-Schmid, 2013).

Genetic and environmental factors play significant roles in the development of FTD. It is estimated that 40% of FTD cases are familial, often with an autosomal dominant inheritance pattern where mutations in several genes, including *MAPT*, *GRN*, and *C9orf72*, are implicated (Antonioni et al., 2023). *MAPT* mutations, associated with tau deposition, are linked to the familial form of the disease, while mutations in the progranulin (*GRN*) gene are commonly observed in cases with a behavioural variant of FTD. The *C9orf72* gene is another critical component, particularly in cases associated with amyotrophic lateral sclerosis (Olszewska et al., 2016). Understanding these genetic factors has opened the door to potential targeted therapies and genetic screening for affected families.

In addition to genetic mutations, environmental factors are believed to contribute to the development of FTD. Studies have highlighted the role of neuroinflammation,

autoimmune disorders, and head trauma as potential risk factors for FTD (Onyike & Diehl-Schmid, 2013).

Biomarkers for FTD remain a topic of active research. Although clinical diagnosis often relies on neuroimaging techniques such as MRI or PET scans, recent advances in fluid biomarkers, such as CSF and blood-based markers, have shown promise in improving diagnostic accuracy. Elevated levels of tau in cerebrospinal fluid have been proposed as potential biomarkers for the disease, helping to distinguish FTD from other neurodegenerative conditions such as Alzheimer's disease (Antonioni et al., 2023).

2.1.3 Alzheimer's disease

Alzheimer's disease is a progressive neurological condition and is the third leading cause of dementia globally (Pavlíková, 2024). This degenerative tauopathy begins in the temporal and parietal lobes and is defined by abnormal hyperphosphorylation of tau, leading to deteriorating cognitive function, memory loss, behavioural changes, and functional deficiency (Annadurai, 2023; Avila et al., 2004).

It is hypothesized that the accumulation of tau-containing neurofibrillary tangles (NFTs) and insoluble amyloid- β ($A\beta$) plaques interfere with healthy neuronal function and impair communication within the brain (Singh et al., 2024).

The assembly of tau into the characteristic paired helical filaments (PHFs) in AD has been shown to depend on a specific hexapeptide motif $^{306}\text{VQIVYK}^{311}$, which adopts a β -sheet conformation essential for pathological aggregation (Von Bergen et al., 2000). This conformational change enhances tau's self-association, contributing to the formation of NFTs and further driving disease progression.

Current pharmacological treatments for Alzheimer's disease (AD) primarily offer symptomatic relief and fail to significantly alter disease progression. Commonly used medications, such as acetylcholinesterase inhibitors, provide only modest and temporary cognitive benefits (Annadurai, 2023). Consequently, attention has shifted toward disease-modifying strategies, including the repurposing of antineoplastic agents originally developed for cancer treatment. These compounds have demonstrated neuroprotective properties in preclinical models by modulating key processes involved in AD pathology, such as tau phosphorylation, $A\beta$ accumulation, and neuroinflammation. Furthermore,

advances in drug delivery systems, particularly those designed to cross the blood–brain barrier, may enhance the effectiveness and safety of these therapies in future clinical applications (Das et al., 2024).

2.2 Tau protein

2.2.1 Structure and function

Tau protein is a member of the microtubule-associated protein family found across different animal species. In humans, it is predominantly expressed in neuronal cells but can also be present in other cell types, including glial cells and astrocytes (Pavlíková, 2024). The *MAPT* gene, located on chromosome 17q21, encodes tau protein and is regulated by various factors, including alternative splicing (Caillet-Boudin et al., 2015). Alternative splicing of the *MAPT* gene leads to the production of six different tau isoforms (Fig. 1), which vary in the number of microtubule-binding repeats and N-terminal inserts. The isoforms have different roles in brain development, with three-repeat (3R) tau being more common during foetal development and a balanced 3R:4R tau ratio in adults (Avila et al., 2004; Caillet-Boudin et al., 2015).

The structure of tau contains a specific sequence, known as the hexapeptide motif ³⁰⁶VQIVYK³¹¹, which plays a critical role in tau protein's ability to aggregate into paired helical filaments, a hallmark of Alzheimer's disease (Von Bergen et al., 2000). The different isoforms of tau, with variations in their structure, influence their tendency to aggregate, contributing to the development of tau-related diseases (Annadurai, 2023).

Tau protein's primary function is to stabilize microtubules, essential components of the cell's cytoskeleton (Avila et al., 2004). In neurons, tau plays a critical role in axonal transport, which is responsible for the proper communication between neurons. Tau protein's interaction with microtubules is regulated by phosphorylation, which can either enhance or impair its binding. Under normal conditions, tau maintains the stability of microtubules by promoting their assembly (Annadurai, 2023). However, when tau undergoes hyperphosphorylation, it loses its affinity to microtubules, resulting in microtubule destabilization. This disruption of microtubule dynamics is a pathological process observed in various neurodegenerative disorders, with Alzheimer's disease being a prominent example (Annadurai, 2023; Avila et al., 2004).

Several disease-associated mutations in the tau protein are located in microtubule-binding domain and have been linked to frontotemporal dementia and related tauopathies. Among the most studied are the mutations at position 301, particularly P301L and P301S, where the original proline residue is replaced by leucine or serine. These mutations are known to reduce tau's affinity for microtubules and enhance its tendency to misfold and aggregate. Both P301L and P301S have been widely used in experimental models of tauopathy and serve as key examples of how single-point mutations can accelerate tau aggregation and neurotoxicity (Annadurai, 2023).

