Details of the Research Work

Sun Pharma Science Scholars Fellowships 2024

Multipronged strategies to mitigate amyloid associated toxicities and ferroptosis in Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a multifaceted, progressive neurodegenerative disorder and a primary contributor to dementia, accounting for 70-80% of cases globally.[1,2] The prevalence of dementia afflicts more than 55 million people worldwide, with projections expected to cross 153 million by the year 2050.[3] While mortality rates from various chronic ailments show a declining trend, AD shows a concerning increase in prevalence.[1,2]

Clinically AD manifests as a spectrum of debilitating symptoms, encompassing learning and memory deficits, cognitive impairment, and ultimately, fatality.[4] Pathologically, AD is hallmarked by the accumulation of extracellular amyloid β (Aβ) senile plaques and neurofibrillary tangles (NFTs) comprised of hyperphosphorylated tau protein, concomitant with brain atrophy and progressive neurodegeneration.[5,6,7] The Aβ monomer aggregate to form toxic oligomers, protofibrillar and fibrillar species that cause neuronal toxicity. Similarly, hyperphosphorylated tau aggregate to form toxic NFTs. Furthermore, the disease progression by these amyloid proteins is exacerbated by metal dyshomeostasis, oxidative stress, biomolecular damage, mitochondrial dysfunction, and neuroinflammation.[8,9] The amyloid toxicity further encompasses cholinergic toxicity, immune outrage, Ca^{2+} dyshomeostasis, neurovascular toxicity, lymphatic dysfunction, apoptosis dysregulation, impairment in telomerase activity, microbial infection and imbalance, glucose hypometabolism, endoplasmic reticulum

stress, autophagy dysfunction, genetic risk and ferroptosis among others that contribute in the disease progression.[2]

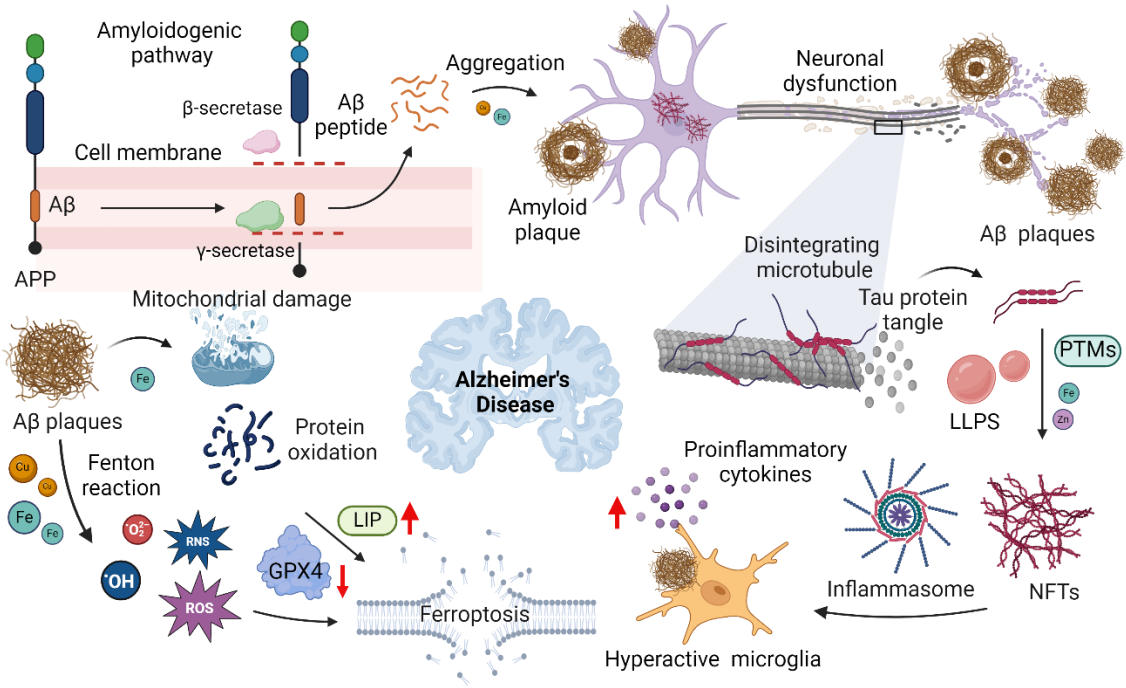

Figure 1. Schematic illustration indicating multiple aetiologies of AD. (created with BioRender.com).

