

CAPITAL UNIVERSITY OF SCIENCE AND  
TECHNOLOGY, ISLAMABAD



# Pharmacological Investigation of INCA-6 in Animal Models of Scopolamine-induced Memory Deficit

by

Babar Bin Hamid

A thesis submitted in partial fulfillment for the  
degree of Master of Philosophy

in the

Faculty of Pharmacy

Department of Pharmacology

2026

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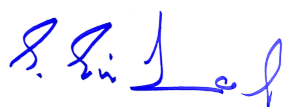
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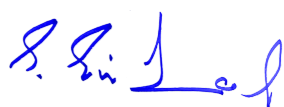
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## Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline, synaptic dysfunction, and neuronal degeneration. Impairment of cholinergic neurotransmission, oxidative stress, and neuroinflammation are considered major contributors to memory deficits associated with AD. The objective of the current study was to investigate the pharmacological effects of INCA-6, a potential neuroprotective and anti-inflammatory compound, in animal models of scopolamine-induced memory deficit. The study employed a multidisciplinary approach integrating *in vivo* behavioral assessments, biochemical analysis, histopathological evaluation, and *in silico* computational modeling to comprehensively assess cognitive performance and neuroprotective potential.

Behavioral assessments were conducted using the Morris water maze and Y-maze tests to evaluate spatial learning, memory retention, and working memory. INCA-6 treatment significantly improved cognitive performance, as evidenced by reduced escape latency and increased time spent in the target quadrant in the Morris water maze, along with enhanced spontaneous alternation behavior in the Y-maze test. Scopolamine caused clear memory impairment and neuroinflammation in the mice, and INCA-6 reversed both in a dose-dependent way. The 0.1 and 0.5 mg/kg doses gave partial recovery, while 1 mg/kg restored performance to near control levels ( $p < 0.01$  vs. scopolamine), matching or beating Donepezil. Docking showed strong binding to AChE (-8.4 kcal/mol, vs. -8.1 for Donepezil), BuChE and muscarinic receptors. *In silico* ADME and toxicity looked favorable, and acute toxicity testing showed no mortality or abnormal behavior or body weight in the mice.

Taken together, these findings suggest that INCA-6 improves memory and cognitive function in scopolamine-induced memory deficit by modulating cholinergic dysfunction and inhibiting neuroinflammatory signaling pathways. Therefore, INCA-6 may serve as a promising therapeutic candidate for the management of memory impairment and neurodegenerative disorders due to its dual neuroprotective and anti-inflammatory properties.

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# Abbreviations

|                              |                                                         |
|------------------------------|---------------------------------------------------------|
| <b>A<math>\beta</math></b>   | Amyloid-beta                                            |
| <b>A<math>\beta</math>40</b> | Amyloid-beta 40                                         |
| <b>A<math>\beta</math>42</b> | Amyloid-beta 42                                         |
| <b>Ach</b>                   | Acetylcholine                                           |
| <b>AChE</b>                  | Acetylcholinesterase                                    |
| <b>AD</b>                    | Alzheimer's Disease                                     |
| <b>ANOVA</b>                 | Analysis of Variance                                    |
| <b>APP</b>                   | Amyloid Precursor Protein                               |
| <b>BACE</b>                  | $\beta$ -Site Amyloid Precursor Protein Cleaving Enzyme |
| <b>BBB</b>                   | Blood-Brain Barrier                                     |
| <b>BSA</b>                   | Bovine Serum Albumin                                    |
| <b>CBC</b>                   | Complete Blood Count                                    |
| <b>CDCP</b>                  | Centers for Disease Control and Prevention              |
| <b>ChAT</b>                  | Choline Acetyltransferase                               |
| <b>CNS</b>                   | Central Nervous System                                  |
| <b>CRP</b>                   | C-Reactive Protein                                      |
| <b>CSF</b>                   | Cerebrospinal Fluid                                     |
| <b>CSF1R</b>                 | Colony-Stimulating Factor 1 Receptor                    |
| <b>CUST</b>                  | Capital University of Science and Technology            |
| <b>DAB</b>                   | 3,3'-Diaminobenzidine                                   |
| <b>DMSO</b>                  | Dimethyl Sulfoxide                                      |
| <b>DNA</b>                   | Deoxyribonucleic Acid                                   |
| <b>ELISA</b>                 | Enzyme-Linked Immunosorbent Assay                       |
| <b>EOAD</b>                  | Early-Onset Alzheimer's Disease                         |