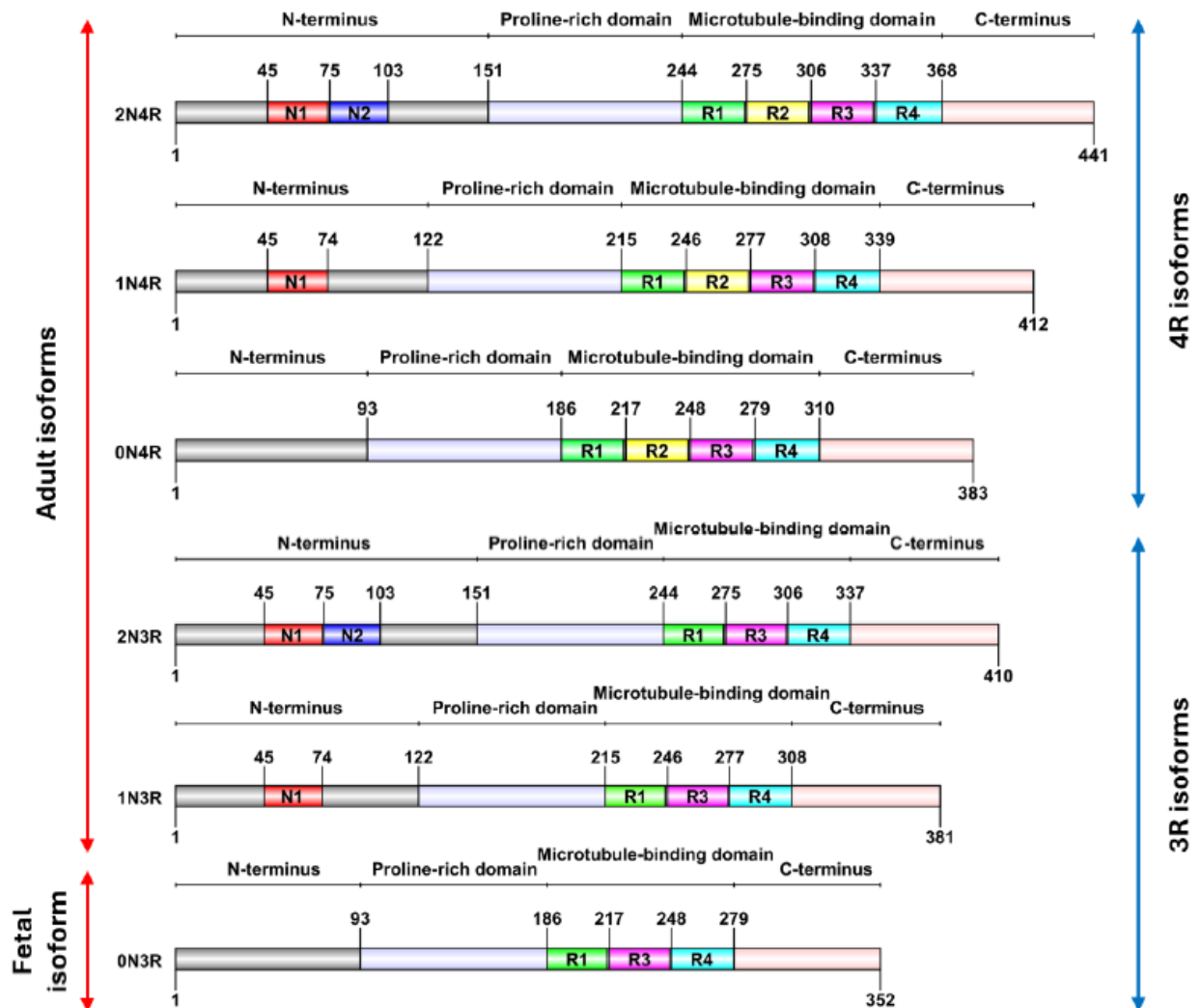


Figure 1. Illustration of the six human CNS tau isoforms. These isoforms comprise an N-terminal region (referred to as the projection domain), a proline-rich domain, a microtubule-binding domain, and a C-terminal tail, serving as the core component of the protein. The distinction among the isoforms arises from variations in the number of repetitive sequences (repeats) within the microtubule-binding domain (3R or 4R) and the presence of inserts at the N-

terminal region of the protein (0N, 1N, or 2N). (Adapted from (Pirscoveanu et al. 2017; Nizynski et al. 2017))

2.2.2 Post-translational modifications

Like all proteins, tau undergoes post-translational modifications (PTMs), with phosphorylation being the most prevalent and functionally significant (Avila et al., 2004). These modifications profoundly influence tau's structure, activity, and intracellular distribution. In healthy neurons, PTMs help regulate tau's normal physiological roles; however, in neurodegenerative disorders such as Alzheimer's disease, dysregulated PTMs contribute to pathological tau aggregation and toxicity (Pavlíková, 2024).

Phosphorylation occurs mainly on threonine and serine residues, but tyrosine can be affected too (Avila et al., 2004; Hamano et al., 2021). These phosphorylation sites are commonly divided into two categories: those targeted by proline-directed kinases such as GSK-3, CDK5, MAP kinases (e.g., JNK, p38), and those modified by non-proline-directed kinases like PKA, PKC, CaMKII, or CKII, which often target residues near acidic amino acids, especially within exons 2 and 3 (Avila et al., 2004).

Research suggests that hyperphosphorylated tau is the primary constituent of PHFs found in neurofibrillary tangles (Medina & Avila, 2020). Abnormal hyperphosphorylation induces the dissociation of tau from microtubules, leading to microtubule destabilization and disassembly, and accumulation of tau in the cytoplasm (Götz et al., 2019). Subsequently, dissociated tau undergoes conformational changes aggregating into fibrillary tangles that accumulate in the brain, contributing to neuropathological changes associated with tauopathies like AD and FTD (Pavlíková, 2024).

Tau undergoes other PTMs like acetylation, ubiquitination, truncation or methylation. Acetylation is a post-translational modification that plays a significant role in tau polymerization and toxicity, particularly at lysine residues involved in electrostatic and hydrophobic interactions (Hamano et al., 2021). Acetylated tau protein is regulated by histone acetyltransferases and can be reversed with histone deacetylases (Pavlíková, 2024). Under normal conditions, tau is cleared through autophagy-lysosomal and ubiquitin-proteasomal pathways, like other proteins. However, improper ubiquitination of tau can lead to its aggregation and the formation of neurofibrillary tangles. SUMOylation and acetylation has been shown to inhibit tau ubiquitination and

degradation, indicating its potential pathogenic role in tauopathies (Islam et al., 2022b). Ubiquitin, a 76-amino acid protein, attaches to substrates for degradation in an ATP-dependent manner. Tau can undergo ubiquitination, although ubiquitinated tau is primarily found in abnormal aggregates, such as inclusion bodies seen in Pick's or Parkinson's diseases, or in some forms of PHFs found in Alzheimer's disease (Avila et al., 2004). Tau truncation refers to the cleavage of tau at glutamic acid residue 391, a modification that may promote abnormal tau aggregation. Additionally, tau can undergo deamidation at asparagine or glutamine residues, a process that may also contribute to tau aggregation (Avila et al., 2004).

2.2.3 VQIVYK and VQIINK motifs

The aggregation of tau protein is a hallmark of several neurodegenerative diseases and is strongly driven by specific short hexapeptide sequences within its microtubule-binding domain. Among these, the motifs ³⁰⁶VQIVYK³¹¹ and ²⁷⁵VQIINK²⁸⁰ are critical for tau self-assembly into β -sheet-rich fibrils in hydrophilic environment (Annadurai, 2023). These motifs facilitate the formation of pathological tau aggregates through intermolecular interactions and are highly conserved across tau isoforms. Due to their central role in fibrillization, they represent key targets for therapeutic intervention and are frequently studied in structural and drug development research (Ganguly et al., 2015).

The ³⁰⁶VQIVYK³¹¹ motif, commonly referred to as PHF6, is a six-amino-acid sequence located at the beginning of the third repeat (R3) region of the tau protein and is present in every tau isoform (Ganguly et al., 2015). This highly aggregation-prone hexapeptide fragment forms the structural core of tau fibril formation, mediating the self-assembly of tau by promoting intermolecular interactions between tau molecules (Pavlíková, 2024). Despite tau's random coil appearance in solution, as indicated by spectroscopic and x-ray studies, it forms well-defined fibers, such as PHFs, through a nucleation-condensation mechanism. This aggregation process can be accelerated by oxidation of SH groups and the presence of polyanions like heparin, nucleic acids or arachidonic acid (D. Chen et al., 2019; Von Bergen et al., 2000).

