# EVALUATING THE EFFECTS OF RITONAVIR ON BACE GENE EXPRESSION, COGNITION AND DEPRESSION IN ALUMINIUM CHLORIDE-INDUCED MOUSE MODEL OF ALZHEIMER'S DISEASE

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**NIGERIA**

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**MATRICULATION NUMBER: PHA1908459**

**UNDER THE SUPERVISION OF**

**Dr. (Mrs) A. AKHIGBEMEN**

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**TO THE**

**DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY,**

**FACULTY OF PHARMACY,**

**UNIVERSITY OF BENIN,**

**BENIN CITY, EDO STATE**

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## CERTIFICATION

This is to certify that this project work titled "EVALUATION OF THE EFFECTS OF RITONAVIR ON BACE GENE EXPRESSION, COGNITION AND DEPRESSION IN ALUMINIUM CHLORIDE-INDUCED MOUSE MODEL OF ALZHEIMER'S DISEASE" was carried out by Micheal Oluwasegun Aladekoye in the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria in partial fulfilment of the award of the Doctor of Pharmacy Degree in the University of Benin, Edo state, Nigeria.

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(Head of Department)

**CERTIFICATION OF THESIS ON PLAGIARISM**

We the undersigned attest and declare that the thesis of Micheal Oluwasegun Aladekoye titled "EVALUATION OF THE EFFECTS OF RITONAVIR ON BACE GENE EXPRESSION, COGNITION AND DEPRESSION IN ALUMINIUM CHLORIDE-INDUCED MOUSE MODEL OF ALZHEIMER'S DISEASE" has successfully passed the anti-plagiarism test and does not violate any copyright regulations.

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## DEDICATION

I dedicate this project to almighty God, the one who has protected, guided and sustained me throughout my journey in the university.

This work is also dedicated to everyone that has contributed to the advancement of pharmacy profession and general public health.

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**Aβ**-Amyloid-Beta

**Aβ42** -Amyloid-Beta 42

**AChEIs** - Acetylcholinesterase Inhibitors

**AD** - Alzheimer's Disease

**AGEs** - Advanced Glycoxidation End Products

**$AlCl_3$** - Aluminium Chloride

**APOE** - Apolipoprotein E

**APP** - Amyloid Precursor Protein

**ANOVA**- Analysis of Variance

**BACE-1** - Beta-site APP Cleaving Enzyme 1

**BBB** - Blood-Brain Barrier

**BPSD** - Behavioural and Psychological Symptoms of Dementia

**CCL2** - C-C Motif Chemokine Ligand 2

**CCL3** - C-C Motif Chemokine Ligand 3

**CDC** - Centers for Disease Control and Prevention

**CMP** - Comprehensive Metabolic Panel

**CNS** - Central Nervous System

**COX-2** - Cyclooxygenase-2

**CSF** - Cerebrospinal Fluid

**CT** - Computed Tomography

**CVD** – Cardiovascular-Disease

**CXCL8** - C-X-C Motif Chemokine Ligand 8

**DI** - Discrimination Index

**DMTs** - Disease-Modifying Therapies

**ECG** - Electrocardiogram

**EDTA** - Ethylenediaminetetraacetic Acid

**EEG** - Electroencephalogram

**EOAD** - Early-Onset Alzheimer's Disease

**ER** - Endoplasmic Reticulum

**FDA** - Food and Drug Administration

**fMRI** - Functional Magnetic Resonance Imaging

**HNE** - 4-Hydroxy-2,3-nonenal

**HPA** - Hypothalamic-Pituitary-Adrenal

**IFN-γ** - Interferon-Gamma

**IHME** - Institute for Health Metrics and Evaluation

**IL-1β** - Interleukin-1 Beta

**IL-6** - Interleukin-6

**IL-18** - Interleukin-18

**iNOS** - Inducible Nitric Oxide Synthase

**LOAD** - Late-Onset Alzheimer's Disease

**LOX** - Lipoxygenase

**mAbs** - Monoclonal Antibodies

**MCI** - Mild Cognitive Impairment

**MDA** - Malondialdehyde

**MMSE** - Mini-Mental State Examination

**MoCA** - Montreal Cognitive Assessment

**MRI** - Magnetic Resonance Imaging

**NF-κB** - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

**NFTs** - Neurofibrillary Tangles

**NIH** - National Institutes of Health

**NICE** - National Institute for Health and Care Excellence

**NMDA** - N-methyl-D-aspartate

**NOR** - Novel Object Recognition

**PAF** - Platelet-Activating Factor

**PET** - Positron Emission Tomography

**PI** - Protease Inhibitor

**PSEN1/PSEN2** - Presenilin 1 / Presenilin 2

**p-tau** - Phosphorylated tau

**RNS** - Reactive Nitrogen Species

**ROS** - Reactive Oxygen Species

**SEM** - Standard Error of the Mean

**SPECT** - Single-Photon Emission Computed Tomography

**TNF-α** - Tumour Necrosis Factor-Alpha

**t-tau** - Total tau

**TST** - Tail Suspension Test

**WHO** - World Health Organization

**γ-secretase** - Gamma-secretase

## ABSTRACT

**Background:** Alzheimer's disease (AD) is a progressive neurodegenerative condition marked by the accumulation of amyloid-beta plaques, a process largely influenced by the activity of β-site amyloid precursor protein cleaving enzyme 1 (BACE-1). Current therapeutic options are mainly symptomatic and do not alter the course of the disease, highlighting the need for treatments that can target its underlying pathology. Protease inhibitors have therefore emerged as promising candidates for modulating this key pathological mechanism.

**Objective:** This study aimed to investigate the influence of the protease inhibitor Ritonavir on BACE-1 gene expression, cognitive performance, and depression-like behaviours in a mouse model of Alzheimer's disease induced by aluminium chloride ($AlCl_3$).

**Methods:** Fifty-six Swiss albino mice were allocated into seven groups: a control group, three groups treated with Ritonavir at doses of 100, 200, and 400 mg/kg, an $AlCl_3$-induced Alzheimer's model group, and two positive control groups administered Donepezil (5 mg/kg) and Ascorbic Acid (100 mg/kg). After 28 days of treatment, molecular analysis was carried out to quantify BACE-1 gene expression. Cognitive function and depression-related behaviours were assessed using the Novel Object Recognition (NOR) test and the Tail Suspension Test (TST), respectively.

**Results:** The high dose of Ritonavir (400 mg/kg) significantly suppressed $AlCl_3$-induced BACE-1 gene overexpression, demonstrating efficacy comparable to the positive controls. However, this robust molecular effect did not translate into significant improvements in the behavioural assays. No significant differences were observed in the NOR test discrimination index or in the immobility duration during the TST across all treatment groups.

**Conclusion:** The findings suggest that Ritonavir is effective in normalizing BACE-1 gene expression at a high dose, indicating potential disease-modifying properties at the molecular level. However, its inability to reverse cognitive deficits or depression-like behaviour in this model suggests that BACE-1 inhibition alone may be insufficient for comprehensive functional recovery. Further investigation in chronic models and exploration of combination therapies is warranted.

# CHAPTER ONE

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Definition of Alzheimer's Disease

Alzheimer's disease (AD) is an irreversible and progressively worsening neurodegenerative condition that predominantly impairs memory, cognitive abilities, and behaviour (Scheltens *et al*., 2021). As the foremost cause of dementia, it mainly impacts the elderly population (aged 65 and older) and is distinguished by the abnormal buildup of amyloid-beta plaques together with neurofibrillary tangles made up of hyperphosphorylated tau protein in the brain (Alzheimer's Association, 2024). However, it should be emphasized that a less common, early-onset variant of the disease may develop in individuals younger than 65 years, representing roughly 5–6% of the total reported cases (Scheltens *et al*., 2021). It typically presents with subtle memory impairment that gradually worsens over time, leading to language difficulties, disorientation, personality change, and functional decline (Alzheimer's Association, 2023). Data from the Centres for Disease Control and Prevention (CDC) indicate that in 2022, Alzheimer's disease was the seventh leading cause of mortality in the United States. Prior to the COVID-19 pandemic, it held the position of the sixth leading cause of death in the United States (Ahmad *et al*., 2022). Although not currently ranked within the top ten causes of death in Nigeria, Alzheimer's disease and other dementias are becoming a public health issue of growing importance, a trend largely attributable to the nation's swiftly aging demographic (IHME, 2024). The disease does not directly lead to mortality, but it significantly heightens susceptibility to other complications that ultimately prove fatal. The typical form of Alzheimer's disease, which appears after the age of 65, is termed late-onset Alzheimer's disease (LOAD). In contrast, early-onset Alzheimer's disease (EOAD), developing before the age of 65, is less prevalent and accounts for approximately 5% of all patients. EOAD frequently presents with non-typical symptoms, and its diagnosis is often delayed, resulting in a more aggressive progression of the illness (Mendez, 2017).

A defining feature of Alzheimer's disease (AD) is a steady and worsening deterioration of neuronal health caused by widespread nerve cell loss, where the initial pathological alterations frequently begin in the entorhinal cortex, part of the hippocampal formation (Scheltens *et al*., 2021; Long and Holtzman, 2019). In addition to its clinical symptoms, the condition places a significant emotional and psychological strain on the families of those

affected and imposes a considerable socioeconomic impact on society at large (Alzheimer's Association, 2024; WHO, 2021).

### 1.2 Risk Factors

The following risk factors has been identified to contribute to the development of AD: Advancing age represents the predominant risk factor, with increasing age serving as the principal contributor to disease development (Mendez, 2017).

Genetic factors account for approximately 70% of the susceptibility to developing AD. Mutations in the genes for amyloid precursor protein, presenilin 1, and presenilin 2 (PSEN1 and PSEN2) are typically responsible for early-onset AD, while late-onset AD is primarily linked to a polymorphism in the apolipoprotein E (APOE) gene, particularly the E4 allele (Giri *et al*., 2016).

Cardiovascular diseases (CVD) are also identified as substantial risk factors for AD. They not only elevate the risk of developing AD but also contribute to dementia risk associated with strokes or vascular pathology. CVD is increasingly acknowledged as a modifiable risk element for AD (Livingston *et al*., 2020).

Obesity and diabetes are significant modifiable risk factors for the onset of Alzheimer's disease (Scheltens *et al*., 2021). Increased body weight can impair glucose regulation and raise the probability of developing type 2 diabetes. Chronic high blood sugar and insulin resistance promote cognitive deterioration by encouraging β-amyloid buildup and perpetuating neuroinflammatory pathways (Nguyen *et al*., 2020). Furthermore, obesity intensifies this susceptibility via the release of pro-inflammatory cytokines from fat tissue, which together worsen systemic insulin resistance and brain inflammation (Guzman-Martinez *et al*., 2019).

Additional potential risk factors for AD encompass traumatic brain injury, dyslipidemia, depression, advanced parental age at the time of birth, smoking, a family history of dementia, and carrying the APOE ε4 allele (Nicolas G *et al*., 2018). An individual's risk of developing the disease increases by 10% to 30% if they have a first-degree relative with Alzheimer's disease (Liljegren *et al*., 2015). Those with two or more siblings affected by LOAD encounter a risk that is three times greater than that of the general population (Tong *et al*., 2018).