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|                                |                                                            |
|--------------------------------|------------------------------------------------------------|
| <b>FDA</b>                     | Food and Drug Administration                               |
| <b>GSK-3</b>                   | Glycogen Synthase Kinase-3                                 |
| <b>H&amp;E</b>                 | Hematoxylin and Eosin                                      |
| <b>HRP</b>                     | Horseradish Peroxidase                                     |
| <b>IFN-<math>\gamma</math></b> | Interferon-gamma                                           |
| <b>IL</b>                      | Interleukin                                                |
| <b>INCA-6</b>                  | Inhibitor of Calcineurin–NFAT Association-6                |
| <b>LTD</b>                     | Long-Term Depression                                       |
| <b>LTP</b>                     | Long-Term Potentiation                                     |
| <b>MAO</b>                     | Monoamine Oxidase                                          |
| <b>MBT</b>                     | Multitarget-Based Therapeutics                             |
| <b>MCP-1</b>                   | Monocyte Chemoattractant Protein-1                         |
| <b>MRP</b>                     | Muscle Re-education Program                                |
| <b>MWM</b>                     | Morris Water Maze                                          |
| <b>NADPH</b>                   | Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form) |
| <b>NSAIDs</b>                  | Non-Steroidal Anti-Inflammatory Drugs                      |
| <b>NFAT</b>                    | Nuclear Factor of Activated T Cells                        |
| <b>NFT</b>                     | Neurofibrillary Tangle                                     |
| <b>NIH</b>                     | National Institutes of Health                              |
| <b>NMDA</b>                    | N-Methyl-D-Aspartate                                       |
| <b>OD</b>                      | Optical Density                                            |
| <b>PBS</b>                     | Phosphate-Buffered Saline                                  |
| <b>PSEN</b>                    | Presenilin                                                 |
| <b>ROS</b>                     | Reactive Oxygen Species                                    |
| <b>Rpm</b>                     | Revolutions Per Minute                                     |
| <b>SEM</b>                     | Standard Error of the Mean                                 |
| <b>TKI</b>                     | Tyrosine Kinase Inhibitor                                  |
| <b>TLR</b>                     | Toll-Like Receptor                                         |
| <b>TNF-<math>\alpha</math></b> | Tumor Necrosis Factor-alpha                                |
| <b>TNFR1</b>                   | Tumor Necrosis Factor Receptor 1                           |
| <b>USP</b>                     | United States Pharmacopeia                                 |
| <b>WHO</b>                     | World Health Organization                                  |

# Chapter 1

## Introduction

### 1.1 Background

Conditions of the nervous system fall under the label of neurological disorders; this field encompasses a variety of conditions, such as neurodevelopmental ones [1]. Some of the commonly found symptoms of neurodegeneration include synapse impairment, interruption of neural circuitry, and buildup of abnormal protein aggregates in the brain. Neurological disorders comprise various conditions, yet notable disorders that are associated with neurodegenerative processes are prion diseases, Huntington's disease, motor neuron disease, and amyotrophic lateral sclerosis (ALS) [2]. Among them, Alzheimer's disease (AD) is the most prevalent form of dementia, accounting for 60–80% of cases globally, and is marked by progressive cognitive decline, memory loss, and behavioral changes [3]. The development of AD is a complicated process since many different factors play a vital role in this process, comprising molecular, genetic, and environmental factors. The main characteristics of AD are amyloid-beta plaques and tau tangles whose accumulation leads to interruption of neuronal communication and death of neurones. However, the new research emphasizes the importance of neuroinflammation in the development of AD. The role of activated microglia and astrocytes, important to the release of pro-inflammatory cytokines such as  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$ , is particularly

emphasized as these substances lead to the synaptic dysfunction, neuronal injury, and cognitive impairment [4, 5].