Computational analyses, such as density functional theory, have highlighted the importance of glutamine (Q) within the sequence, as it facilitates both peptide crystallization and the initiation of amyloid formation. Interestingly, multiple polymorphic forms of the resulting fibrils have been observed, indicating variability in

the sidechain packing while retaining the core β -sheet structure (Plumley & Dannenberg, 2011).

Phosphorylation of tau can expose or enhance the aggregation-prone nature of the $^{306}\text{VQIVYK}^{311}$ region, thereby accelerating pathological tau aggregation and contributing to disease progression (D. Chen et al., 2019). Therefore, $^{306}\text{VQIVYK}^{311}$ is a potential drug target for preventing abnormal aggregation of tau in tauopathies (Annadurai, 2023).

The $^{275}\text{VQIINK}^{280}$ sequence, also referred to as PHF6*, is a six-residue hexapeptide motif located within the second repeat (R2) region of the tau protein and is present exclusively in the 4-repeat (4R) isoforms of tau (Ganguly et al., 2015). Despite the predominantly hydrophilic nature of full-length tau, PHF6* exhibits significant aggregation propensity due to its content of hydrophobic amino acids and its high tendency to form β -sheet structures. This makes $^{275}\text{VQIINK}^{280}$ a crucial driver of tau aggregation and a core component in the formation of paired helical filaments in tauopathies such as Alzheimer's disease (Annadurai, 2023). Unlike the related PHF6 motif, no crystal structure has yet been reported for the $^{275}\text{VQIINK}^{280}$ sequence, making its precise aggregation mechanism structurally less defined despite its proven role in fibrillization (Plumley & Dannenberg, 2011).

2.3 Inhibitors

2.3.1 Catechols

Drug repurposing is a pharmaceutical approach for identifying new uses of approved generic drugs (Pushpakom et al., 2019). It's a low-cost and promising strategy of discovering potential effective disease-modifying treatments with known pharmacokinetic, pharmacodynamic and toxicity properties against tauopathies (Das et al., 2024).

Catechols are naturally occurring benzene derivatives featuring two adjacent hydroxyl groups, commonly employed in the treatment of Parkinson's disease due to their neuroprotective properties (Wu et al., 2020). Catechols include drug tolcapone (Fig. 2A), a selective catechol-O-methyltransferase (COMT) inhibitor used in the treatment of Parkinson's disease. This compound works by inhibiting COMT, the enzyme responsible for the degradation of dopamine in the brain, thereby increasing dopamine concentrations

in synaptic clefts and improving motor function in patients with Parkinson's disease. (Artusi et al., 2021). Tolcapone also exhibits anti-inflammatory and neuroprotective properties that may be useful in the treatment of tauopathies. Research indicates that tolcapone may reduce tau protein aggregation, thereby contributing to slowing the progression of Alzheimer's disease and other tauopathic conditions (Yoon et al., 2024).

Similarly to tolcapone, entacapone (Fig. 2B) is also a COMT inhibitor employed in Parkinson's disease treatment. Entacapone increases the concentration of dopamine in the brain by inhibiting its metabolism, thereby promoting motor function. In addition to its effects on Parkinson's disease, entacapone also exhibits anti-inflammatory and neuroprotective properties that may be beneficial in the treatment of NDDs such as Alzheimer's disease and other tauopathies. This compound may help to reduce the toxic effects associated with tau protein aggregation, thereby mitigating neurodegeneration (Mohamed et al., 2013). In addition to neurodegenerative diseases, entacapone is also being investigated for potential use in cancer therapies, as some studies suggest its ability to inhibit tumour cell growth (Duarte et al., 2021).

3-nitro-1,2-benzenediol is a catechol derivative (Fig. 2C) known for its antioxidant and anti-inflammatory properties. This compound has been studied in the context of neurodegenerative diseases since evidence shows its ability to inhibit tau protein aggregation. 3-nitro-1,2-benzenediol also acts to reduce oxidative stress and inflammation in the brain, which may help slow the progression of these diseases (Candido Júnior et al., 2022).

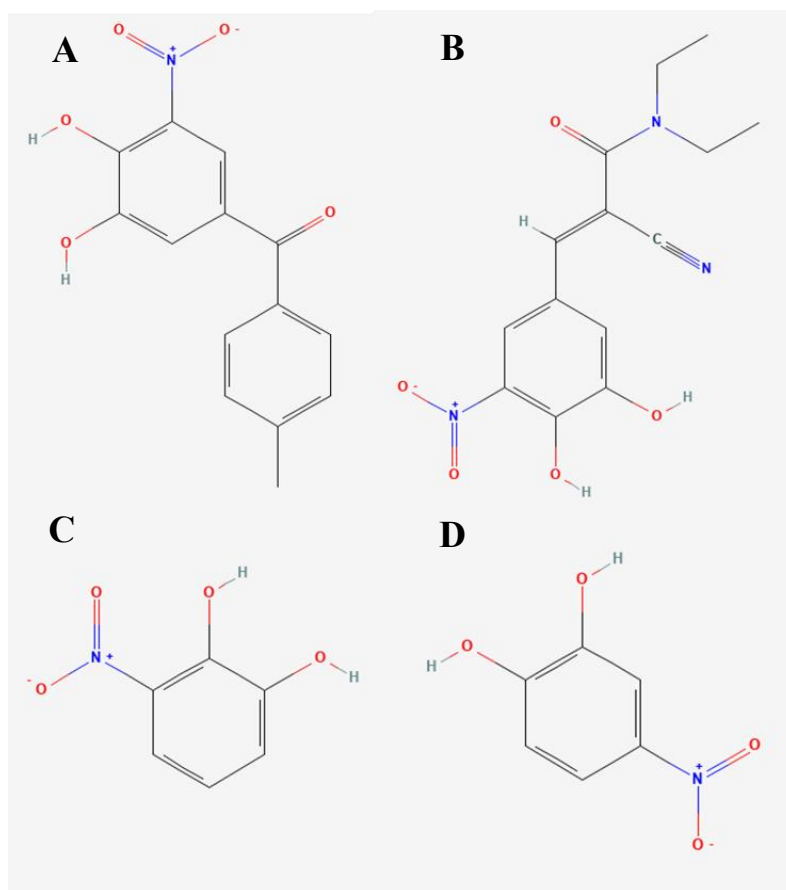


Figure 2. Chemical structures of catechol inhibitors. Tolcapone (A), entacapone (B), 3-nitro-1,2-benzenediol (C), 4-nitrocatechol (D). Chemical structures were adapted from PubChem.

4-nitrocatechol is another catechol derivative (Fig. 2D) that is being researched for its neuroprotective and anti-inflammatory effects. Like other catechol compounds, 4-nitrocatechol shows the ability to inhibit tau protein aggregation, which is key in Alzheimer's disease and other tauopathic diseases. It also appears that 4-nitrocatechol may reduce oxidative stress and inflammation in nerve tissue, thereby protecting nerve cells from degenerative processes (Xu et al., 2024).