### 1.3 Signs and Symptoms of Alzheimer's Disease

Alzheimer's disease follows a slowly evolving course, with its severity increasing over time as clinical signs mirror a continuing deterioration in cognitive, functional and behavioural capacities (Scheltens *et al*., 2021; Petersen *et al*., 2022). These signs and symptoms are generally classified into three progressive phases: early (mild), middle (moderate), and late (severe) stages (Alzheimer's Association, 2024).

A. Early-stage Symptoms

During the earliest phase, clinical manifestations are frequently mild and can be misattributed to the normal aging process. Typical characteristics encompass memory impairment, particularly affecting recently acquired information, challenges with word retrieval or maintaining conversations, difficulties in planning and organizing activities, disorientation in time or familiar settings, and mood alterations including apathy, depressive states, or increased irritability (Scheltens *et al*., 2021; Apostolova, 2016).

B. Middle Stage Symptoms

As Alzheimer's disease advances into the middle stage, cognitive impairments become more severe, and the individual's capacity to manage daily activities declines markedly. This stage is typified by heightened confusion and memory lapses, often involving difficulty recalling the names of close relatives and an inability to perform complex tasks such as financial management (Scheltens *et al.,* 2021; Apostolova, 2023). In addition, behavioural and psychological disturbances emerge more prominently, manifesting as sleep disruptions, wandering, and notable personality alterations such as increased suspicion, irritability, aggression, or agitation (Canevelli *et al.,* 2021). Repetitive speech and stereotyped actions are also frequently exhibited during this phase as part of the broader behavioural symptomatology (Alzheimer's Association, 2024).

C. Late-stage Symptoms

In the advanced stage of Alzheimer's disease, affected individuals progressively lose the capacity to interact with their surroundings or engage in meaningful communication. This terminal phase is characterized by profound memory deterioration, often accompanied by the inability to recognize close relatives, alongside a severe decline in

physical functioning, including impairments in mobility, swallowing, and the control of bladder and bowel activities. Consequently, patients become entirely dependent on caregivers for all aspects of daily living (Scheltens *et al.,* 2021). The management of such debilitating symptoms, particularly dysphagia and overall functional decline constitute a central component of palliative care strategies for individuals with late-stage dementia (Van der Steen *et al*., 2023).

Although the specific manifestations of Alzheimer's disease may differ slightly across individuals, the condition generally follows a consistent and predictable trajectory of progressive neurological deterioration (Scheltens *et al*., 2021; Apostolova, 2023). This highlights the crucial importance of early detection and timely intervention, as these measures can facilitate effective symptom management, ensure access to supportive care and novel therapeutic options, and ultimately enhance the quality of life for both patients and their caregivers (Livingston *et al.,* 2020; Apostolova, 2023).

### 1.4 Causes of Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder with a complex and multifactorial origin, whose exact causative mechanisms are not yet fully understood. However, comprehensive investigations have identified a series of interrelated biological, genetic, and environmental elements that work in concert to drive its underlying disease processes (Scheltens *et al*., 2021):

A. Amyloid-Beta Plaque Accumulation

A prominent theoretical framework in Alzheimer's disease pathogenesis centers on the progressive buildup of amyloid-beta peptides. These peptides clump together to create insoluble deposits known as extracellular plaques within the brain. These plaques are known to impair signaling between nerve cells, prompt an inflammatory response, and set off a chain of events that results in synaptic failure and neuronal loss (Selkoe and Hardy, 2019; Scheltens *et al*., 2021).

B. Neurofibrillary Tangles (Tau Protein)

Another hallmark feature of the disease is the abnormal addition of phosphate groups to tau protein, leading to the formation of neurofibrillary tangles inside neurons. These twisted protein aggregates undermine the stability of microtubules, disrupt the transport of essential materials along nerve fibers, and ultimately cause the breakdown of nerve cells (Vogel *et al*., 2021).

C. Genetic Factors

Hereditary factors are particularly influential in early-onset Alzheimer's disease, where pathogenic mutations in the APP, PSEN1, or PSEN2 genes frequently play a deterministic role (Sims *et al*., 2020; Andrews *et al*., 2023). For the more common late-onset form, the APOE ε4 allele represents the primary genetic risk factor, which is linked to heightened amyloid pathology and an earlier manifestation of the disease (Giri *et al*., 2016).

D. Neuroinflammatory and Oxidative Stress

Persistent neuroinflammatory activity, driven by the chronic activation of microglial cells and astrocytes, plays a key role in damaging neurons and driving the progression of neurodegeneration in Alzheimer's disease. Also, oxidative stress resulting from mitochondria dysfunction and free radical accumulation exacerbates neuronal damage (Leng and Edison, 2021; Cai *et al.,* 2021; Sorrentino *et al.,* 2023).

E. Vascular Contribution

There is a growing body of evidence implicating cerebrovascular pathology, diminished blood flow to the brain, and impairment of the blood-brain barrier as significant factors in the pathogenesis of Alzheimer's disease. These vascular issues may impair clearance of amyloid-beta and accelerate neurodegeneration (Iadecola, 2020; Montagne *et al.,* 2020).

F. Lifestyle and Environmental Factors

Lifestyle elements, including insufficient exercise, an unhealthy nutritional intake, a history of head trauma, and limited mental stimulation, are similarly linked to a heightened vulnerability to developing Alzheimer's disease (Livington *et al*., 2020).

### 1.5 Diagnosis of Alzheimer's Disease

The diagnosis of Alzheimer's disease is clinical, supported by neuropsychological assessments, neuroimaging, and biomarker analysis (Scheltens *et al*., 2021). A definitive diagnosis of Alzheimer's disease during a patient's lifetime cannot be achieved by a single test, with confirmation ultimately relying on postmortem brain examination. Therefore, clinical diagnosis depends on assessing a combination of established criteria, observable symptoms, and the systematic elimination of other potential conditions (Rabinovici, 2021).

#### 1.5.1 Clinical Assessment

The evaluation of a patient with suspected Alzheimer's disease in a primary care setting involves a structured, multi-faceted process. The initial evaluation should involve a detailed exploration and verification of both the patient's medical and family history, alongside a careful examination of current medications to identify agents that might induce or exacerbate cognitive decline (NICE, 2018). Objective evaluation of cognitive performance is essential and can be achieved through standardized bedside assessments such as the Mini-Mental State Examination (MMSE) or the Montreal Cognitive Assessment (MoCA) (Creavin *et al*., 2016; Carson *et al*., 2018). Additionally, ordering relevant blood tests to dismiss reversible causes of cognitive dysfunction such as metabolic or endocrine abnormalities represents a critical component of the diagnostic workup (Apostolova, 2023; NICE, 2018).

Standard laboratory evaluations, including complete blood count, comprehensive metabolic panel, thyroid-stimulating hormone assessment, and vitamin B12 measurement, typically do not show disease-specific abnormalities in individuals with Alzheimer's disease They are conducted to rule out any other potential causes of cognitive impairment (Haapasalo, Hiltunen, 2018; Peterson, 2018).

#### 1.5.2 Neuropsychological Testing

In-depth neuropsychological evaluation designed to examine specific cognitive functions, including memory, language, visuospatial processing, and executive control. This helps distinguish Alzheimer's disease from other types of dementia (Albert *et al.,* 2018; Jack *et al.,* 2018; Dubois *et al.,* 2021). Among clinical tools, neuropsychological testing remains the most sensitive and dependable method for detecting mild cognitive impairment (MCI) in its initial phases (Edmonds *et al*., 2019).

### 1.5.3 Neuroimaging

Imaging helps exclude other structurer causes of dementia (e.g stroke, tumour) and supports diagnosis by identifying characteristic changes in Alzheimer's disease (Jack *et al*., 2018):

- A computed tomography (CT) scan of the brain may demonstrate evidence of cerebral atrophy along with enlargement of the third ventricle in patients diagnosed with Alzheimer's disease. Nevertheless, these radiological signs remain suggestive rather than diagnostic, since similar changes can appear in various other dementing illnesses or as part of typical brain aging in healthy older adults (Förster *et al*., 2018).
- Magnetic resonance imaging (MRI) offers superior anatomical resolution compared with CT when assessing individuals with cognitive decline or suspected Alzheimer's disease. In confirmed Alzheimer's cases, MRI frequently reveals characteristic sequential atrophy beginning in the entorhinal cortex and progressing to involve the hippocampi and broader medial temporal cortex (Frisoni *et al*., 2022).
- Although neither MRI nor CT can independently confirm a diagnosis of Alzheimer's disease, MRI proves particularly helpful in recognizing distinctive atrophy patterns associated with alternative neurodegenerative conditions causing dementia, such as frontotemporal lobar degeneration, or specific imaging hallmarks of multisystem atrophy, Creutzfeldt-Jakob disease, and progressive supranuclear palsy (Sobański *et al*., 2020).
- In recent years, volumetric MRI has gained prominence as a precise quantitative technique for tracking regional brain volume loss. In Alzheimer's disease, this method reliably documents progressive shrinkage of the medial temporal lobe structures, especially the hippocampus—a highly characteristic finding that strongly correlates with declining memory performance (Frisoni *et al*., 2021).
- Advanced functional neuroimaging modalities, including positron emission tomography (PET), functional MRI (fMRI), and single-photon emission computed tomography (SPECT), are increasingly capable of mapping hypometabolism or altered activity in circumscribed regions of the medial temporal and parietal lobes. Although these techniques show considerable promise for early identification and longitudinal monitoring of Alzheimer's disease, their role in providing a definitive diagnosis remains under investigation (Leuzy *et al*., 2019).

- Amyloid PET imaging enables direct visualization of cerebral amyloid-β plaques and is primarily employed in research protocols or highly selected clinical scenarios. It offers greater pathological specificity for Alzheimer's disease than for many other neurodegenerative conditions. Its diagnostic utility is tempered, however, by the fact that amyloid deposition can also occur in cognitively intact older individuals. Amyloid PET is most valuable when the goal is to differentiate dementias driven by amyloid-β pathology from those caused by alternative proteinopathies, such as tau-related disorders (Jack *et al*., 2018; Villemagne *et al*., 2018; Pontecorvo *et al*., 2019).
- Electroencephalography (EEG) generally contributes little diagnostic value in Alzheimer's disease and related neurodegenerative syndromes. Recordings typically reveal only diffuse slowing without focal or epileptiform features that would point specifically to Alzheimer's pathology (Babiloni *et al*., 2020).

### 1.5.4 Cerebrospinal Fluid (CSF) and Biomarkers

Over the last ten years, remarkable advances have occurred in identifying fluid and imaging biomarkers capable of detecting Alzheimer's disease during its preclinical and prodromal phases. Despite these breakthroughs, most of these biomarkers remain largely confined to research environments and are not yet routinely accessible in everyday clinical practice (Hansson, 2021). The recent emergence of disease-modifying therapies, particularly monoclonal antibodies directed against amyloid-beta, has dramatically heightened the clinical relevance of accurate early biomarker detection (Hansson *et al*., 2023).