Genetic aspects such as mutations of APP, PSEN1 and PSEN2 can put someone at risk of having early-onset AD and, on the other hand, lifestyle factors like nutrition, exercise, and mental activity are responsible for modifying the operation of inflammation pathways and risk of the illness itself. Modern approaches are based on stopping symptoms but don't stop the disease itself. Cholinesterase inhibitors (donepezil, rivastigmine) and memantine (NMDA-receptor antagonist) have a small optimistic result on cognition but they are not aimed at the core neuroinflammatory processes. The new drugs aducanumab and lecanemab are made to help eliminate amyloid beta but their effect is not very reliable and they can provoke some side effects like amyloid-related imaging abnormality (ARIA) [6]. Similarly, BACE inhibitors targeting beta-secretase have faced setbacks due to safety concerns and limited efficacy [3, 6].

The limitations of current treatments highlight the need for treatments that talk about the inflammatory components of AD. Modulating neuroinflammation may offer a promising avenue for decelerating disease development and improving cognitive outcomes in affected individuals [7, 8].

INCA-6's efficacy in treating memory deficit may be ascribed to its capacity-6 inhibits the transcription of pro-inflammatory to suppress the calcineurin/NFAT signaling pathway. INCA seem minor at the start; they can affect the day-to-day lives of people with dementia.

Dementia occur when the brain is injured by diseases or a succession of strokes. Many diseases can results in dementia, with Alzheimer's being the prevalent. Other than the cause, dementia can be scary, which makes it problematic to discover the appropriate help [9]. Cytokines including IL-1 $\beta$  and TNF- $\alpha$  by targeting calcineurin and preventing the dephosphorylation of NFAT, impeding its translocation to the nucleus. In the context of memory deficit, the primary factors contributing to nerve injury and pain hypersensitivity are inflammation and oxidative stress. The Quinone configuration of INCA-6 enables it to regulate oxidative

stress, potentially contributing to the reduction of neuroinflammation. Through its dual method of redox modulation and calcineurin inhibition, INCA-6 has great potential as a candidate for memory deficit.

The distinctive structural characteristics of INCA-6 enable it to offer therapeutic alleviation by specifically addressing the inflammatory and oxidative stress pathways that are included in the onset and continuation of AD.

## 1.2 Memory and Memory Loss

The ability to remember things is called memory and it is vital for the existence of human, this enable the individuals to connect their past with the present. Memory also ensures the permanency of the knowledge gained in the past. Memory is typically divided into four types: sensory, short-term (STM), long-term (LTM), and working (WM). Short-term memory is a temporary memory that recalls information that is needed for cognitive tasks. Whereas the long-term memory stores the knowledge acquired in the past and experiences of life till the present.

The long-term memory has two types, i.e., declarative and non-declarative memory. The declarative memory include the episodic memory and semantic memory. This type of memory is a conscious type of memory storing facts and information. It can be voiced. Whereas the non-declarative memory includes the procedural memory. This memory operates unconsciously and retrieve skills and habits to perform a task. It is understood in both ways i.e. encoding and retrieval [10].

Dementia is a general term that is characterized by symptoms that include loss of memory, changes in mood, problem-solving, complications with thinking, and language. The condition may seem to be not problematic in start but it can disturb one's daily life. The cause for dementia can be a disease that have potential to cause damage to the brain or the multiple acts of stroke (cerebrovascular accidents). There are lots of condition due to which dementia is caused, out of which AD is the most common condition causing dementia specially in old aged people [9].