2.3.2 Natural inhibitors

Natural inhibitors include various compounds with promising therapeutic potential, particularly for AD. These include purpurin (Fig. 3A), a trihydroxyanthraquinone derived from the anthracene family. A natural compound found in *Rubia argyi* and *Rubia wallichiana*. Purpurin exhibits significant antioxidant, anti-inflammatory and neuroprotective properties that make it a promising candidate for the treatment of neurodegenerative diseases (Rustage et al., 2025). Studies suggest that purpurin may

modulate the aggregation of tau protein. In this way, it may contribute to the reduction of neurotoxicity caused by pathological tau aggregates, thus providing promising therapeutic potential in the treatment of neurodegenerative diseases (Viswanathan et al., 2020).

Oleocanthal (Fig. 3B), an anti-inflammatory compound present in extra-virgin olive oil, exhibits properties akin to the nonsteroidal anti-inflammatory drug ibuprofen (Parkinson & Keast, 2014). In the context of neurodegenerative diseases, particularly tauopathies, oleocanthal shows the ability to inhibit tau protein aggregation (Giusti et al., 2018). In addition, oleocanthal exhibits neuroprotective effects by reducing oxidative stress and inflammation in the brain, which is key in preventing nerve cell damage. Thus, this natural remedy appears to be a promising candidate for therapeutic use in the treatment of neurodegenerative diseases (Parkinson & Keast, 2014).

Rosmarinic acid is a polyphenolic compound (Fig. 3C) found in plants such as rosemary, sage, mint and other herbs. This compound is known for its potent antioxidant, anti-inflammatory and neuroprotective properties, which make it attractive for research in the field of neurodegenerative diseases (Wu et al., 2020). Rosmarinic acid exhibits the ability to inhibit tau protein aggregation. In addition to its potent antioxidant, anti-inflammatory and neuroprotective effects, rosmarinic acid also exhibits antineoplastic properties, which are manifested in its ability to inhibit tumour growth and promote apoptosis in tumour cells. Studies show that rosmarinic acid can inhibit cancer cell proliferation, particularly in breast and lung cancer, through various mechanisms such as reducing inflammation and regulating genetic pathways responsible for tumour growth (Messeha et al., 2020). Furthermore, it has the potential to improve cognitive function by reducing inflammation and oxidative stress in the brain. Due to these effects, rosmarinic acid may act as a neuroprotective agent that protects nerve cells from damage and helps slow the progression of neurodegenerative diseases (Nadeem et al., 2019).

Curcumin, a beta-diketone (Fig. 3D) sourced from the root of *Curcuma longa*, is known for its anti-inflammatory, antioxidant and neuroprotective properties (Aggarwal et al., 2007). Curcumin also exhibits antineoplastic properties that make it a promising candidate in cancer therapy. Curcumin can inhibit tumour cell growth and suppress and metastasis by regulating various molecular mechanisms such as pathways responsible for cell proliferation, apoptosis, and immune response. Studies show that curcumin has

potential in the treatment of a variety of cancers, including colon, liver, pancreatic and lung cancers (Giordano & Tommonaro, 2019). In the field of neurodegenerative diseases, curcumin shows the ability to inhibit tau protein aggregation and reduce oxidative stress, which may help slow the progression of diseases such as Alzheimer's disease. The combination of these antineoplastic and neuroprotective effects makes curcumin very attractive for research and development of therapeutic approaches that could address not only neurodegenerative diseases but also cancer (Hu et al., 2015).

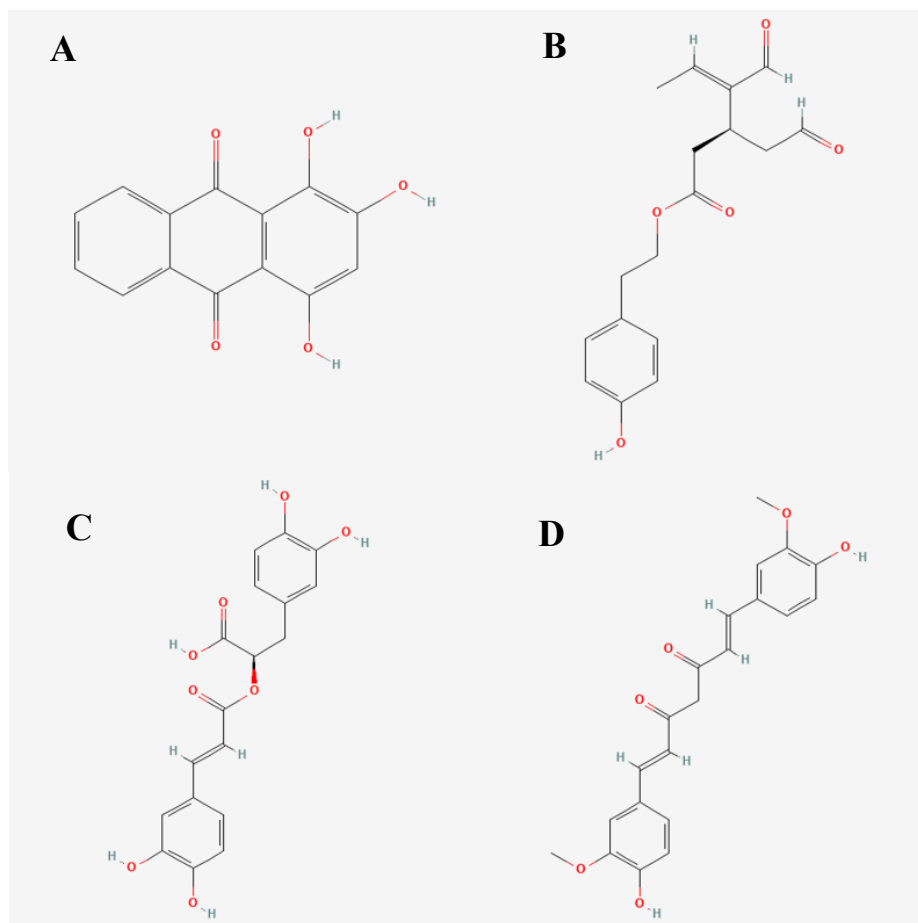


Figure 3. Chemical structures of natural inhibitors. Purpurin (A), oleocanthal (B), rosmarinic acid (C), curcumin (D). Chemical structures were adapted from PubChem.

2.3.3 Other inhibitor

Palmatine chloride is a natural isoquinoline alkaloid (Fig. 4) extracted from various medicinal plants, including *Hydrastis canadensis* and *Coptis chinensis*, that has gained attention for its broad-spectrum pharmacological effects (Long et al., 2019). This compound has shown therapeutic potential across a diverse range of conditions. It is

effective in inhibiting the growth of *Helicobacter pylori*, a key pathogen implicated in gastric ulcers and gastric cancer, likely through disruption of bacterial membrane integrity and suppression of urease activity (X. Chen et al., 2020).

Additionally, palmatine chloride has been shown to exert hepatoprotective effects, reducing hepatic inflammation and oxidative damage in models of hepatitis and cirrhosis. In relation of metabolic dysfunction, palmatine chloride improves insulin sensitivity and reduces hyperglycemia in diabetic rodent models by modulating glucose transporter expression and reducing oxidative stress in pancreatic tissues (Tarabasz & Kukula-Koch, 2020).