Analysis of cerebrospinal fluid (CSF) represents one of the most valuable approaches for identifying Alzheimer's pathology at a preclinical stage, often revealing characteristic molecular changes years before overt cognitive symptoms appear (Hansson *et al*., 2022). That said, CSF markers provide limited information about current disease severity or clinical stage. The core CSF biomarkers currently employed for Alzheimer's disease are amyloid-beta 1–42 (Aβ42), phosphorylated tau (p-tau), and total tau (t-tau). The typical Alzheimer's pattern consists of reduced Aβ42 concentrations alongside elevated levels of both p-tau and t-tau. The rise in CSF p-tau and t-tau directly mirrors the intraneuronal aggregation of hyperphosphorylated tau and neuronal injury occurring in the brain (Blennow and Zetterberg, 2018).

Recent advances have led to development of blood tests detecting phosphorylated tau and other markers, offering a less invasive diagnostic tool with growing clinical potential (Hampel *et al*., 2021).

### 1.5.5 Genetic Studies

Genetic testing is not part of standard diagnostic workups for Alzheimer's disease. It is typically reserved for rare situations involving familial early-onset Alzheimer's disease with a clear autosomal-dominant inheritance pattern across multiple generations (Goldman *et al*., 2019).

Even after thorough clinical assessment, detailed neuropsychological evaluation, and extensive ancillary testing, a conclusive diagnosis of Alzheimer's disease is not always achievable during life. Certain individuals present with subjective memory concerns that can be confirmed objectively on testing, yet the degree of cognitive deficit remains insufficient to significantly disrupt everyday activities (Jack *et al*., 2018). In these instances, the appropriate diagnostic label is usually mild cognitive impairment (MCI) rather than dementia. MCI occupies a transitional zone between the minor cognitive changes expected with normal aging and the more profound dysfunction characteristic of Alzheimer's disease and related dementias. A substantial proportion of individuals diagnosed with MCI progress to overt dementia most commonly Alzheimer's disease within five to seven years (Porsteinsson *et al*., 2021).

## 1.6 Pathogenesis of Alzheimer's Disease

The pathogenesis of Alzheimer's disease (AD) involves a multifaceted interplay of biological processes, with the progressive cerebral accumulation of extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein representing the defining histopathological features. Contemporary research has significantly deepened our insight into these core mechanisms, particularly by elucidating the pivotal role of β-site amyloid precursor protein cleaving enzyme 1 (BACE-1). Elevated BACE-1 activity not only drives increased Aβ production but has also been strongly linked to accelerated cognitive deterioration and the emergence of depressive symptomatology in AD (Sun *et al*., 2022). Alzheimer's disease is neuropathologically defined by two cardinal lesions: senile plaques primarily composed of aggregated amyloid-β peptides and neurofibrillary tangles consisting of abnormally hyperphosphorylated tau protein (Alzheimer's Association, 2025).

i. Amyloid-beta (Aβ) Plaques: These are extracellular deposits composed of Aβ peptides, which exhibit neurotoxic properties, particularly in their soluble oligomeric forms.
ii. Neurofibrillary Tangles (NFTs): These are intracellular accumulations of hyperphosphorylated tau protein that disrupt neuronal transport, resulting in synaptic dysfunction and eventual neuronal death.

These pathological alterations are believed to develop decades before the onset of clinical symptoms, initiating a progressive cascade that culminates in neurodegeneration, synaptic loss, and brain atrophy (Scheltens *et al*., 2021).

The shrinkage of the hippocampus and cerebral cortex in patients with Alzheimer's disease results from the progressive loss of neurons within these regions. This neurodegeneration tends to worsen with disease advancement and increasing age (Pini *et al*., 2016).

Furthermore, the progressive loss of cognitive abilities in Alzheimer's disease is closely linked to both the atrophy of the hippocampus and the abnormal accumulation of Tau protein within neurons. As Tau proteins aggregate, they form neurofibrillary tangles that disrupt normal cell function and communication. Tau pathology in Alzheimer's disease propagates along a highly predictable anatomical sequence. It begins in the transentorhinal and pre-olfactory regions, subsequently involving the entorhinal cortex and the CA1/subiculum sectors of the hippocampal formation (Vogel *et al*., 2021). As the disease advances, neurofibrillary tangles progressively infiltrate multimodal association cortices, with pronounced involvement of the temporal, parietal, and frontal lobes in later stages. This hierarchical, temporospatial expansion of tau deposition closely mirrors the characteristic clinical evolution of Alzheimer's disease, in which episodic memory deficits emerge early and are later compounded by increasing impairment in judgment, executive control, language, and visuospatial abilities (Dos Santos *et al*., 2018).

### 1.6.1 The Amyloid and Tau Hypothesis

In the amyloidogenic pathway, the amyloid precursor protein (APP) is first cleaved by an enzyme called β-secretase (BACE-1) and then by γ-secretase, which produces the amyloid-beta (Aβ) peptide (Nasb, *et al*., 2024). The Aβ42 peptide, which is 42 amino acids long, tends to clump together easily and is considered the most toxic form. Recent studies suggest that soluble Aβ oligomers (small clumps) are mainly responsible for damaging synapses, even more than the larger, insoluble Aβ plaques (Abeysinghe *et al*., 2020; Nasb, *et al*.,

2024). Over time, these small oligomers join together to form senile plaques, which are visible outside neurons in the brains of Alzheimer's disease (AD) patients (National Institute on Aging, 2023). The accumulation of β-amyloid deposits depends on the stability of its molecular structure. When mutations occur that destabilize β-amyloid, proper aggregation is impaired, altering its pathological potential. The Aβ42 isoform, produced through the previously described cleavage processes, exhibits particularly strong cytotoxic effects on neurons. This neurotoxicity promotes the production of reactive oxygen species (ROS), which are harmful to nerve cells. The damaging effects are largely associated with the disruption of calcium homeostasis caused by alterations in neuronal cell membrane lipids, eventually leading to neuronal injury and death (Pendin *et al*., 2021).

Another element of the plaque formation process is the Tau protein. The buildup of Aβ is thought to trigger abnormal hyperphosphorylation (excess addition of phosphate groups) of the Tau protein. Tau protein plays a critical role in promoting the proper assembly of tubulin, a structural protein essential for maintaining neuronal integrity. Tubulin molecules polymerize to form microtubules, which serve as vital components of the cell's internal framework. These microtubules create pathways that facilitate the movement of motor proteins responsible for transporting cellular materials and also play a central role during cell division by forming the mitotic spindle (Guan *et al*., 2021). Normally, Tau helps stabilize microtubules, which are structures that support nerve cells and help transport materials within them (Nasb *et al*., 2024; Saporito *et al*., 2025). When Tau becomes hyperphosphorylated, it seperates from microtubules, causing them to fall apart and disrupting transport inside the neuron. The faulty Tau proteins then aggregate to form neurofibrillary tangles (NFTs), which are toxic and strongly linked to the level of cognitive decline in Alzheimer's disease (Zhang *et al*., 2024). The neurotoxic effects of Tau protein occur through two principal mechanisms. First, the loss of Tau's normal physiological function results in the destabilization of microtubules, disrupting intracellular transport and structural stability within neurons. Second, abnormal Tau can acquire toxic properties that directly harm neurons, triggering a cascade of cellular stress responses that ultimately lead to apoptosis. Together, these processes contribute significantly to the neurodegenerative changes, characteristic of Alzheimer's disease (Sebastián-Serrano Á *et al*., 2018).

### 1.6.2 Neuroinflammation and Immune Dysregulation

A substantial body of research has firmly established neuroinflammation as a pivotal driver in both the initiation and progression of Alzheimer's disease (Jagust, 2018). This chronic inflammatory state arises from the coordinated activation of glial cells, primarily microglia and astrocytes along with the sustained release of numerous pro-inflammatory mediators, including cytokines, chemokines, complement proteins, and reactive oxygen and nitrogen species. Collectively, these processes disrupt neuronal homeostasis, induce oxidative damage, trigger synaptic dysfunction, and ultimately promote widespread neuronal loss through apoptotic and necroptotic pathways, thereby giving rise to the progressive cognitive decline that defines the clinical syndrome of AD (Fakhoury, 2020).

The presence of neuroinflammatory changes in Alzheimer's disease was originally first documented more than thirty years ago (Ozben *et al*., 2019). Despite considerable advances in characterizing the key cellular players and molecular signals involved, the exact triggers that initiate this inflammatory cascade and the mechanisms governing its self-perpetuating nature remain only partially elucidated (Mauersberger *et al*., 2021). From a pathophysiological perspective, the inflammatory reaction observed in AD exhibits striking parallels with peripheral inflammatory responses, particularly in the repertoire of soluble mediators that orchestrate the process (Denver *et al*., 2018).

The principal pro-inflammatory molecules implicated in Alzheimer's-related neuroinflammation encompass a broad array of cytokines (including IL-1β, IL-6, IL-18, TNF-α, and IFN-γ), chemokines (such as CCL2, CCL3, and CXCL8), complement cascade components (e.g., C1q and C5), transcription factors (most notably NF-κB), vasoactive peptides (e.g., bradykinin), and inducible enzymes (COX-2, iNOS, and lipoxygenase family members). This inflammatory milieu is further intensified by additional mediators such as platelet-activating factor (PAF) and other coagulation-associated factors, all of which collectively amplify and sustain the neuroinflammatory response within the central nervous system (Newcombe *et al*., 2018).

The build-up of abnormal proteins in Alzheimer's disease (AD) triggers further cellular damage, which speeds up brain cell loss and cognitive decline. The aggregation of Aβ and hyperphosphorylated Tau activates immune cells in the brain; microglia and astrocytes, leading to long-term neuroinflammation (Nasb *et al*., 2024). At the early stages of AD,

microglia in their M2 protective form help by clearing Aβ. However, with ongoing exposure to protein aggregates, they shift to the M1 inflammatory form, releasing harmful pro-inflammatory cytokines. This causes a continuous cycle of inflammation, nerve cell damage, and reduced Aβ clearance (He *et al*., 2023).

Numerous studies have reported decreased levels of regulatory factors responsible for suppressing neuroinflammation in patients with Alzheimer's disease. This reduction is believed to be associated with the accumulation of β-amyloid deposits in the brain, which not only trigger inflammatory responses but also sustain them over time, leading to chronic inflammation. Understanding this mechanism requires examining the origins and underlying processes that initiate and perpetuate neuroinflammation in Alzheimer's disease (Kinney *et al*., 2018).

### 1.6.3 Synaptic Dysfunction and Neurodegeneration

One of the earliest and most immediate causes of memory impairment in Alzheimer's disease is synaptic injury. Soluble amyloid-β oligomers and hyperphosphorylated tau directly impair synaptic plasticity – the brain's capacity to modify synaptic strength – thereby disrupting the neural basis of learning and memory consolidation (Zhang *et al*., 2024). Loss of cholinergic neurons in the basal forebrain also plays a significant role in memory decline. Although the cholinergic hypothesis is among the oldest proposed mechanisms in AD, it retains strong relevance, as cholinergic deficits are closely tied to the toxic effects of accumulating Aβ and tau pathology (Breijyeh and Karaman, 2020).