### 1.2.1 Prevalence and Global Burden

The WHO says that if proper preventive measures would not be taken to reduce the prevalence of the dementia the number of individuals affected by dementia caused due to Alzheimer's would be 11 crores 50 lakh persons globally by 2050. Today approximately 69 lakh people in US who are more the 65 years of age are suffering from AD. In 2060, it is expected that this count could reach approximately 13.8 million, if the preventive measure according to their medical history are not taken to prevent the worsening of condition [11].

According to CDCP statistics, In 2022, Alzheimer's is the 7th chief reason of mortality in the US, after COVID-19 which was the 4th chief cause of death. Before the COVID-19 epidemic, Alzheimer's was the 6th predominant reason of mortality. On the bases of the onset AD is of two types out of which, Late-onset Alzheimer's typically occurs in later age of 65 years. Whereas, Early-onset Alzheimer's occurs earlier in age of 65 years. It is relatively uncommon, accounting for approximately 5% of all Alzheimer's disease cases [12].

## 1.3 Alzheimer's Disease

AD is a type of neurodegenerative ailment that advances gradually over time. The occurrence of Alzheimer's disease was identified first by Alois Alzheimer in the year 1906 [13]. The AD is considered as the most usual cause of trouble in memory and thinking among the patients of dementia [14].

Histologically, the AD is defined by two unique features; first one is the neurofibrillary tangles (NFTs) formation which is believed to be caused due to deposition of Tau protein inside the neuronal tissues. These feature involves aggregation of Amyloid beta ( $A\beta$ ) deposits in the fibers which occurs in the extra cellular space [15].

The most seen manifestation of AD is the deterioration of short-term memory, which later leads to the loss of both types of memory in terms of different functions [16]. The disease also has a negative impact on the cognitive processes, such

as the process of solving problems and the ability to reason abstractly. The ability to perform more than one task at a time, as well as the ability to carry out cognitive operations, is impaired due to the dysfunctions. In the beginning, the impact of the disease can be observed in daily activities, such as driving, eating, balancing an account, and other operations [17].

### 1.3.1 Etiology of Alzheimer's Diseases

AD has no definite etiology. The two potential factors that could be behind the ailment are genetic and non-genetic causes. Various researches on the epidemiology of AD stated that genetic and non-genetic factors could be the factor that would determine the form of AD a patient would develop based i.e. early-onset AD and late-onset AD. Studies conducted on the genes show that the mutations in PSEN1 and PSEN2 genes and APP gene are passed down from generation to generation based on Mendelian inheritance, however extensive studies have found other genes that might be responsible for late-onset AD.

In terms of genetics involved in the disease have been inherited, however non-genetic factors like jobs that expose individuals to harmful products like pesticides, electromagnetic fields and organic solvents could also be responsible for AD [18].

Identifying a risk factor in the early stages could be helpful not only in preventing and treating the disease but also in preventing the condition from progressing. However, further research needs to be done to find more Alzheimer's risk factors, as well as to study the relationship between genetic and non-genetic factors, so that effective treatment can be ensured and prevention established. Furthermore, it is important to organize awareness campaigns aimed at educating people about how to address non-genetic risk factors, as these factors can put society at risk of getting AD [19].

### 1.3.2 Prevalence of Alzheimer's and Gender Distribution

The studies have shown that female genders are more prone to have Alzheimer's than male, about two-thirds of AD patients are women. In general population

1 in 5 women are affected by AD whereas 1 in 10 men are diagnosed with AD. Alzheimer's was first diagnosed in a female in 1901 [20].

### 1.3.3 Types of Alzheimer's Disease

There are two forms of AD known till now which are as follows:

Familial type follows an autosomal dominant inheritance pattern. The person exhibits early onset of the disease (EOAD) before the age of 65. This form is present in about one to five percent of sufferers and is defined by impairment in certain genes. Approximately seventy percent of familial Alzheimer's cases show mutations in presenilin-1 (PSEN1, 14q24.2). Less frequent are mutations in the presenilin-2 gene (PSEN2, 1q42.13) and in the amyloid precursor protein gene (APP, 21q21.3) [20].