Palmatine chloride has demonstrated notable antineoplastic effects across various cancer types, including prostate, colon, and liver cancer. These effects are largely attributed to its ability to suppress tumour growth and promote cancer cell death through interference with key cellular signalling mechanisms (Long et al., 2019).

Although traditionally not associated with neurological disorders, recent evidence suggests potential neuroprotective properties of palmatine chloride. In AD models, it has been shown to reduce A β deposition, enhance autophagy, and relieve neuroinflammation, resulting in improved cognitive performance. Nevertheless, its application in neurodegenerative diseases remains underexplored, and further studies are needed to clarify its efficacy and mechanisms in NDDs (Utpal et al., 2025).

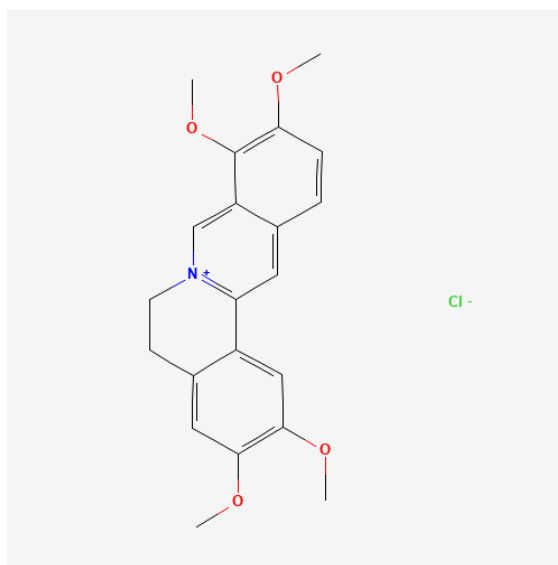


Figure 4. Chemical structure of other inhibitor. Palmatine chloride. Chemical structure was adapted from PubChem.

3 EXPERIMENTAL PART

3.1 Materials

3.1.1 Chemicals, solutions and peptides

Chemicals

Alexa Fluor® 647 anti-Tau, 210-230 Antibody (Thermo Fisher Scientific (Invitrogen), United States)

8-Anilino-1-naphthalenesulfonic acid (Sigma Aldrich, Merck, Burlington, MA, USA)

Ethanol

Heparin (Sigma Aldrich, Merck, Burlington, MA, USA, #H4784-1G)

Thioflavin T (ThT) (Sigma Aldrich, Merck, Burlington, MA, USA, #T3516-5G)

4RT Anti-Tau Antibody (Thermo Fisher Scientific (Invitrogen), United States)

Solutions

Aggregation buffer

20 mM Tris-HCl (pH 7,4), 100 mM NaCl, and 1 mM EDTA were dissolved in sterile water and filtered using a syringe filter. pH was adjusted to 7 with HCl. The solution was kept in the cold room.

1x PBS buffer

100 mL·L⁻¹ of Phosphate-Buffered Saline (PBS) 10x concentrated was diluted with 900 mL·L⁻¹ of sterile water to PBS 1x concentrated. The solution was filtered using a syringe filter and kept in a cool and dark place.

1x TBST buffer

100 mL of Tris-Buffered Saline (TBS) was mixed with 900 mL of distilled water and 1 mL of 0,1% Tween-20. The solution was kept in a cool and dark place.

5% BSA blocking solution

2,5 g of Bovine Serum Albumin (BSA) was diluted in 50 mL of distilled water. The solution was kept in a cool and dark place.

Peptides

The peptides used in the experiments (Table 1), were obtained from AnaSpec (Fremont, CA, USA) and Genscript Biotech (Piscataway, NJ, USA). They were reconstituted in sterile water and stored at -80 °C until further use.

Table 1. Tau peptides used in experiments. Blue indicates amyloid motifs, green cysteine and red present mutation.

Peptide	Sequence
Wild type	VQIINKKLD LSNVQSKCGS KDNIKHVPPG GSVQIVYKPV DLSKVTSKCG SLGNIHHKPG GGQ
S285R/R2	VQIINKKLD L ^R SNVQSKCGS KDNIKHVPPG GSVQIVYKPV DLSKVTSKCG SLGNIHHKPG GGQ

3.1.2 Inhibitors

3-Nitro-1,2-benzenediol (cat. no. N1555, CAS 603-11-2)

4-Nitrocatechol (cat. no. N1555, CAS 3316-09-4)

Curcumin (cat. no. C1386, CAS 458-37-7)

Entacapone (cat. no. E9284, CAS 130929-57-6)

Oleocanthal (cat. no. O1383, CAS 289030-99-5)

Palmitine chloride (cat. no. SMB00472, CAS 10605-02-4)

Purpurin (cat. no. P3889, CAS 81-54-9)

Rosmarinic acid (cat. no. R4033, CAS 20283-92-5)

Tolcapone (cat. no. T5515, CAS 134308-13-7)

3.1.3 Laboratory instruments and equipment

Agilent PlateLoc Thermal Microplate Sealer (Agilent Technologies, Santa Clara, CA, USA)

Biohazard Herasafe KS (Thermo Fisher Scientific, Waltham, MA, USA)

Cell Carrier 384 well plates (PerkinElmer, Waltham, MA, USA)

Centrifuge 5810R (Eppendorf, Hamburg, Germany)

EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA)

Eppendorf tubes

Mini Centrifuge MiniSpin® (Eppendorf, Hamburg, Germany)

Nitrocellulose membrane

Parafilm

Pencil

Petri dish

Pipettes (Eppendorf, Hamburg, Germany)

Plastic containers

Scissors

Sterile glass bottle

Sterile syringe filter (0.22 µM) (TPP Techno Plastic Products AG, Trasadingen, Switzerland)

Sterile tips

Stratagene Hybridizer 700 Hybridization Oven (Agilent Technologies, Santa Clara, CA, USA)

Thermo-Shaker PST-60HL (Biosan, Riga, Latvia)

Tweezers

Vortex V-1 Plus (Biosan, Riga, Latvia)

3.1.4 Software

GraphPad Prism 10 (Dotmatics, Boston, MA, USA)

Microsoft Office (Excel, Powerpoint, Word) (Microsoft, Redmond, WA, USA)

ImageJ, ver. 1.4.3.67

3.2 Methods

3.2.1 Tau aggregation assay

Thioflavin T (ThT) is a fluorescent benzothiazole dye commonly used for identification and quantification of amyloid fibrils both *in vitro* and *ex vivo*. Upon binding to a β -sheet-rich sample, ThT emits strong fluorescence at 490 nm, with an excitation maximum at 440 nm. It's an especially widely used technique for determining fibrillation kinetics in real time, however the exact mechanism of interaction between ThT and amyloid fibrils isn't fully understood. It's theorized that ThT intercalates into grooves formed by solvent-exposed side chains of the amyloid fibril (Hudson et al., 2009).

8-anilino-1-naphthalenesulfonic acid (ANS) is a widely used fluorescent dye for characterizing hydrophobic protein binding sites *in vitro*. During ANS excitation, the molecule first reaches an excited state located on the naphthalene ring, known as the nonpolar state. In nonpolar solvents, the emission from this state occurs, and its maximum shifts slightly depending on the solvent's polarity. In more polar solvents, the nonpolar state undergoes an electron transfer, forming the charge-transfer state, which becomes the lower-energy state and emits at longer wavelengths. In aqueous solutions, another

electron transfer happens, involving ionization and the solvation of electrons (Gasymov & Glasgow, 2007).