### 1.6.4 Oxidative Stress

Researchs showed the brain consumes roughly 20% of the body's oxygen despite representing only 2% of body weight, rendering it especially susceptible to damage from reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Hou *et al*., 2020). These highly reactive molecules, characterized by unpaired electrons, cause widespread oxidative injury in Alzheimer's disease (Zabel *et al*., 2018). Neuronal membranes, rich in polyunsaturated fatty acids, are particularly prone to peroxidation by ROS and RNS, resulting in lipid peroxidation, disturbed redox balance of Aβ–metal complexes, mitochondrial impairment, and subsequent neuronal apoptosis. Oxidative damage to lipids

and DNA also accelerates neuronal senescence and cortical atrophy, thereby driving AD progression (Bennett *et al*., 2018).

Lipid peroxidation of polyunsaturated fatty acids produces toxic aldehydes and isoprostanes, including 4-hydroxynonenal (HNE), malondialdehyde (MDA), and F2-isoprostanes. These by-products promote tau hyperphosphorylation, disrupt neuronal calcium homeostasis, and activate apoptotic cascades (Cassidy *et al*., 2020).

Oxidative stress further contributes to AD pathology through the formation of advanced glycation end products (AGEs) via reactions between ROS and glycoproteins. These AGEs are neurotoxic, induce pro-inflammatory cytokines such as IL-1 and TNF-α, and intensify neuroinflammation. Additionally, oxidative modifications convert normal tau into AGE-tau species that aggravate neurodegeneration (Song *et al*., 2021).

### 1.6.5 Genetic factors

While genetic factors are not considered the sole cause of Alzheimer's disease, certain mutations significantly increase disease susceptibility (Bellenguez *et al*., 2022). Among the most prominent genetic risk factors are mutations in the amyloid precursor protein (APP) gene and the presence of the ε4 allele of apolipoprotein E (ApoE), both of which have been strongly linked to amyloid pathology and neurodegeneration (Zhu *et al*., 2015). Approximately 20% of AD patients carry the ApoE ε4 allele, accounting for nearly 65% of total cases. Individuals with this allele have nearly a threefold increased risk of developing the disease. Although the exact mechanisms through which the ApoE ε4 allele contributes to AD remain under investigation, evidence suggests it impairs β-amyloid clearance, promoting plaque accumulation in the brain (Sienski *et al*., 2021). Additionally, mutations in the presenilin genes, PSEN1 (located on chromosome 14) and the less commonly affected PSEN2 (on chromosome 1) are well-established causes of familial early-onset Alzheimer's disease. Mutations in PSEN1 are found in 18–50% of such cases, while PSEN2 mutations are comparatively rarer. These genetic abnormalities collectively influence amyloid processing, neuronal signals, and mitochondrial function, thereby accelerating neurodegenerative changes (Van Giau *et al*., 2019).

### 1.6.6 Mitochondrial Dysfunction

Mitochondrial impairment represents a central pathological hallmark of Alzheimer's disease, resulting from compromised antioxidant defenses that typically shield mitochondria against reactive oxygen species (ROS) (Giorgi *et al*., 2018). Evidence shows that ROS predominantly target mitochondrial complexes I and II, thereby interfering with energy production and intensifying oxidative stress. Normally, mitochondria possess antioxidant enzymes, including cytochrome c oxidase, that counteract oxidative damage (Gowda *et al*., 2022). Nevertheless, research reveals that individuals with Alzheimer's disease display markedly lower cytochrome c oxidase activity in the hippocampus, which further exacerbates mitochondrial dysfunction.

In addition to oxidative stress, β-amyloid oligomers have been shown to enter mitochondria through enhanced expression of endoplasmic reticulum (ER); mitochondrial membrane proteins or via the outer membrane translocase complex (Huang *et al*., 2020). Once inside, β-amyloid obstructs the electron transport chain, leading to excessive ROS production and exacerbating mitochondrial dysfunction. This self-perpetuating cycle of oxidative damage and impaired energy metabolism contributes to neuronal apoptosis and the broader neurodegenerative process underlying Alzheimer's disease (Song *et al*., 2023).

### 1.6.7 Cholinergic Alterations

Acetylcholine serves as a major neurotransmitter in the human brain and is prominently active in the cerebral cortex, basal ganglia, and basal forebrain areas (Picciotto *et al*., 2022). It is critically involved in supporting neuroplasticity, synchronizing neuronal activity, and sustaining efficient neuronal signaling, all of which are indispensable for learning, memory, and cognitive performance (Zhang *et al*., 2018). The cholinergic hypothesis, initially put forward in 1976, stands as one of the first explanations for the cognitive decline seen in Alzheimer's disease (Hampel *et al*., 2018).

This hypothesis attributes the cognitive symptoms of AD to atrophy and the loss of synaptic connections, leading to impaired neurotransmission within cholinergic pathways. Both nicotinic (ionotropic) and muscarinic (metabotropic) receptors in the cortex of the cerebrum are affected (Gatta *et al*., 2020).

As Alzheimer's disease advances, cholinergic neurons in the basal nucleus and cortex undergo substantial degeneration, with late stages exhibiting more than 90% loss of neurons in the basal nucleus (Vitanova *et al*., 2019). This degeneration reduces receptor binding efficiency, contributing to the neuropsychiatric and cognitive symptoms characteristic of AD (Sultzer *et al*., 2022). Although receptor-binding reductions can also occur in normal aging, they are more closely associated with mild memory decline rather than the extensive neuronal loss seen in Alzheimer's disease (Lebois *et al*., 2018).

### 1.7 Treatment of Alzheimer's Disease

For nearly twenty years, available treatments for Alzheimer's disease were largely confined to symptomatic medications, including acetylcholinesterase inhibitors (such as donepezil) and N-methyl-D-aspartate (NMDA) receptor antagonists (such as memantine), which received approval prior to 2006. Although these agents provided temporary improvement in cognitive symptoms, they did not prevent or slow the progression of the underlying neurodegenerative processes. (Alzheimer's Association, 2022). The treatment of Alzheimer's disease (AD) has advanced greatly in recent years, shifting from only managing symptoms to including new disease-modifying therapies (DMTs). Today, effective care involves both modern drugs that attack the root causes of the disease and supportive non-drug approaches for complete patient care (WHO, 2025). The actual neurodegenerative process begins twenty to thirty years before first clinical symptoms. Amelioration of the pathological alterations such as Aβ plague accumulation and neurofibrillary tangles before their expansion is crucial for an actual cure (WHO, 2025). The approval of anti-amyloid monoclonal antibodies (mAbs) by the Food and Drug Administration (FDA) starting in 2021 marked a major milestone in Alzheimer's disease research. This development supported the amyloid cascade hypothesis as a viable therapeutic target and provided new optimism for slowing disease progression, especially among individuals in the early stages of the disease, such as those with mild cognitive impairment (MCI) or mild dementia related to Alzheimer's disease (Brockmann *et al*., 2023).

#### 1.7.1 Current Pharmacological Therapies in Alzheimer's Disease

Existing Alzheimer's disease (AD) therapies primarily aim to restore neurotransmitter balance in the brain. The acetylcholinesterase inhibitors (AChEIs) authorised for AD

treatment are donepezil, galantamine, and rivastigmine (Cummings *et al*., 2019). These drugs operate on the cholinergic hypothesis, which posits that progressive degeneration of cholinergic projections in the limbic system and neocortex is largely responsible for the impairment of memory, learning, attention, and other higher-order cognitive abilities. Moreover, neuronal loss in the basal forebrain is thought to induce dysfunction and subsequent death of cholinergic neurons, resulting in extensive cholinergic deficit. By blocking acetylcholine breakdown, AChEIs elevate synaptic acetylcholine levels and have demonstrated the ability to delay cognitive deterioration in AD patients (Hampel *et al*., 2018).

In clinical investigations, amyloid-beta (Aβ) and tau proteins continue to serve as the main targets for disease-modifying therapies (DMTs) in Alzheimer's disease (Thompson *et al*., 2020). From a therapeutic standpoint, AD could potentially be prevented or controlled by decreasing Aβ and tau production, blocking their aggregation or misfolding, neutralising or removing their toxic oligomeric and fibrillar species, or combining these approaches (Nguyen and Roberts, 2021). Besides Aβ plaques and neurofibrillary tangles (NFTs), multiple interrelated pathological processes have been recognised that either promote or result from these core lesions, including persistent neuroinflammation, oxidative stress, disrupted iron regulation, altered cholesterol metabolism, and blood–brain barrier (BBB) impairment (Garcia *et al*., 2019).

Currently, the only medications approved by the United States Food and Drug Administration (FDA) for Alzheimer's disease are the acetylcholinesterase inhibitors donepezil, galantamine, and rivastigmine, together with the N-methyl-D-aspartate (NMDA) receptor antagonist memantine (Anderson *et al*., 2020). AChEIs work by inhibiting acetylcholinesterase within the synaptic cleft, thereby reducing acetylcholine degradation and strengthening central cholinergic transmission (Huang and Patel, 2019). This action helps mitigate cognitive decline, especially within the first year, although disease progression ultimately resumes. Stopping AChEI treatment is frequently associated with swift worsening of cognition and daily function, as well as a higher risk of institutionalisation (Lee *et al*., 2022). No meaningful differences in clinical effectiveness have been observed among the three AChEIs (Gomez and Tan, 2021). Donepezil and rivastigmine are licensed for mild, moderate, and severe AD, while galantamine is approved only for mild-to-moderate stages (Walker *et al*., 2020).

Memantine, a low-affinity, non-competitive NMDA receptor antagonist, regulates glutamatergic signalling to protect against excitotoxicity caused by overactivation of NMDA receptors (Nguyen *et al*., 2021). It is FDA-approved for moderate-to-severe AD, either alone or combined with an AChEI (Chen *et al*., 2020). Clinical trials indicate that memantine monotherapy provides short- and long-term improvements in cognition, activities of daily living, and behavioural and psychological symptoms of dementia (BPSD) in patients with moderate-to-severe AD (Garcia and O'Brien, 2019).

Combination therapy with memantine and an AChEI has been shown to yield additive therapeutic effects without a corresponding increase in adverse reactions, owing to their complementary mechanisms of action (Li and Zhou, 2020). Moreover, sustained monotherapy or combination therapy with optimised dosing in patients with moderate to advanced AD is associated with improved global function and overall clinical outcomes (Kim *et al*., 2022).

### 1.7.2 Currently Studied Disease-Modifying Therapies (DMTs) for Alzheimer's Disease

Recent advances in Alzheimer's disease (AD) research have shifted the focus from symptomatic management to the development of disease-modifying therapies (DMTs) aimed at altering the underlying pathophysiological processes. Most investigational DMTs target amyloid-beta (Aβ) and tau pathologies; the two major hallmarks of AD, while others explore alternative mechanisms such as neuroinflammation, oxidative stress, and synaptic dysfunction (Cummings *et al*., 2021).