Sporadic (late-onset) Alzheimer's, in turn, is caused by the affected person's age—they have to be over sixty-five years old. Age determines the risk of developing the condition, but there are several other factors that may contribute to the illness. Female gender, traumatic brain injury, environmental pollution, lack of physical activity, inadequate social interaction, depression and low academic level may serve as possible risk factors [21].

### 1.3.4 General Pathophysiology

The major and important neuropathological symbols of Alzheimer's are the aggregation of amyloid- $\beta$  ( $A\beta$ ) protein in parenchyma of brain and vasculature of cerebrum, this is accompanied by intraneuronal neurofibrillary tangles and loss of neuronal connections (synapsis). The factors which are involved in the progression of the disease and triggering factors are still unclear till day irrespective of multiple studies done to find these factors. Alzheimer's have a complex molecular mechanism due to which the pathophysiology of it have not been fully understood. Multiple pathways or factors are identified which are affected by Alzheimer's, these pathway includes, vascular abnormalities, damaging changes to mitochondrial functions, oxidative stress, reduction in glucose utilization by brain, and

neuroinflammation are considered to be the culprit of triggering and worsening of Alzheimer's disease [21].

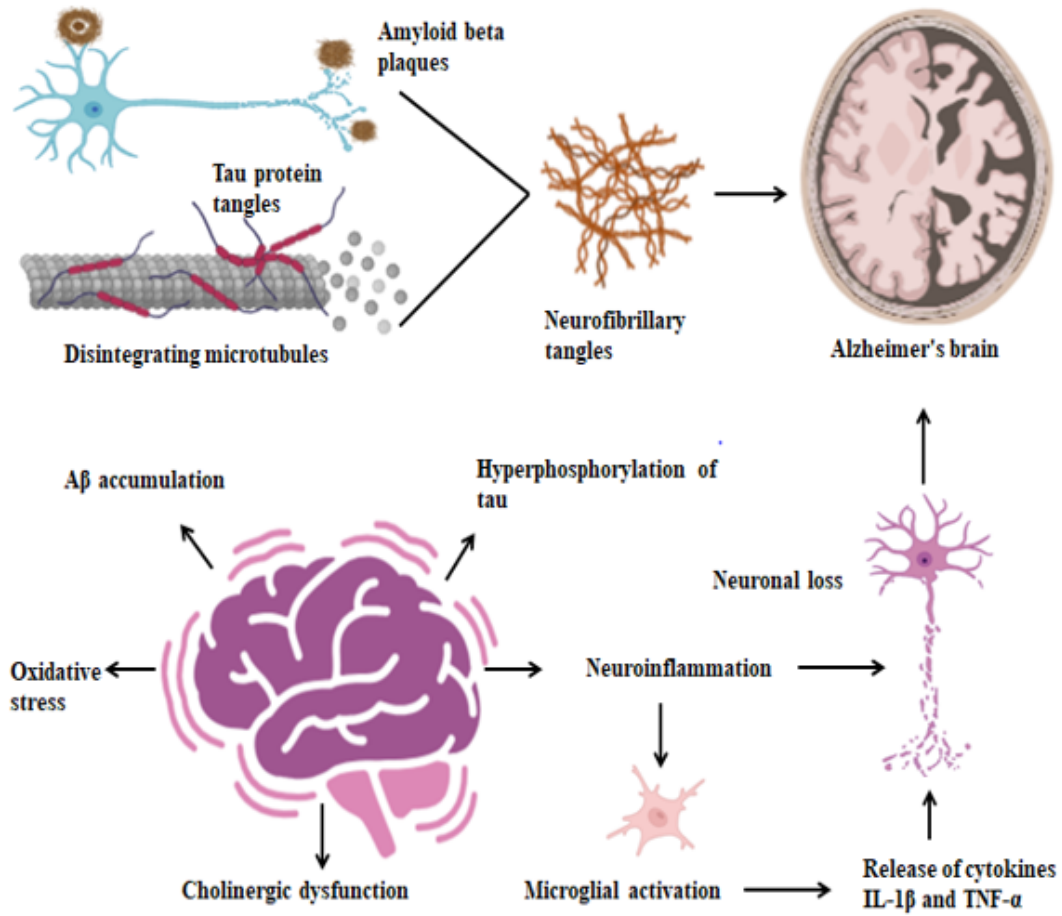


FIGURE 1.1: Pathophysiology of Alzheimer's