The samples were prepared with a reaction mixture of PBS and aggregation buffer in an Eppendorf tube. Heparin was added to enhance aggregation, along with peptide R2 or R3 and finally ThT. Inhibitors were not included in the control sample. The prepared mixture was transferred to a dark 367-well plate. The plate was then placed in an EnSpire® Multimode Plate Reader, where the fibrillation process was monitored for 48 hours at 37 °C. Fluorescence measurements were taken at regular 5-minute intervals to track the fibrillation kinetics, based on the increase in ThT fluorescence intensity (Annadurai et al., 2022).

For the ANS assay, the samples were prepared in the same manner as for the ThT assay, with the only difference being the use of ANS dye. The protocol followed was identical to that of the ThT assay, with excitation measured at 390 nm and emission at 475 nm (Annadurai et al., 2022).

3.2.2 Pelleting assay

Replicate wells of the same inhibitor and control from the tau aggregation assay were transferred to microcentrifuge tubes and subjected to centrifugation at 13,600 RPM at 4 °C for 1 hour. The supernatant was discarded, and the pellet was resuspended in aggregation buffer (pH 7). Following another centrifugation cycle under the same conditions, the pellet was resuspended in aggregation buffer (pH 7) to make a 20x concentrated pellet sample. Finally, the microcentrifuge tubes containing pellet samples were stored in a cold room at 4 °C until further use (Hill et al., 2022).

3.2.3 Immuno-dot blot assay

Pellet samples prepared with pelleting assay were kept on ice as 2 µL of the sample were applied onto a nitrocellulose membrane in sterile conditions. The membrane was placed in an incubator at 37 °C for 5 minutes to allow the peptides to bind to the membrane (Jin et al., 2022). The process was repeated before the membrane was washed 3 times at 10-minute intervals with 1x TBST and blocked with 5% BSA for 1 hour at room temperature (RT) (Liu et al., 2023). Subsequently, the membrane was imaged and incubated anti-tau 4-repeat isoform RD4 overnight in the cold room (4 °C). After the membrane was rewashed with 1x TBST, secondary antibody AlexaFluor 647 anti-mouse (1:2000) was

applied for 1 hour at RT. Finally, the membrane was washed with 1x TBST and imaged. Membrane images were captured using a scanner equipped with AlexaFluor 488 and AlexaFluor 647 detection channels.

4 RESULTS

4.1 Evaluation of tau fibrillization kinetics via Thioflavin T Fluorescence Assay

The aggregation behaviour of tau peptides in the presence of selected inhibitors was investigated using the Thioflavin T (ThT) fluorescence assay. The tested compounds included tolcapone, entacapone, 3-nitro-1,2-benzenediol, 4-nitrocatechol, purpurin, oleocanthal, rosmarinic acid, curcumin, and palmatine chloride, as listed in the Materials section. The aim of this analysis was to assess their ability to modulate amyloid-like aggregation of both wild-type and mutant form of the peptide.

Fluorescence intensity was measured after 48 hours of incubation, and the results were compared to the control sample containing peptide without any inhibitor. The data were summarized in four graphs: one showing the aggregation profiles of all inhibitors with the wild-type peptide, and another displaying the same data with statistical significance (p-values). An analogous pair of graphs was generated for the mutant peptide S285R/R2. The comparative analysis of these results enabled the identification of compounds with potential inhibitory effects on tau aggregation in both peptide variants.

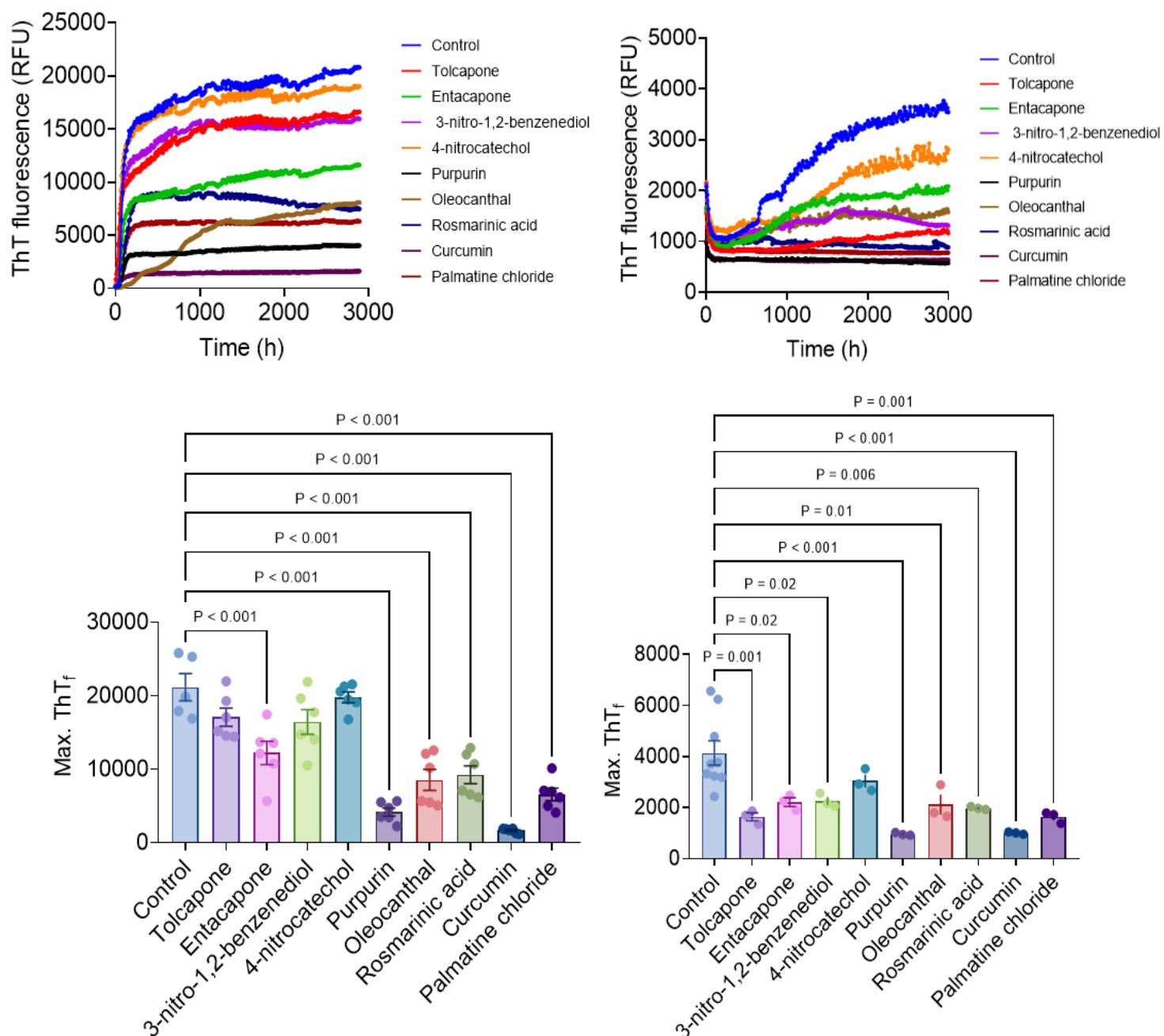


Figure 5. ThT fluorescence kinetics of wild-type and S285R/R2 mutant tau peptide in the presence of inhibitors. The figure presents Thioflavin T (ThT) fluorescence kinetics to evaluate the fibril formation of wild-type tau peptide (top and bottom left panels) and the S285R/R2 mutant peptide (top and bottom right panels). The fluorescence intensity reflects β -sheet-rich fibril formation over time. The graphs compare samples treated with specific inhibitors to untreated controls. Statistical analysis was performed using one-way ANOVA. Significant differences (p-values) are indicated for conditions where inhibitors effectively reduced ThT fluorescence, confirming their inhibitory effect on tau aggregation.