Among amyloid-targeted approaches, monoclonal antibodies have gained important attention for their ability to bind and promote the clearance of Aβ aggregates. Agents such as aducanumab, lecanemab, and donanemab have demonstrated varying degrees of efficacy in reducing amyloid plaque burden and modestly slowing cognitive decline in clinical trials (van Dyck *et al*., 2023; Mintun *et al*., 2021). In parallel, β-secretase (BACE) and γ-secretase inhibitors, designed to reduce Aβ production, have been extensively studied; however, many have failed to show clinical benefit due to safety concerns and off-target effects (Henley *et al.,* 2020).

Tau-directed therapies represent another promising avenue of investigation. These include inhibitors of tau aggregation, modulators of tau phosphorylation, and immunotherapies that

facilitate clearance of pathological tau species (Bai *et al*., 2022). Furthermore, emerging DMTs are being developed to target secondary pathological mechanisms implicated in AD progression, such as microglial activation, mitochondrial dysfunction, lipid metabolism imbalance, and vascular impairment (Zhou *et al*., 2023).

Although several of these investigational compounds have reached late-stage clinical evaluation, consistent and clinically meaningful cognitive benefits remain limited. Ongoing trials continue to explore combination therapies targeting multiple molecular pathways, with the goal of achieving greater disease modification and improved long-term outcomes (Cummings and Zhong, 2022).

### 1.8 Rationale for the Study

Alzheimer's disease (AD) continues to be the leading neurodegenerative disorder worldwide, distinguished by gradual deterioration of cognitive abilities, behavioural changes, and neuropsychiatric manifestations (Long and Holtzman, 2019). Despite decades of research, the absence of effective disease-modifying therapies underscores the complexity of AD pathogenesis. A central mechanism implicated in AD involves aberrant proteolytic processing of amyloid precursor protein (APP) by β-site amyloid precursor protein cleaving enzyme 1 (BACE-1), which initiates the amyloidogenic pathway leading to excessive accumulation of amyloid-β (Aβ) peptides and subsequent plaque deposition (Hampel *et al*., 2021; Zhang *et al*., 2022). Consequently, pharmacological inhibition of BACE-1 has emerged as a promising therapeutic strategy aimed at reducing Aβ burden and mitigating amyloid-associated neurotoxicity (Yan *et al*., 2019).

Protease inhibitors, through modulation of BACE-1 and related enzymatic cascades, have the potential to alter both amyloid metabolism and downstream neurodegenerative processes (Coimbra *et al*., 2018). Nevertheless, multiple investigations have expressed concerns about the long-term neurocognitive safety of BACE-1 inhibition, since pronounced suppression of BACE-1 function has been linked to impairments in synaptic plasticity, axonal guidance, and cognitive abilities. These findings underscore the importance of maintaining a precise equilibrium between therapeutic benefits and possible off-target consequences when modulating protease activity in the brain (Filser *et al*., 2019; Kennedy *et al*., 2020).

Recent evidence also suggests that BACE-1 expression is sensitive to stress, oxidative damage, and inflammatory stimuli; factors that are not only central to AD pathology but also linked to affective disturbances such as depression (Zhao *et al*., 2020; Du *et al*., 2021). Experimental studies have shown that stress-induced upregulation of BACE-1 may exacerbate Aβ generation, synaptic dysfunction, and memory impairment. These findings indicate a possible mechanistic overlap between protease dysregulation, amyloid accumulation, and mood alterations observed in AD (Zhou *et al*., 2019). Therefore, interventions targeting protease activity may have dual implications: potentially reducing amyloid pathology while influencing depressive and cognitive outcomes

Given these interrelationships, investigating the effects of protease inhibitors on BACE-1 gene expression, cognitive function, and depression-like behaviour in an AD mouse model represents a crucial step toward elucidating the broader impact of protease modulation on neurodegenerative and neuropsychiatric outcomes. Such an investigation could clarify whether protease inhibition exerts neuroprotective or detrimental effects at molecular and behavioural levels. Moreover, understanding how protease inhibitors regulate BACE-1 transcription and translation may support the development of more selective and safe therapeutic approaches.

Ultimately, this study seeks to connect molecular and behavioural research by combining biochemical, genetic, and neurobehavioural measures. By clarifying the impact of protease inhibition on both amyloidogenic and affective pathways, it could offer important insights into the dual involvement of proteases in cognitive and mood regulation, thereby supporting the development of more targeted next-generation disease-modifying treatments for Alzheimer's disease.

### 1.9 Aim and Objectives

#### 1.9.1 Aim:

The primary aim of this study is to evaluate the effects of protease inhibitors (RITONAVIR) on BACE-1 gene expression, cognitive performance, and depression-like behaviours in a mice model of Alzheimer's disease (AD).

### 1.9.2 Objectives

The objectives of this study were to:

i. To determine the effect of protease inhibitor administration on BACE-1 gene expression in the brain tissues of Alzheimer's disease-induced mice, using molecular and biochemical assays.

ii. To assess the impact of protease inhibitors on cognitive function through behavioural paradigms such as the Novel Object Recognition Test, which evaluate spatial learning, memory, and executive function.

iii. To evaluate depression-like behaviours following protease inhibitor treatment using validated behavioural assessments such as the Tail Suspension Test.

iv. To compare the therapeutic effects of protease inhibitors with standard Alzheimer's treatments (e.g donepezil), where applicable, to determine their relative efficacy and safety profile.

# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Experimental Animals

This study utilized a total of fifty-six Swiss albino mice sourced from the institutional animal facility of the Department of Pharmacology and Toxicology, University of Benin, Nigeria. The mice, with body weights ranging from 17 to 30 grams, were maintained under controlled environmental conditions. These included a 12-hour light/dark cycle, a constant ambient temperature of 25°C, and unrestricted access to both standard rodent feed and water. Cage bedding, consisting of wood shavings, was replaced daily to maintain hygiene. Prior to the commencement of the experimental protocols, the animals underwent a two-week acclimatization period to adjust to the housing environment. All research activities were performed in strict compliance with the internationally recognized principles for the ethical use of laboratory animals, as outlined in the National Institutes of Health guide for care and use (NIH Publication No. 80-23, revised 2002).

### 2.1.2 Drugs and Chemicals

All drugs and chemicals used in this study were of analytical grade and obtained from standard commercial sources. Drugs were solubilized in distilled water and were freshly prepared daily for administration.

### 2.1.3 Experimental Design

Protease inhibitors (PI) play a complex role in Alzheimer's disease (AD). They can contribute to the pathology and progression of the disease of the while also acting as a potential therapeutic agent. Some research suggested that protease inhibitors that target BACE-1 gene may be valuable in AD treatment. This research aims to evaluate the effect of a known PI (Ritonavir) on Alzheimer's Disease induced by Aluminium Chloride; determining its effect on BACE-1 gene expression, cognition and depression. The study was conducted using a total of 56 Swiss albino mice, weighing between 17-30g, obtained from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy,

University of Benin, Nigeria. The mice were randomly assigned into seven groups of 8 mice each as follows:

1. Group 1 (Control): mice in this group received 0.2mL of distilled water only.
2. Group 2 (PI 100 mg/kg): mice in this group received 100 mg/kg orally of PI (Ritonavir).
3. Group 3 (PI 200 mg/kg): mice in this group received 200 mg/kg orally of PI (Ritonavir).
4. Group 4 (PI 400 mg/kg): mice in this group received 400 mg/kg orally of PI (Ritonavir).
5. Group 5 ($AlCl_3$ 100 mg/kg): mice in this group received 100 mg/kg orally of $AlCl_3$.
6. Group 6 (Donepezil 5mg/kg): mice in this group received 5 mg/kg orally of Donepezil, an approved drug for the management of AD.
7. Group 7 (Ascorbic Acid 100 mg/kg): mice in this group received 100 mg/kg orally of Ascorbic Acid.

### 2.2 Methods

#### 2.2.1 Behavioural Tests

The behavioural tests were used to investigate the impact of the protease inhibitor (Ritonavir) on cognitive function and depressive-like behaviour, two domains frequently impaired in Alzheimer's disease. To assess these parameters, two distinct behavioural paradigms were employed: the Novel Object Recognition (NOR) test for evaluating memory and cognition, and the Tail Suspension Test (TST) for quantifying depressive-like states. Both behavioural assessments were conducted following a 28-day drug administration period.

#### 2.2.2 Novel Object Recognition (NOR) Tests

The NOR experiment was performed in a rectangular open-field apparatus measuring 40 × 40 × 40 cm, constructed with opaque walls to block external visual cues. Testing took place in a dimly lit room, maintaining illumination. The arena’s position and orientation were kept constant throughout all sessions to ensure consistency in environmental cues. The items used as test objects were comparable in size to minimize bias due to object dimensions. Preliminary trials confirmed that the mice showed no inherent preference for the objects used as familiar or novel. Objects likely to retain scent or be damaged by gnawing were

excluded. During the sample phase, two identical replicas of the familiar object were presented to the animals. To reduce handling stress, the mice were accustomed to human contact for three days before behavioural testing began. They were then habituated to the empty open-field arena for 10 minutes per day over three consecutive days. During this period, animals were allowed to move freely within the space without the presence of any objects.

The mice were acclimated to the testing environment for seven days before experimentation. They were further habituated to the open-field apparatus (40 × 40 × 40 cm) for 10 minutes per day over three consecutive days, during which no objects were present.

The Novel Object Recognition (NOR) test consisted of a 10-minute sample phase in which two identical objects were presented. During the subsequent 5-minute test phase, one of the familiar objects was replaced with a novel one.

Exploratory behaviour; defined as the mouse's nose approaching within 2 cm of, or making direct contact with an object, was recorded. The discrimination

index (DI), calculated as *(*Tnovel − Tfamiliar) / (Tnovel + Tfamiliar*)*, served as the main behavioural outcome measure. The discrimination index (DI) yields values ranging from −1 to +1, where a positive score reflects a greater exploration of the novel object, indicative of preserved recognition memory.

Between trials, the arena and all objects were cleaned with 70% ethanol to eliminate residual odour cues.

### 2.2.3 Tail Suspension Test (TST)

Depressive-like behaviour was evaluated with the Tail Suspension Test (TST). Individual mice were suspended by the tail using adhesive tape affixed roughly 1 cm from the tip, with the tape attached to a horizontal bar located 50 cm above the table surface. The test lasted 6 minutes and was video-recorded for later scoring.

Immobility, defined as the total absence of active movements was quantified over the last 4 minutes of the test. Between trials, the apparatus was cleaned with 70% ethanol to remove any lingering olfactory cues.

Prolonged immobility duration was interpreted as an indicator of depression (behavioural despair).

### 2.2.4 Statistical Analysis

All results are presented as mean ± standard error of the mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test, with statistical significance set at $P < 0.05$. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

# CHAPTER THREE

# RESULTS

## 3.1 Effect Protease Inhibitors (Ritonavir) on BACE-1 Gene Expression in Mouse Model of Alzheimer's Disease.

The results show significant variation in the relative expression of the BACE gene across the seven experimental groups. The significant differences were observed between multiple treatment groups, as indicated by the adjusted p-values. Distinctly, significant differences were found between certain treatment groups, denoted by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$), while some comparisons were not significant ($p>0.05$).