Tau, a microtubule-associated protein (MAP), plays a pivotal role in regulating the maintenance and dynamics of microtubules within mature neurons.[10] However under pathological conditions, this intrinsically disordered protein undergoes various post translational modifications (PTMs) which drive its aggregation, culminating in the formation of NFTs and toxic intracellular paired helical filaments (PHFs).[11–13] Recent research has unveiled that the aggregation mechanism of tau is initiated through a process involving a metastable state known as liquid-liquid phase separation (LLPS).[14–17] LLPS is a biological phenomenon wherein proteins segregate and coalesce to form membrane-less biomolecular condensates.[18,19] In physiological contexts, these phase-separated tau species facilitate the nucleation of microtubule bundles. However,

aberrant phase transitions augment the local concentrations of tau, fostering the formation of toxic aggregates.[20–22] These toxic tau aggregates thus formed impair axonal transport, induce synaptic dysfunction, and inflict mitochondrial damage, ultimately leading to neuronal cell death. Notably, the tau pathway exhibits significant overlap with that of Aβ underscoring their interdependency in AD pathogenesis. Furthermore, tau aggregates have been implicated in microglial activation and neuroinflammation, exacerbating the neurodegenerative cascade observed in AD.[23,24] The development of molecular tools capable of modulating LLPS to disrupt the aberrant phase transitions to inhibit the aggregate formation holds paramount importance in the formulation of novel therapeutic strategies.[25]

The landscape of drug development for AD has predominantly revolved around targeting the cholinergic and amyloid pathways over the past three decades.[26] Presently marketed AD therapeutics offer symptomatic relief, albeit without directly addressing the underlying pathological mechanisms.[27] Notably, the recent approval of monoclonal antibodies targeting Aβ, marks a significant milestone in therapeutic intervention for AD.[28] However, many Aβ-targeting immunotherapies have failed due to a lack of specificity to various isoforms and limited efficacy in the late stages of the disease, which trigger various downstream toxic pathways. Additionally, there is a growing interest in targeting tau protein, inhibiting its aggregation, and facilitating its clearance, with several candidates undergoing clinical trials, albeit with limited success.[29]

Despite targeting these amyloid markers, AD therapeutics have achieved only limited success. The setbacks in developing Aβ and tau-targeted drugs underscore the need to elucidate novel disease mechanisms. This understanding may guide the development of multifunctional molecules (MFM) that can target multiple aetiologies.[6,3,30–33] In this work, we delineate AD as a multifactorial disorder, expounding upon each well-

documented mechanism. Additionally, we venture into two emerging paradigms, namely ferroptosis and tau liquid-liquid phase separation (LLPS), which have garnered prominence in tackling AD (Figure 1).

Ferroptosis drives neurodegeneration in AD

Ferroptosis, an iron-regulated lipid peroxidation mediated cell death has emerged as a key area of research due to its involvement in various chronic illness including cancer and neurodegeneration.[34] Intracellular labile iron levels catalyse Fenton-type reactions, leading to increased lipid peroxidation and cell death. Iron also enhances enzymatic lipid peroxidation by serving as a cofactor for lipoxygenase (LOX) enzymes, which oxidize polyunsaturated fatty acids (PUFAs) into hydroperoxyl derivatives.[35,36] Additionally, ferroptosis is associated with mitochondrial dysfunction, lipid peroxidation, and reduced activity of the antioxidant enzyme glutathione peroxidase 4 (GPX4). GPX4, a master regulator of ferroptosis, reduces toxic lipid peroxides generated from PUFAs and reactive oxygen species (ROS) to non-toxic lipid alcohol. GPX4 activity is influenced by its cofactor, glutathione (GSH).[37] Ferroptosis can also occur independently of GPX4 inhibition, such as through tumor protein 53 (TP53) or Cytochrome P450 reductase (POR). Ferroptosis Suppressor Protein 1 (FSP1) has been identified as another regulator, functioning both with and without GPX4.[38]