4.2 Structural rearrangement monitoring in tau aggregation using ANS assay

The 8-anilino-1-naphthalene-sulfonic acid fluorescence assay was performed to assess surface hydrophobicity and conformational changes in tau peptides. ANS binds to exposed hydrophobic regions of proteins, and its fluorescence increases upon interaction, making it a sensitive probe for conformational changes associated with misfolding and aggregation. Fluorescence intensity was measured after 48 hours of peptide incubation with inhibitors, and the results were compared to the control sample containing peptide without any inhibitor.

The results were presented in two separate graphs: one for the wild-type peptide and one for the S285R/R2 mutant peptide. Each graph included selected tested compounds. These inhibitors were chosen due to their promising performance in the ThT assay, indicating a potential effect on tau aggregation. This assay complemented the ThT fluorescence data by providing insight into structural alterations and exposure of hydrophobic domains induced by the inhibitors, thus helping to elucidate their potential modulatory effects on tau peptide folding and aggregation.

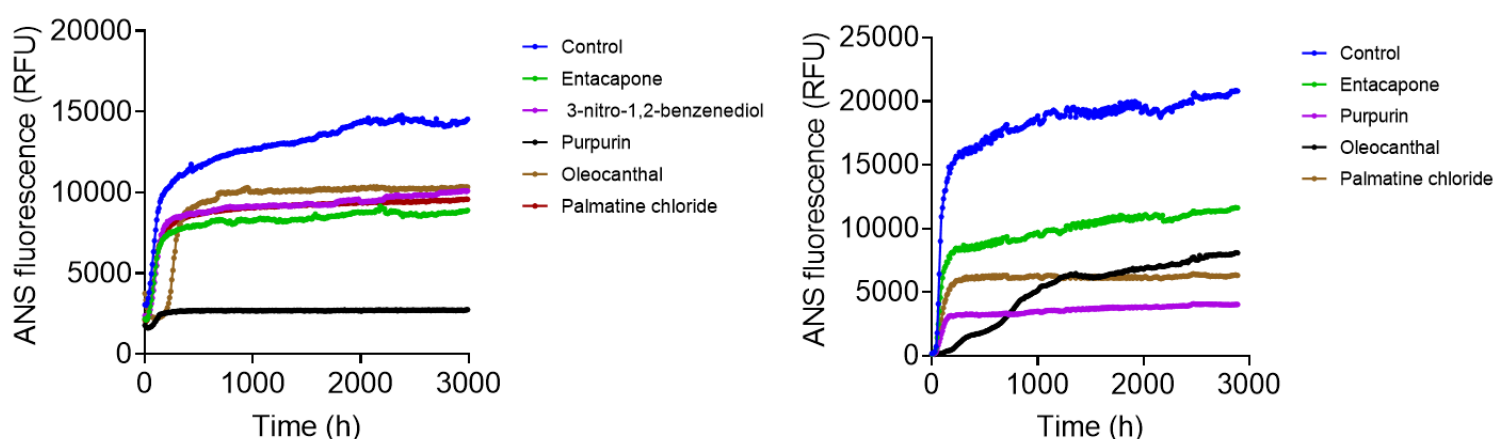


Figure 6 ANS fluorescence of wild-type and S285R/R2 mutant tau peptides in the presence of selected inhibitors. The figure illustrates ANS fluorescence intensity as a measure of surface hydrophobicity in tau peptides. The wild-type samples (left panel) and S285R/R2 mutant samples (right panel) were incubated with various inhibitors. ANS binding reveals the extent of hydrophobic surface exposure, which reflects structural compactness or unfolding. Increased fluorescence in mutant samples indicates enhanced hydrophobicity and conformational instability. The 3-nitrocatechol condition was excluded due to irregular data.

4.3 Immuno-dot blot-based characterization of conformational changes in tau

Immuno-dot blot analysis was employed to assess tau aggregation levels in wild-type tau peptide and S285R/R2 tau peptide samples treated with selected inhibitors. After incubation, samples were applied to a nitrocellulose membrane and probed using the tau-specific 4RT primary antibody, followed by a fluorescently labelled secondary antibody. Signal intensity was quantified using ImageJ software by calculating integrated density for each sample.

The results were visualized in two separate bar graphs, each corresponding to a single blot: one for the wild-type peptide and one for the mutant peptide S285R/R2. Each graph displays absolute integrated density values for the individual inhibitor-treated samples and for control. This provided a direct, membrane-based comparison of aggregation levels under different treatment conditions and supported the fluorescence-based findings obtained from ThT. As only one dot per condition was analysed ($n = 1$), no statistical analysis was performed. Data are presented descriptively to visualize the relative inhibitory effects on tau aggregation.

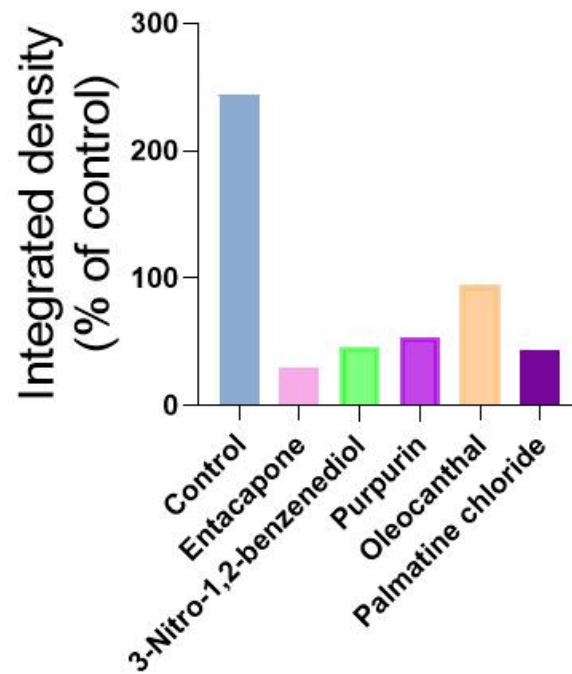
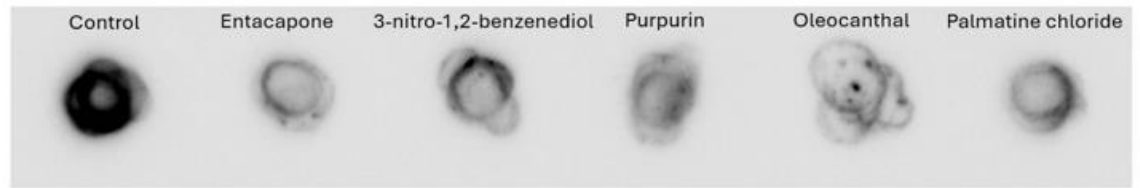


Figure 7. Dot blot analysis of wild-type tau peptide aggregates compared to control. Representative dot blot image of tau samples treated with various inhibitors. Bar graph showing integrated density values (% of control), calculated using ImageJ. Results indicate decreased tau signal intensity in the presence of most inhibitors, suggesting potential anti-aggregative effects.