Figure 3.1 shows the relative expression of BACE gene across the different treatment groups. Data is presented as Mean ± SEM. The statistical analysis involves a one-way ANOVA with Tukey's multiple comparison.

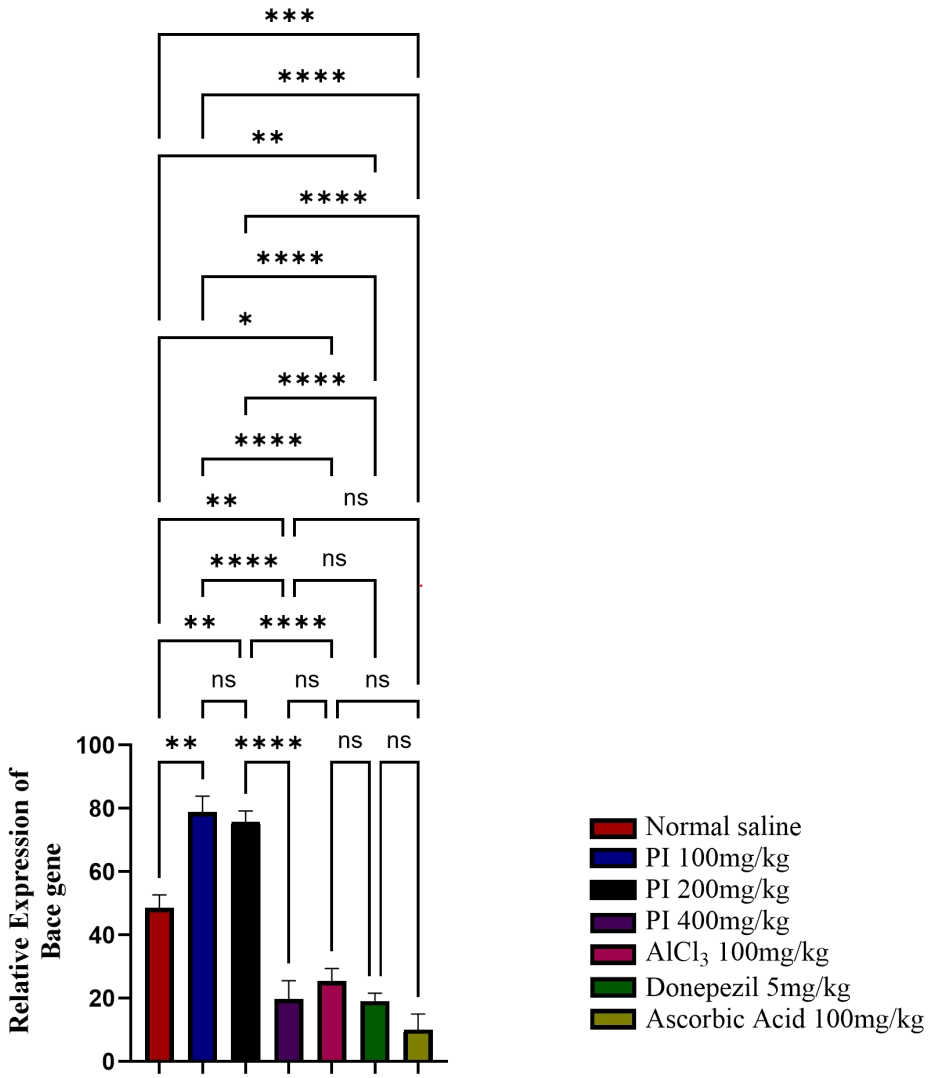


**Figure 3.1** shows the relative BACE gene expression in the cortex of mouse model of Alzheimer's disease. Data is presented as Mean ± SEM (n=2). Statistical analysis: one-way ANOVA with Tukey's multiple comparisons. Significant differences: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$, while some comparisons were not significant ($p > 0.05$). ANOVA: $F = 83.22$, $p < 0.0001$.

### 3.2 Tail Suspension Test: Effect of Protease Inhibitors (Ritonavir) on Depression in Mouse Model of Alzheimer's Disease.

The results show that the administration of a protease inhibitor (i.e. Ritonavir) at different doses produce no significant differences between any of the treatment groups that they were administered. Comparison across all groups (ns = no significant) showed that no group is statistically distinct from any other.

Figure 3.2 shows the duration of mobility across the different treatment groups. Data is presented as Mean ± SEM. The statistical analysis involves a one-way ANOVA with Tukey's multiple comparison.

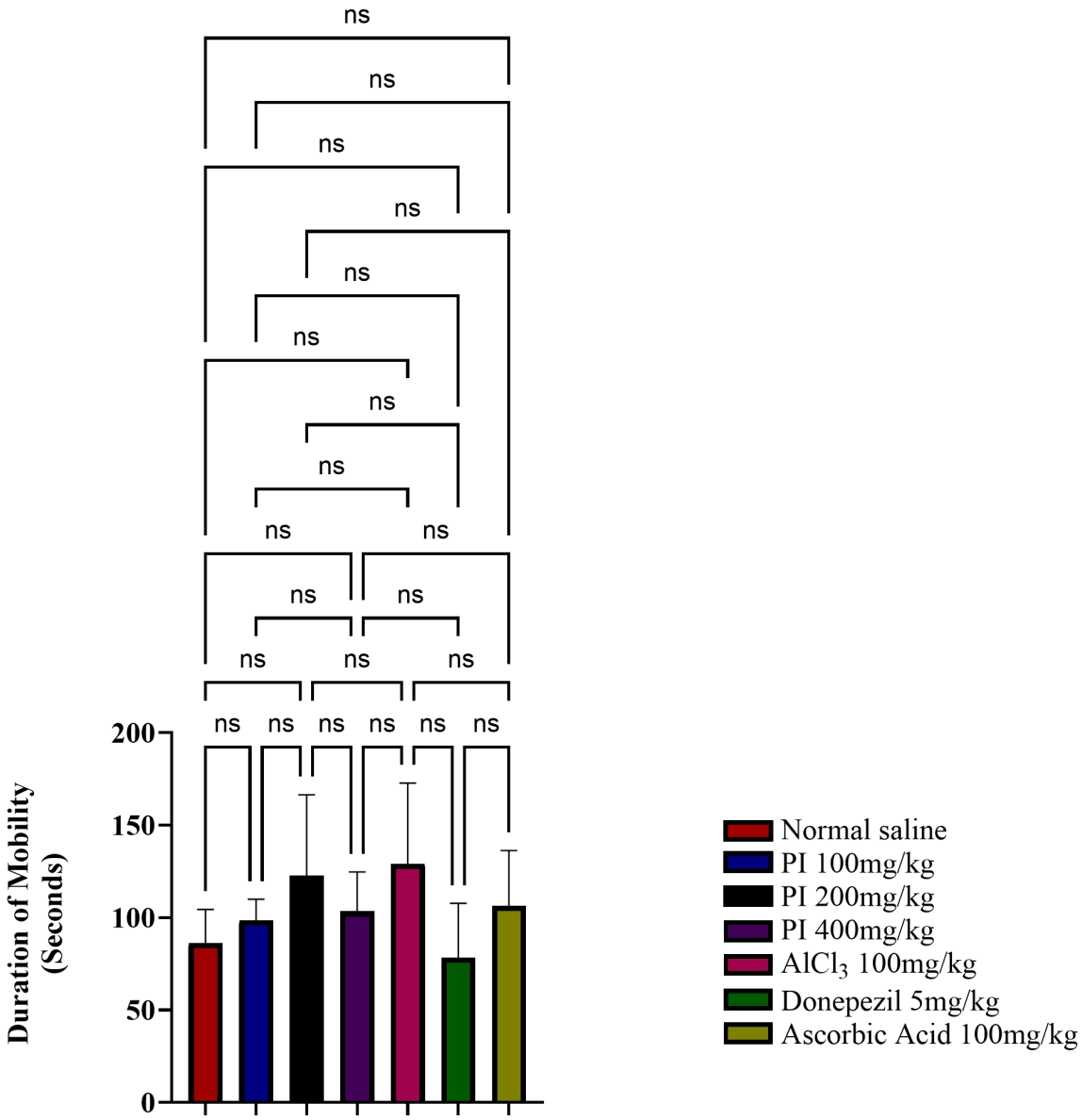


**Figure 3.2** shows the duration of mobility in mouse Model of Alzheimer's disease. Data is presented as Mean ± SEM (n=6). Statistical analysis: one-way ANOVA with Tukey's multiple comparisons. Significant differences: comparisons were not significant (ns) ($p > 0.05$). ANOVA: $F = 2.254$, $P = 0.0606$.

### 3.3 Tail Suspension Test: Effect of Protease Inhibitors (Ritonavir) on Depression in Mouse Model of Alzheimer's Disease.

The results show that the administration of a protease inhibitor (i.e. Ritonavir) at different doses produce no significant differences between any of the treatment groups that they were administered. Comparison across all groups (ns = no significant) showed that no group is statistically distinct from any other.

Figure 3.3 shows the duration of immobility across the different treatment groups. Data is presented as Mean ± SEM. The statistical analysis involves a one-way ANOVA with Tukey's multiple comparison.