In the context of neurodegenerative diseases, ferroptosis plays a detrimental role by exacerbating disease progression. Conditions like AD, stroke, Parkinson's disease, and Huntington's disease have been linked to ferroptosis. Elevated iron levels and lipid peroxidation are observed in AD, with high amyloid plaque loads correlating with increased cortical iron and AD risk.[39,40,41] Ferroptosis inhibitors have shown promise in protecting neurons and restoring cognitive function in stroke models. Additionally, GPX4 knockout in mice leads to age-dependent neuronal loss. Aβ42 treatment has been shown to increase tissue

iron and decrease GPX4 levels in the hippocampus, suggesting Aβ42 might induce ferroptosis.[42–44] Additionally oxidative stress and ROS are significant contributors to both AD and ferroptosis. Antioxidants capable of neutralizing ROS are potential dual

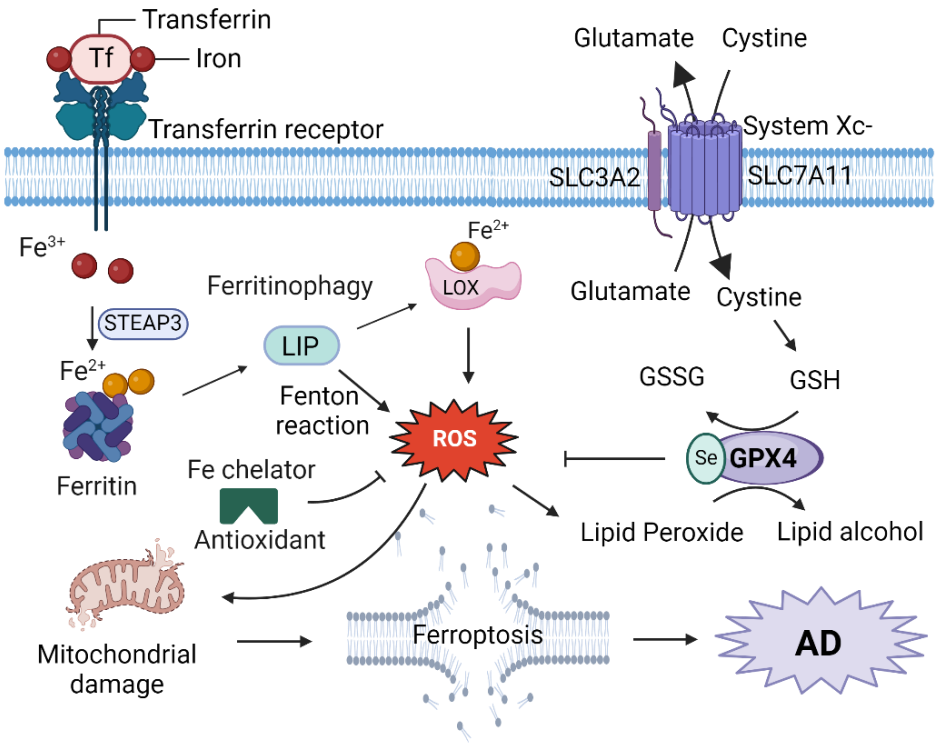

Figure 2. Schematic representation of the mechanism of ferroptosis implicated in AD. (created with BioRender.com)

therapeutic targets. Despite these insights, few therapeutics address both ferroptosis and AD. Most ferroptosis inhibitors focus on iron chelation and antioxidant activity, while the activation of GPX4 remains underexplored. Developing multifunctional molecules that target both ferroptosis and AD, and enhance GPX4 activity, may offer new strategies to mitigate the complex pathology of both conditions. There is an untapped avenue in development of multifunctional modulators that can tackle the pathological nexus between ferroptosis, amyloid associated toxicities in AD.[3]

Multifunctional therapeutic molecules for AD

AD being a multifactorial disorder necessitates for therapeutic molecules that target multiple etiologies.[31,45–47] Recent insights into the various factors associated with AD have paved the way for the design of tools capable of addressing multiple aspects of the disease pathology. Therapeutic strategies targeting BACE1, Aβ and tau aggregation, oxidative stress, metal dyshomeostasis, mitochondrial dysfunction, and neuroinflammation have garnered considerable attention.[3]