### 1.3.5 Neurotransmitter and Cholinergic Hypothesis

The cholinergic neurons secrete a neurotransmitter named as acetylcholine which is involved in signal transmission which is necessary for storage of memory and ability to learn in AD. In 1980 it was proposed that decline in the levels of acetylcholine (ACh) in the neural pathways of the hippocampus and cortex is responsible for causes decline in cognitive processes in AD [22].

The cholinergic hypothesis was the first ever theory which was proposed for the pathogenesis of the AD. As it was known that the atrophy of cholinergic pathways and decline in cognitive processes are every evident in age related neurodegeneration, as it occurs in AD too. Moreover the abnormal changes in central cholinergic

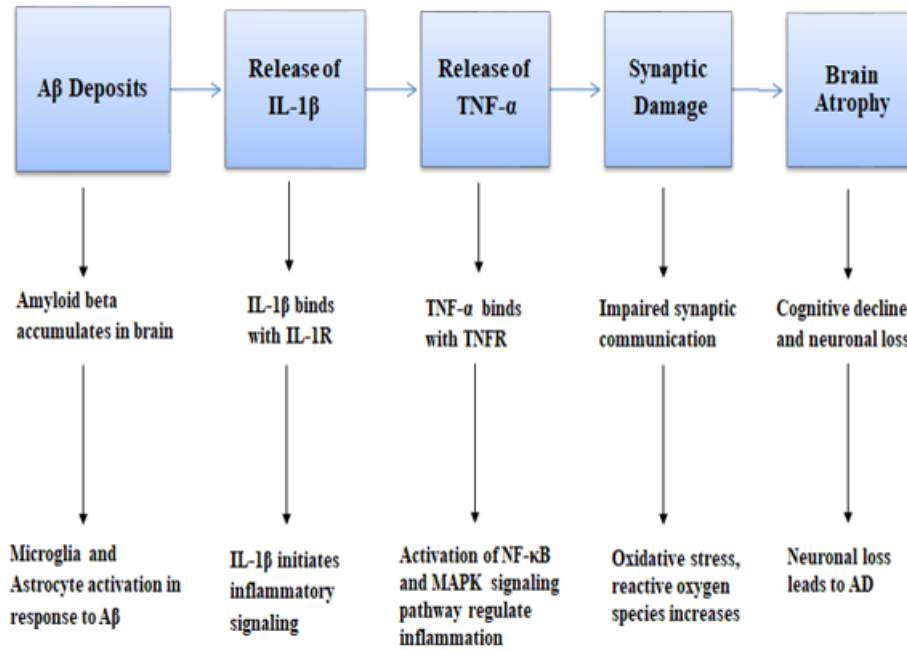


FIGURE 1.2: Signaling pathways of AD

pathways can also lead to accumulation of unusual phosphorylation of tau protein, inflammation of nerve cell, cell apoptosis, and other pathological phenomena, but the exact mechanism of action is still unclear [23].

### 1.3.6 Hypothesis of Pathophysiological Processes

Many hypotheses for pathophysiological processes of AD. As mentioned earlier that pathological processes in AD are assumed to be pathways that are Amyloid- $\beta$  pathway, Tau protein pathway and Neuroinflammatory pathway, which are found to be affected in the course of the disease, these involve several the cholinergic, glutamatergic, amyloid, tau protein, inflammatory, microbiota-gut-brain axis, oxidative stress, metal ion, and abnormal autophagy. Hypotheses on each pathway have been explain below that how they are involved in the initiation and deterioration of the disease.