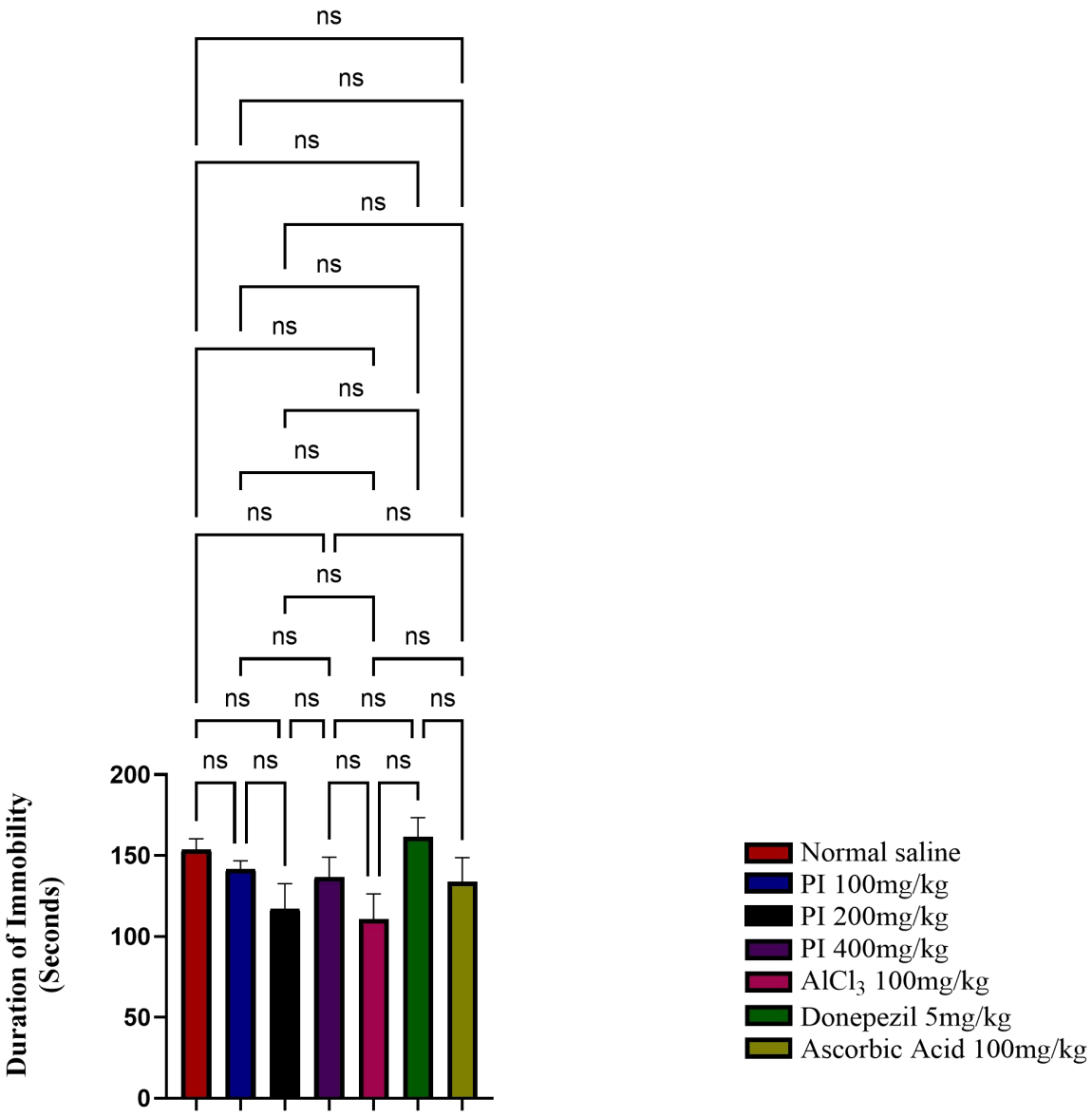


**Figure 3.3** shows the duration of immobility in mouse Model of Alzheimer's disease. Data is presented as Mean ± SEM (n=6). Statistical analysis: one-way ANOVA with Tukey's multiple comparisons. Significant differences: comparisons were not significant (ns) ($p > 0.05$). ANOVA: $F = 2.254$, $P = 0.0606$.

### 3.4 Novel Object Recognition (NOR) Test: Effect of Protease Inhibitors (Ritonavir) on Cognition in Mouse Model of Alzheimer's Disease.

The statistical results for this test are clear and consistent. There is no evidence from this Novel Object Recognition test that the protease inhibitor Ritonavir, at any of the administered doses, had a significant effect on the discrimination index, a measure of recognition memory, in this mouse model of Alzheimer's disease.

Figure 3.4 shows the discrimination index across the different treatment groups. Data is presented as Mean ± SEM. The statistical analysis involves a one-way ANOVA with Tukey's multiple comparison.

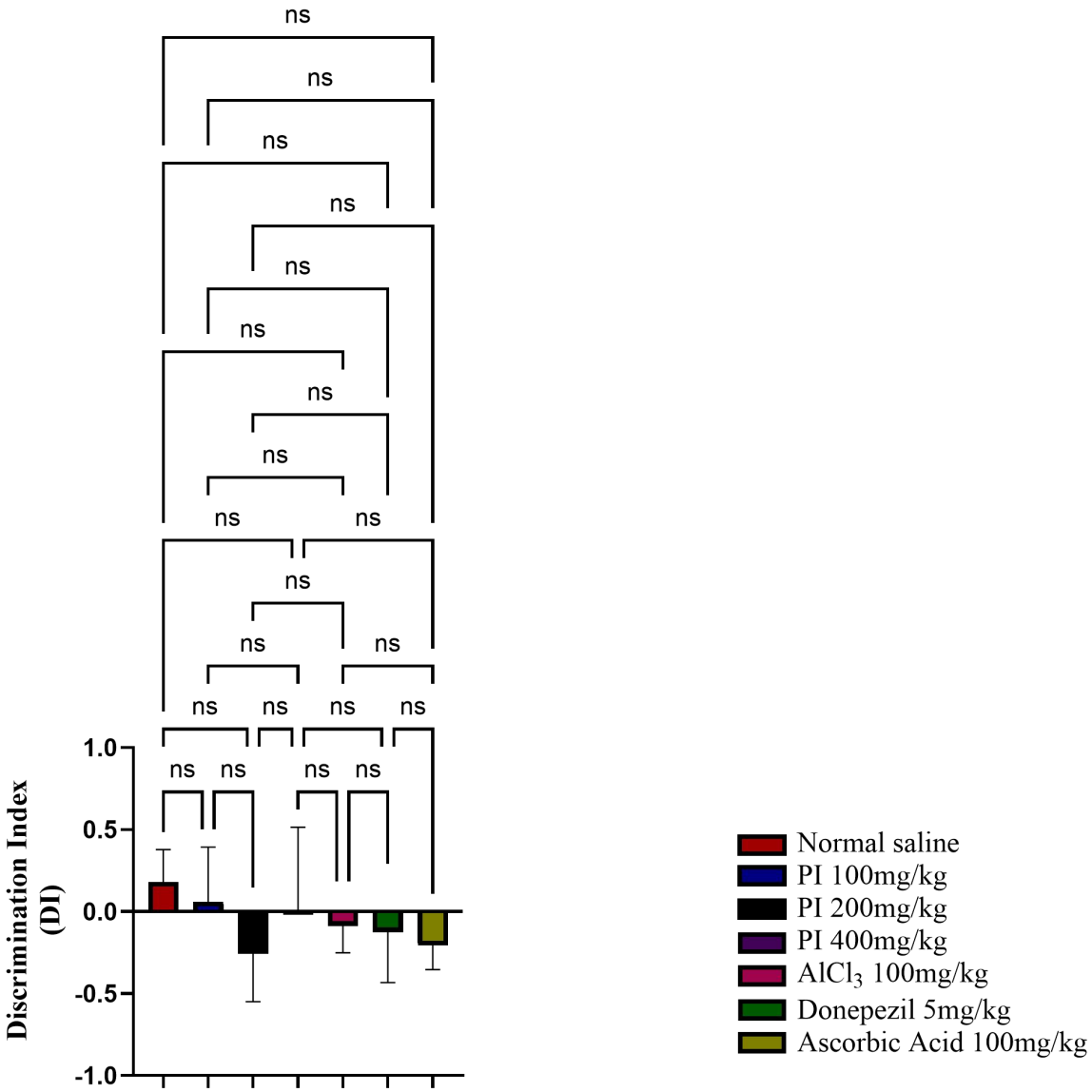


**Figure 3.4** shows the discrimination index in mouse model of Alzheimer's Disease. Data is presented as Mean ± SEM (n=6-7). Statistical analysis: one-way ANOVA with Tukey's multiple comparisons. Significant differences: Comparisons were not significant (ns) ($p > 0.05$). ANOVA: $F = 0.3615$, $P = 0.8986$.

# CHAPTER FOUR
# DISCUSSION

This chapter details the experimental outcomes of protease inhibitors (in this case Ritonavir) in an $AlCl_3$-induced mouse model of Alzheimer's pathology. The data, which encompass molecular, cognitive, and affective domains, are systematically delineated and critically evaluated in the subsequent sections to contextualize their significance within the broader field of AD research.

## 4.1 Effect on BACE Gene Expression

A central finding of this investigation was the profound suppression of $AlCl_3$ induced BACE-1 upregulation by the high-dose (400 mg/kg) protease inhibitor. Given that BACE-1 is the critical rate-limiting enzyme responsible for the amyloidogenic processing of Amyloid Precursor Protein (APP), its pathological upregulation represents a central driver of Alzheimer's disease pathogenesis by directly promoting excessive generation of neurotoxic Aβ peptides (Scheltens *et al*., 2021).

The complete prevention of this key event by the high-dose PI strongly supports its possible classification as a promising disease-modifying agent.

The analysis revealed a critical dose-response relationship for the protease inhibitor. Notably, the lower doses (100 and 200 mg/kg) not only failed to confer protection but were associated with a paradoxical increase in BACE-1 expression. In contrast, the 400 mg/kg regimen demonstrated robust molecular efficacy. This pattern underscores the existence of a precise therapeutic threshold, a finding consistent with the documented clinical challenges of BACE inhibition, where narrow therapeutic windows and inadequate target engagement have historically contributed to late-stage trial failures (Mullard, 2022). The mechanistic basis for the elevated BACE-1 at sub-therapeutic doses remains to be fully elucidated but may involve compensatory feedback loops or off-target effects.

Most compellingly, the high-dose PI achieved a level of BACE-1 suppression equivalent to that of the positive controls, Donepezil (an acetylcholinesterase inhibitor) and Ascorbic Acid (a potent antioxidant). Despite possessing a distinct primary mechanism, potentially through the inhibition of proteases that govern BACE-1 expression or stability, this convergence on a common pathological endpoint reinforces the validity of targeting the amyloid pathway. It further posits this novel protease inhibitor as a potential therapeutic alternative or adjunct to existing modalities (Wang *et al*., 2016).

### 4.2 Effect on Cognitive Function (Novel Object Recognition Test)

Contrary to the pronounced molecular benefits, behavioural phenotyping using the Novel Object Recognition (NOR) paradigm failed to reveal any cognitive enhancement. Post-hoc analysis via Tukey's test confirmed a lack of statistically significant pairwise differences among the treatment groups. This dissociation between a robust molecular outcome and a null cognitive effect represents the most critical and complex finding of this investigation. Such a disconnect, while challenging, is a recognized phenomenon in translational neuroscience, underscoring the formidable obstacles in converting molecular intervention into functional recovery.

Several plausible, non-exclusive explanations may account for this observed discrepancy. Firstly, the validity of the behavioural model itself must be scrutinized. Although the $AlCl_3$ model effectively induce acute oxidative stress and amyloid-beta pathology, its capacity to induce the profound, progressive synaptic degradation within memory-critical regions such as the perirhinal cortex and hippocampus, which is essential for NOR performance, may be limited (Li *et al*., 2023). Consequently, the induced cognitive deficit might be too subtle or inconsistent, creating a potential "floor effect" where the absence of a measurable impairment precludes the demonstration of any therapeutic rescue.

Secondly, a temporal disconnect between molecular and systems-level processes is a pivotal consideration. The treatment protocol was adequate to prevent the initial molecular injury of BACE-1 upregulation. However, the cognitive impairments observed in Alzheimer’s disease represent a downstream consequence of an extended pathological process that encompasses synaptic dysfunction and the eventual breakdown of neural circuits. It is therefore plausible that the acute molecular protection conferred by the PI would necessitate a substantially longer duration to manifest as measurable cognitive stabilization, suggesting that a single treatment is insufficient to alter a slowly evolving pathological trajectory (Cline *et al*., 2018).