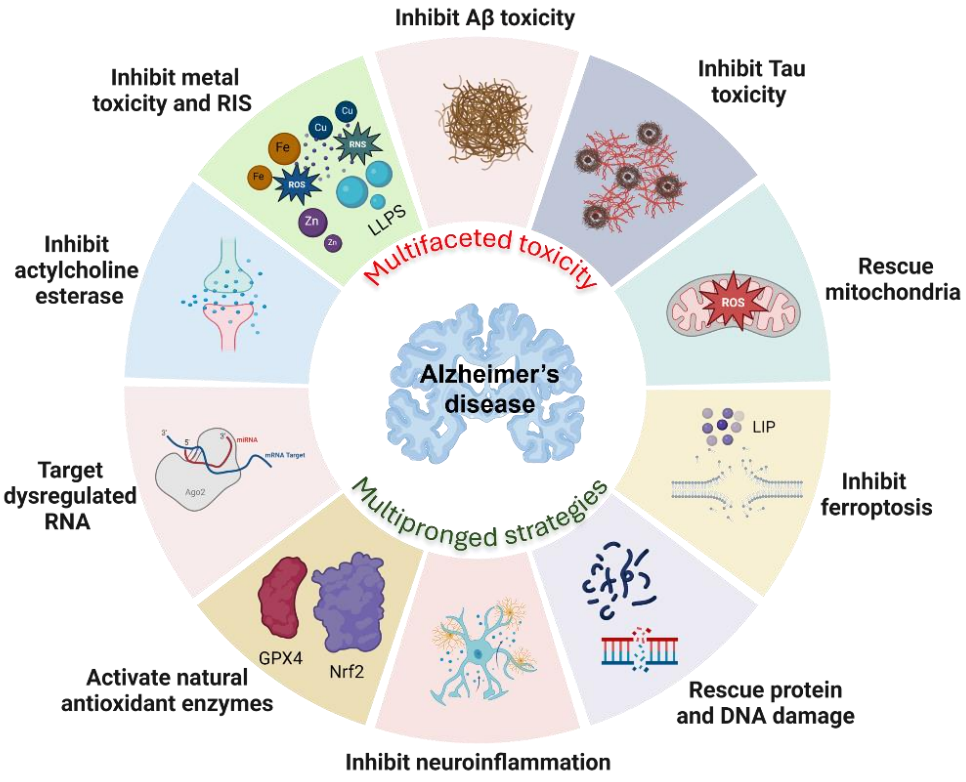

Figure 3. Therapeutic strategies targeting various pathological features of AD. (created with BioRender.com)

In this context, our laboratory has developed a multifunctional peptidomimetic modulator, P6, by conjugating the natural Cu chelator glycyl-L-histidyl-L-lysine (GHK) tripeptide with sarcosine.[48] P6 demonstrates the ability to inhibit Aβ aggregation, and sequester Cu from the Aβ-Cu inclusion complex to reduce ROS generation and mitigate

DNA damage. Hybrid multifunctional molecule (HMM) was developed by integrating the 8-hydroxyl quinoline moiety and polyphenolic core from EGCG, imparting antioxidant and metal chelation properties.[49] Among the molecules studied, TGR86 stands out for its remarkable ability to inhibit Cu-dependent Aβ aggregation and quench ROS generation, thereby preventing protein and mitochondrial damage. Furthermore, we modified a natural product berberine to polyphenolic Ber-D which effectively inhibited Aβ-induced toxicity, ROS, biomolecular damage and downregulated apoptosis.[50]

We developed bipyridyl derivatives that modulate metal-independent and -dependent Aβ toxicity, inhibit oxidative stress, and mitigate neuroinflammation observed in AD.[51] In a subsequent follow-up study, we developed NMI-based MFM that synergistically modulates metal-independent and -dependent Aβ toxicity, scavenges ROS and regulates Nrf2-mediated stress response.[52] The lead M3 significantly reduces structural and functional mitochondrial damage, lowers cytochrome C levels and rescues cells from apoptosis. Moreover, M3 exhibits anti-inflammatory effects by suppressing microglial activation and neuroinflammation through inhibition of the NF-κβ pathway.