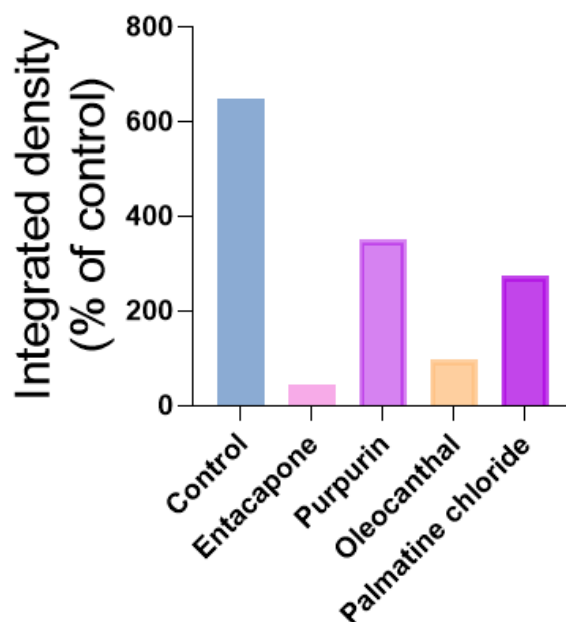


Figure 8. Dot blot analysis of S285R/R2 tau peptide aggregates compared to control. Representative dot blot image of tau samples treated with various inhibitors. Bar graph showing integrated density values (% of control), calculated using ImageJ. Results indicate significantly decreased tau signal intensity in the presence of most inhibitors, suggesting potential anti-aggregative effects. Compound 3-nitro-1,2-benzenediol was excluded from this analysis due to irregular data.

5 DISCUSSION

Tauopathies are a diverse group of neurodegenerative disorders characterized by the misfolding and aggregation of tau protein into toxic fibrillar structures. These aggregates impair normal neuronal function and eventually lead to cell death. A central feature of tau aggregation is the hexapeptide motif ³⁰⁶VQIVYK³¹¹, located in the R3 repeat of the microtubule-binding domain. This motif plays a key role in the formation of paired helical filaments, which are typical of Alzheimer's disease and related disorders (Ganguly et al., 2015; Von Bergen et al., 2000). Hyperphosphorylation is one of the most common post-translational modifications of tau and contributes to aggregation by weakening tau's affinity for microtubules and promoting its self-assembly into insoluble fibrils (Avila et al., 2004; Pavlíková, 2024).

Various tau mutations have been linked to frontotemporal degeneration. Among them, the P301L and P301S mutations in the MAPT gene are well known for increasing tau aggregation by disrupting its normal structure and microtubule-binding capacity (Olszewska et al., 2016). In this study, a mutant peptide S285R/R2 was observed in interaction with selected inhibitors. This mutation replaces serine with arginine, which likely alters the local charge distribution and may enhance tau aggregation. Its proximity to the PHF6*, another aggregation-prone sequence, supports the idea that even subtle changes in this region can significantly influence aggregation behaviour (Annadurai, 2023).

Recent research has focused on developing inhibitors that can interfere with tau aggregation. Compounds such as curcumin, oleocanthal and rosmarinic acid have shown promising anti-aggregation effects, largely due to their ability to disrupt β -sheet structures or stabilize tau in a less aggregation-prone conformation (Wu et al., 2020). In this thesis nine compounds divided into catechols, natural inhibitors and other inhibitor, were tested on both wild-type and S285R/R2 mutant tau peptides to evaluate their effect on tau aggregation.

Thioflavin T fluorescence assays showed that several compounds, including entacapone, purpurin, and palmatine chloride, were effective in reducing the formation of β -sheet-rich fibrils. These findings are in line with previous studies showing that hydroxyl-rich compounds can reduce tau aggregation (Mohamed et al.,

2013; Yoon et al., 2024). Additional insight was provided by ANS fluorescence assays, which detects changes in surface hydrophobicity. Lower ANS fluorescence in inhibitor-treated samples suggested reduced exposure of hydrophobic regions, indicating less misfolded tau. The mutant S285R/R2 peptide generally showed higher ANS fluorescence, which may reflect increased conformational instability, supporting its use as a model for studying pathological tau aggregation.

To further validate the inhibitory effects observed in the fluorescence assays, immuno-dot blot analysis was performed. Integrated density measurements showed that most tested compounds reduced the accumulation of insoluble tau aggregates, especially in the mutant peptide. While only one measurement per condition was taken, which limits statistical verification, the results were consistent with the results observed in ThT and ANS assays. Overall, these findings support the idea that the VQIVYK and VQIINK motifs are promising targets for modulating tau aggregation, and that certain small molecules can affect both wild-type and mutant tau forms.

6 CONCLUSION

This thesis investigated the anti-aggregative effects of selected inhibitors on tau protein using three complementary methods: Thioflavin T fluorescence, ANS fluorescence, and immuno-dot blot analysis. The study focused on wild-type tau peptide and a synthetically modified S285R/R2 mutant peptide, which displayed stronger aggregation tendencies and served as a useful model for tauopathy.

The main results showed that several compounds, particularly entacapone, 3-nitro-1,2-benzenediol, purpurin, and palmatine chloride, reduced tau aggregation and altered its conformational properties. These effects were observed in both wild-type and mutant tau, although the mutant form generally showed higher aggregation. While the study was limited by a low number of replicates, the consistency of results across all methods strengthens the conclusions.

The findings suggest that targeting specific aggregation-driving regions in tau, such as the VQIVYK motif, may be an effective strategy for inhibiting pathological aggregation. Further experiments with more replicates, extensive statistical analysis, and cellular or *in vivo* models will be necessary to evaluate the full therapeutic potential of these compounds in the context of neurodegenerative disease.

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7.2 Website references and hyperlinks

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8 LIST OF ABBREVIATIONS

Aβ	Amyloid- β
AD	Alzheimer's disease
ANS	8-anilinonaphthalene-1-sulfonic acid
COMT	Catechol-O-methyltransferase
CSF	Cerebrospinal fluid
FTD	Frontotemporal dementia
<i>MAPT</i>	Microtubule-associated protein tau
NDD	Neurodegenerative disorder
NFTs	Neurofibrillary tangles
PHF	Paired helical filaments
PTM	Post-translational modification
RT	Room temperature
ThT	Thioflavin T

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