Finally, the hypothesis that solitary BACE-1 inhibition is inadequate to restore cognitive function in this paradigm must be considered. Alzheimer's disease is a multifactorial condition characterized by concomitant tauopathy, neuroinflammation, and neurotransmitter system failure. Targeting amyloid production, while foundational, may require concurrent modulation of these additional pathological pathways to yield observable cognitive benefits, thereby lending support to the emerging paradigm of multi-target therapeutic strategies (Johnson and Lee, 2024).

### 4.3 Effect on Depression-like Behaviour (Tail Suspension Test)

Consistent with the cognitive findings, the assessment of affective behaviour via the Tail Suspension Test (TST) failed to elicit a significant treatment effect. The absence of a positive outcome in this paradigm indicates that neither the $AlCl_3$-induced pathology nor the conferred molecular protection robustly engaged the neural circuits underlying behavioural despair.

The aetiology of depression in Alzheimer's disease is multifaceted and is thought to involve pathologies that are distinct from core amyloidosis. Affective symptoms are more strongly associated with impairments in monoaminergic neurotransmission, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, and persistent neuroinflammation within limbic regions such as the hippocampus and amygdala (Nguyen *et al*., 2020). As the TST is principally sensitive to monoaminergic system function, these results suggest that the acute $AlCl_3$ challenge may not significantly perturb these specific neurobiological systems within the present experimental framework. This further implies that BACE-1-mediated amyloidogenesis is not a primary determinant of the affective phenotype in this model.

Notwithstanding the null result, the observed statistical trend is notable. This marginal finding intimates that a subtle effect may exist, which could be unmasked with greater statistical power or a more sensitive experimental design. It thereby justifies further investigation into the connection between amyloid pathology and affective states, potentially utilizing models with a more pronounced depressive phenotype or an extended disease duration (Johnson *et al*., 2022).

### 4.4 Implications and Future Research

#### 4.4.1 Implications

The results of this study present a complex picture with significant implications for Alzheimer's disease therapeutic development. The most substantial implication stems from the clear dissociation between molecular efficacy and functional outcomes. While the high-dose protease inhibitor demonstrated remarkable potency in normalizing BACE gene expression, achieving complete prevention of $AlCl_3$-induced upregulation, this robust molecular effect failed to translate into measurable cognitive or behavioural improvements.

This dissociation challenges the conventional assumption that successful targeting of a key pathological marker will automatically yield functional recovery. It suggests that the pathway from molecular intervention to behavioural improvement is not linear but is influenced by multiple intervening variables. The findings indicate that BACE inhibition,

while crucial for addressing amyloid pathology, may be insufficient as a standalone therapeutic approach, particularly within acute intervention paradigms.

The differential outcomes across measurement domains underscore the critical importance of multidimensional assessment in preclinical Alzheimer's research. The results demonstrate that relying solely on molecular biomarkers or behavioural endpoints provides an incomplete evaluation of therapeutic potential. This has practical implications for drug development strategies, suggesting that compounds showing strong molecular efficacy deserve further investigation even when initial behavioural results are negative.

Several limitations in the current experimental design may have influenced the observed outcomes. The $AlCl_3$-induced model, while effective for generating acute molecular pathology, may not fully replicate the progressive nature of Alzheimer's disease, particularly in terms of consistent behavioural deficits. The single time-point assessment and acute pre-treatment protocol may have been insufficient to capture potential long-term functional benefits that could emerge with sustained treatment.

The behavioural tests employed, while standard in the field, may have lacked the sensitivity to detect subtle cognitive improvements. The Novel Object Recognition test depends on multiple factors beyond recognition memory, including attention and motivation, which could have masked treatment effects. Similarly, the Tail Suspension Test primarily reflects behavioural despair but may not capture the full spectrum of depression-like behaviours relevant to Alzheimer's pathology.

### 4.4.2 Future Research

The findings and limitations of this investigation delineate several critical pathways for subsequent research to advance the therapeutic development of this protease inhibitor.

I. **Advanced Disease Modelling and Longitudinal Assessment**
   Subsequent studies would benefit from utilizing transgenic models of Alzheimer's disease that recapitulate progressive amyloidosis and tauopathy alongside robust, reproducible cognitive decline. Implementing chronic dosing regimens over extended periods is crucial to ascertain whether sustained BACE-1 suppression ultimately translates into long-term functional preservation. Incorporating serial behavioural assessments at multiple timepoints would be essential to map the temporal dynamics linking molecular efficacy to cognitive and affective outcomes.

II. **Mechanistic Deconvolution and Systems-Level Engagement**

A paramount objective is the clarification of the precise mechanism by which the protease inhibitor modulates BACE-1. Research must discriminate between direct enzymatic inhibition, transcriptional regulation, and post-translational control of protein turnover. A comprehensive systems biology approach, quantifying downstream biomarkers such as Aβ plaque load, pre- and post-synaptic markers (e.g., PSD-95, synaptophysin), and glial activation profiles, is required to fully characterize the compound's target engagement and neuroprotective scope.

III. **Therapeutic Window Refinement and Polypharmacology**

The paradoxical effects observed necessitate a refined dose-ranging study to precisely define the therapeutic index, maximizing efficacy while circumventing adverse events. Given the multifaceted pathology of AD, exploring combination therapies represents a promising frontier. Co-administering the protease inhibitor with agents targeting tau aggregation, neuroinflammation, or fostering synaptic resilience could reveal synergistic effects, potentially overcoming the translational hurdles encountered with mono-therapeutic interventions.

IV. **Behavioural Paradigm Refinement and Circuit Analysis**

Future behavioural phenotyping should encompass a broader battery of tests, specifically probing hippocampal-dependent spatial and episodic memory (e.g., Morris Water Maze). Employing more sensitive, often ethologically-based cognitive tasks, coupled with in vivo electrophysiology or calcium imaging, could detect subtler, circuit-level functional rescue that conventional tests overlook. This would facilitate a more precise mapping between pathology in specific neural circuits and their corresponding behavioural deficits.

V. **Biomarker Discovery and Translational Bridging**

A critical step toward clinical translation is the identification of robust biomarkers that can bridge molecular target engagement to functional outcomes. Future work should integrate fluid biomarkers (e.g., plasma p-tau181), structural and functional neuroimaging (e.g., fMRI, PET), and electrophysiological readouts with behavioural data to establish validated surrogate endpoints. Furthermore, studies initiating treatment at various stages of disease progression will be vital to determine whether the intervention possesses both prophylactic and disease-modifying potential.

# CHAPTER FIVE
# CONCLUSION

## 5.1 Conclusion

This study demonstrates that the high-dose (400 mg/kg) protease inhibitor is a potent and effective agent for preventing $AlCl_3$-induced BACE gene overexpression, performing as well as established reference treatments. However, this clear molecular success did not translate into measurable improvements in cognitive function or depression-like behaviour in the tests employed. This dissociation between molecular and functional outcomes highlights the complexity of Alzheimer's disease treatment and suggests that targeting BACE alone may be insufficient for comprehensive recovery. Despite this, the strong molecular efficacy makes the protease inhibitor a promising candidate worthy of further investigation in more advanced disease models and longer-term studies.

## 5.2 Contribution to Knowledge

This study presents a significant contribution to Alzheimer's disease (AD) therapeutics through a critical pre-clinical evaluation of the antiretroviral drug Ritonavir. Its primary contribution is two-fold;

- First, the research demonstrates that a high dose of Ritonavir (400 mg/kg) exhibits potent, disease-modifying properties by effectively targeting the amyloidogenic pathway upstream. This was evidenced by the complete suppression of $AlCl_3$-induced BACE-1 gene overexpression, an efficacy comparable to established positive controls, thereby identifying Ritonavir as a promising therapeutic candidate.
- Second, and of greater translational significance, the study uncovers a critical dissociation between molecular efficacy and functional outcome. Despite robust BACE-1 suppression, no significant improvements were observed in cognitive or affective behavioral paradigms, as measured by the Novel Object Recognition and Tail Suspension Tests, respectively. This finding fundamentally challenges the conventional linear drug development paradigm in AD, which often presupposes that target engagement directly translates to functional recovery. Consequently, this work underscores the insufficiency of BACE-1 inhibition as a monotherapy and emphasizes the necessity for multi-targeted approaches to address the complex, multifactorial pathology of AD.

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## APPENDIX

## SAMPLE CALCULLATION OF DOSES

1. **RITONAVIR**

A. Required dose = 100 mg/kg

Stock solution = 10 mg/ml

Weight of mouse = 25.5 g

Therefore, dose of Ritonavir required by a 25.5 g mouse will be as follows;

(25.5 x 100) ÷ 1000 = 2.55 mg

Amount of stock solution required will be

(2.55 x 1) ÷ 10 = 0.255 ml

Similar calculations were carried out in all mouse that received Ritonavir 100 mg/kg

B. Required dose = 200 mg/kg

Stock solution = 20 mg/ml

Weight of mouse = 21.5 g

Therefore, dose of Ritonavir required by a 21.5 g mouse will be as follows;

(21.5 x 200) ÷ 1000 = 2.15 mg

Amount of stock solution required will be

(2.15 x 1) ÷ 20 = 0.215 ml

Similar calculations were carried out in all mouse that received Ritonavir 200 mg/kg

C. Required dose = 400 mg/kg

Stock solution = 40 mg/ml

Weight of mouse = 20.7 g

Therefore, dose of Ritonavir required by a 20.7 g mouse will be as follows;

(20.7 x 400) ÷ 1000 = 8.28 mg

Amount of stock solution required will be

(8.28 x 1) ÷ 40 = 0.207 ml

Similar calculations were carried out in all mouse that received Ritonavir 400 mg/kg

**2. DONEPEZIL**

Required dose = 5 mg/kg

Stock solution = 0.5 mg/ml

Weight of mouse = 25.5 g

Therefore, dose of Donepezil required by a 25.5 g mouse will be as follows;

(25.5 x 5) ÷ 1000 = 0.1275 mg

Amount of stock solution required will be

(0.1275 x 1) ÷ 0.5 = 0.255 ml

Similar calculations were carried out in all mouse that received Donepezil 5 mg/kg

**3. ASCORBIC ACID**

Required dose = 100 mg/kg

Stock solution = 10 mg/ml

Weight of mouse = 25.5 g

Therefore, dose of Ascorbic Acid required by a 25.5 g mouse will be as follows;

(25.5 x 100) ÷ 1000 = 2.55 mg

Amount of stock solution required will be

(0.1275 x 1) ÷ 10 = 0.255 ml

Similar calculations were carried out in all mouse that received Ascorbic acid 100 mg/kg

**4. ALUMINIUM CHLORIDE**

Required dose = 100 mg/kg

Stock solution = 10 mg/ml

Weight of mouse = 25.5 g

Therefore, dose of Aluminium Chloride required by a 25.5 g mouse will be as follows;

(25.5 x 100) ÷ 1000 = 2.55 mg

Amount of stock solution required will be

(0.1275 x 1) ÷ 10 = 0.255 ml

Similar calculations were carried out in all mouse that received Aluminium chloride 100 mg/kg