However, there remains a notable gap in the development of therapeutic molecules that can synergistically mitigate the pathological nexus between ferroptosis, amyloid associated toxicity, tau protein LLPS, which has been successfully carried out in this thesis work submitted. To the best of my knowledge this is the first report on development of multifunctional molecule that can tackle Aβ associated toxicity, tau phase separation and ferroptosis synergistically to tackle AD pathophysiology.

Objectives

A) Targeting the amyloid-associated toxicity to tackle the pathology of AD
B) Understanding the role of Ferroptosis in AD and development of therapeutic strategies that can synergistically mitigate their pathological nexus.
C) Strategies to modulate tau Liquid-Liquid Phase Separation (LLPS) in context of AD.

Materials and Methods

Materials: We synthesized a range of biocompatible therapeutic molecules aimed at mitigating amyloid-associated toxicity and ferroptosis in Alzheimer's disease. The compounds developed include small molecules, natural polyphenols, polymer nanocomposites, polymer-drug conjugates, and hydrogels. Each synthesized molecule was thoroughly characterized using nuclear magnetic resonance (NMR) spectroscopy, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, liquid chromatography-mass spectrometry (LC-MS), and high-performance liquid chromatography (HPLC) to ensure structural integrity and purity. Reproducibility was confirmed through resynthesis, and yield optimization was performed to facilitate scale-up reactions.

Methods:

Spectroscopic Techniques: We employed a variety of spectroscopic techniques, including UV-Vis absorption, fluorescence, circular dichroism (CD), infrared (IR), Raman, and NMR spectroscopy, to analyse the structural and biophysical properties of the synthesized molecules.

Molecular Biology Techniques: The molecular biology techniques used include mammalian and neuronal cell culture, RNA and protein isolation, polymerase chain reaction (PCR), reverse transcription quantitative PCR (RT-qPCR), immunoblotting, immunofluorescence, bacterial transformation, plasmid isolation from bacteria, and DNA gel electrophoresis.

Animal Handling: We used Wistar rats for in vivo experiments, including intraperitoneal injections and the collection of skin and blood samples.

Analytical Techniques: Analytical methods such as flow cytometry, microplate reader assays, HPLC, LC-MS, rheological studies, MALDI, and high-resolution mass spectrometry (HRMS) were employed. Isothermal titration calorimetry was used to study molecular interactions.

Microscopy: Microscopy techniques included epifluorescence and confocal microscopy, atomic force microscopy (AFM), cryo-electron microscopy (cryo-EM), transmission electron microscopy (TEM), and field emission scanning electron microscopy (FE-SEM).

Software Tools: Computational and data analysis tools included ChemDraw for molecular visualization, Origin and GraphPad Prism for data analysis, ImageJ for image processing, TopSpin for NMR data analysis, Huygens software for image deconvolution, and Autodock and PyMOL for molecular docking and structural visualization.

Results

This thesis work comprises of experimental works focused on developing biocompatible multipronged therapeutic strategies targeting amyloid-associated toxicities and elucidating the role of ferroptosis in AD. Additionally, it aims to provide insights into modulating tau LLPS for potential therapeutic interventions in AD. We developed cyclic dipeptide (CDP)-based copolymer (CP), which has been explored for its material and biomedical properties. Due to its structural versatility, CDP-CP forms solvent-dependent anisotropic architectures, ranging from dense fibers and mesosheets to vesicles, that interact with dyes and nanoparticles. At low concentrations CP forms polymersome which interacts with gold nanoparticles (GNP) and coacervates with polyoxometalate (POM), to generate faceted architectures (CP-GNP) and a nanocomposite (CP-POM), respectively. CP-GNP and CP-POM exhibit a remarkable ability to inhibit Aβ42 aggregation, disrupt the preformed aggregates, and scavenge reactive oxygen

species (ROS). CP-POM and CP-GNP ameliorate multifaceted amyloid toxicity, rescuing neuronal cells and reducing neuroinflammation in microglial cells (**ACS *Appl. Mater. Interfaces* 2022, 14, 51, 56535–56547**). In the subsequent work, we delved into the pathological relationship between ferroptosis and AD. Further, this work introduces naturally occurring polyphenols (PPs) as dual-acting therapeutic agents aimed at synergistically

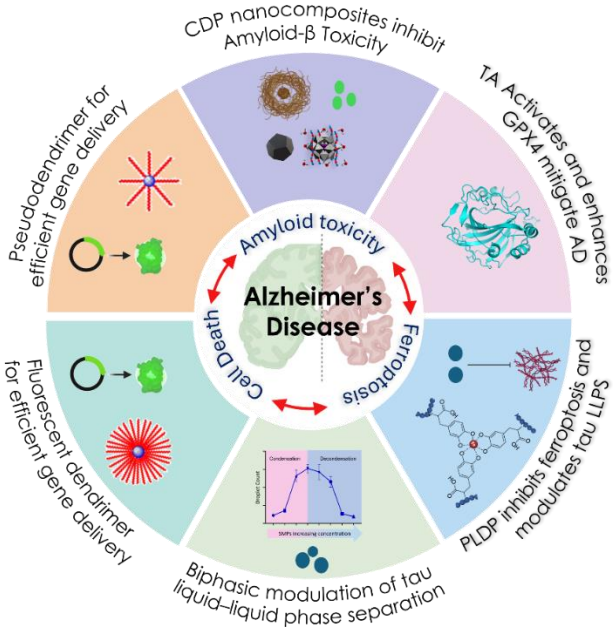

Figure 4. Summary of the work

alleviating ferroptosis and AD. The mechanisms of action involve binding to the labile iron pool, modulation of Aβ and tau cascades, reduction of oxidative stress, mitochondrial rescue, and inhibition of ferroptosis. Specifically, the study reveals that tannic acid (TA), a single multifunctional molecule, binds to the activator site of glutathione peroxidase 4 enzyme (GPX4), enhancing both its activity and cellular levels. This approach offers an innovative and integrated strategy for treating AD via the GPX4–ferroptosis axis. The ability of TA to augment GPX4 levels in the context of AD pathology suggests promising therapeutic avenues for addressing the interplay between ferroptosis and AD (***Chem. Sci.* 2023, 14, 9427–9438**). In the follow up work we focused on the development of polymer-drug conjugates designed to simultaneously target amyloid toxicity and ferroptosis in neuronal cells. This study explores

the potential of polycatechols, specifically PDP and PLDP, which are dopamine and L-Dopa based polymer-drug conjugates, respectively, as multifunctional agents to modulate the pathological nexus between ferroptosis and AD. Polycatechols were found to sequester the labile iron pool (LIP), inhibit Aβ and tau aggregation, scavenge free radicals, protect mitochondria, and prevent ferroptosis, ultimately rescuing neuronal cell death. Notably, PLDP promotes tau LLPS and modulates intermolecular interactions to inhibit the formation of toxic tau aggregates, presenting an innovative approach to addressing tauopathies. This is the first-of-kind polymer-based integrative approach that inhibits ferroptosis, counteracts amyloid toxicity and modulates tau LLPS to mitigate the multifaceted toxicity of AD. (***Mater. Horizons* 2024, 11, 3082-3089**). In the next work we identified small polyphenols as biphasic modulators of tau LLPS. Specifically, we show that gallic acid (GA) is capable of promoting tau LLPS at lower concentrations while disrupting it at higher concentrations, indicative of a reentrant phase transition. Our findings demonstrate GA's ability to expedite the liquid-to-gel transition in tau condensates effectively impedes the formation of deleterious fibrillar aggregates having implications in AD therapeutics (***Chem. Commun.* 2024, 60, 4334–4337**). Last works focuses on nucleic acid-based therapeutics with potential applications in AD (***ACS Appl. Bio Mater.* 2021, 4, 1115–1139**), divided into two parts. First part discusses the development of intrinsically fluorescent dendrimers, up to the third generation, which have demonstrated the ability to condense DNA, protect it from DNase degradation, and exhibit enhanced transfection efficiency in cells. Ongoing research in this area aims to deliver miRNA with therapeutic significance in AD, particularly targeting the inhibition of ferroptosis and neuroinflammation. The second part explores the development of minimalistic pseudodendritic structures (TGP), which can be synthesized in a relatively simpler manner. These TGPs also effectively condense DNA and exhibit enhanced cellular transfection capabilities These findings suggest a

multipronged therapeutic approach targeting ferroptosis, tau LLPS, and amyloid toxicity is generalised framework for effective AD treatment.

Statistical Analysis

All results were statistically validated using one-way ANOVA (non-parametric or mixed) or Student's t-test (non-parametric), with a biological replicate number exceeding three.

Discussion

Alzheimer's disease (AD) is a complex neurodegenerative disorder marked by amyloid-β (Aβ) plaques, tau neurofibrillary tangles (NFTs), synaptic dysfunction, and progressive neurodegeneration. Efforts to develop drugs targeting individual pathological features, particularly Aβ, have largely met with limited success, with many candidates failing in clinical trials. This has underscored the need for novel, multi-targeted therapeutic approaches that can address the multifactorial nature of AD. In addition to Aβ, tau has emerged as a promising target, with some therapeutic candidates, particularly immunotherapies, showing early success in clinical studies. However, the persistent failures of drugs aimed at single targets highlight the necessity for multifunctional therapeutics that can simultaneously address multiple pathological processes driving AD. Recent advances in understanding the various etiological factors of AD have enabled the design of chemical tools that target these diverse disease mechanisms. Natural products and hybrid drug designs have been adapted to create multifunctional molecules that can combat both metal-dependent and independent Aβ toxicity, as well as oxidative stress. We propose the development of multifunctional small molecules as future drug candidates for AD, capable of synergistically addressing the complex pathology of the disease. Furthermore, therapeutic strategies that go beyond the conventional biomarkers and address novel disease mechanisms—such as ferroptosis, an iron-dependent lipid peroxidation-driven cell death, and tau liquid-liquid phase separation (LLPS), a process that

contributes to tau aggregation—are crucial. In addition to these, targeting neuroinflammation alongside amyloid toxicity could provide a more comprehensive approach to treating AD. Despite the urgent need for such approaches, there remains a notable gap in the development of multifunctional molecules capable of simultaneously targeting Aβ-associated toxicity, ferroptosis, and tau LLPS. Our work successfully bridges this gap, demonstrating the potential of these multifunctional small molecules to synergistically address the complex pathology of AD. As traditional approaches focused on core biomarkers have yielded limited results, the rational design of such molecules is a promising direction for future therapeutics. These findings suggest a multipronged therapeutic approach targeting ferroptosis, tau LLPS, and amyloid toxicity for AD treatment.

Impact of the Research

Alzheimer's disease (AD) is a progressive neurodegenerative disorder responsible for over 70% of dementia cases worldwide, affecting more than 55 million people. This number is expected to surge to 139 million by 2050. While deaths from many leading diseases have decreased due to advancements in diagnostics and therapeutic interventions, fatalities from AD have risen by over 145%. Clinically, AD manifests through learning and memory impairments, language difficulties, and cognitive deficits, often leading to death within 5 to 12 years following diagnosis, which is currently based on behavioral and cognitive symptoms. Given the absence of a complete cure and the challenges of early diagnosis, the importance of our research cannot be overstated. Our work opens up new and underexplored avenues in the AD therapeutic pipeline, addressing critical societal needs as AD continues to place an immense burden on patients, families, and healthcare systems worldwide. By focusing on novel therapeutic targets such as ferroptosis, protein phase separation (LLPS), and neuroinflammation, we have designed new therapeutic molecules that adopt a multipronged approach. This strategy aims to

tackle the multifaceted nature of AD, providing a generalized framework for developing effective therapies and ultimately improving the quality of life for those affected.

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- Baruah, P.[+]; **Moorthy, H.**[+]; Ramesh, M.; Padhi, D.; Govindaraju, T. A Natural Polyphenol Activates and Enhances GPX4 to Mitigate Amyloid-β Induced Ferroptosis in Alzheimer's Disease. *Chem. Sci*. **2023**, *14*, 9427–9438. ([+]equal first author contributions). (*Recognized as one of the most popular chemical biology articles of 2023 in Chemical Science, Royal Society of Chemistry*).

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Declaration

The information submitted are accurate and original.

Hariharan