- i. The cholinergic hypothesis states that the loss of cholinergic neurons is a central aspect of the AD pathology and correlates with cognitive dysfunctions. According to the hypothesis, the decline in synthesis and secretion of acetylcholine leads to impairment of its functions needed for learning and

memory. The reduction of choline acetyltransferase (ChAT) activity and the damaging influence of amyloid beta ( $A\beta$ ) on cholinergic signaling results in reduced quantity of acetylcholine. Thus, the decrease of acetylcholine levels causes cognitive impairments in AD patients. To target deficits noticed in AD patients, cholinesterase inhibitors—donepezil, rivastigmine, and galantamine—were developed [22, 23].

- ii. The glutamatergic hypothesis says that abnormal regulations of glutamate which is the main excitatory neurotransmitter in the central nervous system, can play an important role in the pathogenesis of Alzheimer's disease. In Alzheimer's disease, NMDA-receptors are over stimulated, which is responsible for excessive influx of sodium and calcium ions into neurons. This overstimulation can cause swelling of neurons. It also start various calcium-dependent processes which includes the production of reactive oxygen species (ROS), disturbance in mitochondrial function, and moreover it leads to the activation of necrotic or apoptotic pathways, which end in resulting in excitotoxic damage to neurons [24].
- iii. According to the amyloid hypothesis, the accumulation of  $A\beta$  plaques in the brain is a major event in the pathogenesis of Alzheimer's disease which lead to neurodegeneration and declination of cognitive processes. A sequential cleavage is carried out by  $\beta$ -secretase and  $\gamma$ -secretase on amyloid precursor protein (APP) from where the  $A\beta$  is originated, various lengths of  $A\beta$  fragments are made in process. Out of these fragments  $A\beta_{40}$  and  $A\beta_{42}$  are predominant. The hydrophobic C-terminus of  $A\beta_{42}$  is responsible of its aggregation and the formation of senile plaques. The accumulation of  $A\beta$  can be promoted by the mutations in presenilin-1 (PS1), it increases its production and impairs autophagy functions. The relationship between  $A\beta$  levels and FAD mutations is complex. The plaque formation is influenced by a dynamic imbalance between  $A\beta$  production and clearance mechanisms, this occur in sporadic Alzheimer's disease [25].
- iv. The tau protein hypothesis says that the hyperphosphorylation of tau protein can lead to its detachment from microtubules, which results in the formation of neurofibrillary tangles (NFTs) which are a hallmark of Alzheimer's

disease. Normally, the tau protein stabilizes microtubules, but under pathological conditions there is the imbalance of kinases and phosphatases which causes tau to be hyperphosphorylated. This hyperphosphorylation lead the tau protein to aggregate to form paired helical filaments and NFTs, which associate strongly with clinical symptoms of AD. Moreover, the pathology of tau protein is also associated with neuroinflammation. The transcription of tau is increased by pro-inflammatory cytokines, leading to activate inflammatory pathways that further aggravate tau-related neurodegeneration [25].

- v. According to the inflammatory hypothesis, chronic neuroinflammation plays a important role in the pathogenesis of Alzheimer's disease. This hypothesis postulates that various pathological factors, like  $A\beta$  plaques, pro - inflammatory cytokines, and oxidative stress, lead to the activation of microglia, the central nervous system's innate immune cells. After activation, morphological changes occur in microglia and start a range of inflammatory reactions, which can lead to the release of pro-inflammatory mediators that worsen the neurotoxicity. The tenacious activation of microglia results in a failure to reduce inflammation, which is responsible of neuronal damage and the decline cognitive function associated with AD [26].
- vi. The microbiota-gut-brain axis hypothesis proposes a two-way communication system between the gut and the brain which involves metabolic, neural, endocrine, and immune pathways. The factors like dietary habits, use of antibiotic, psychosocial stress, or immune system irregularities have potential to cause disruption of the composition and functionality microbiota this lead to a compromised intestinal epithelial barrier. Due to this the harmful substances and microorganisms enter the bloodstream, eliciting systemic inflammation. This inflammation can cause the inflammatory mediators to cross the blood-brain barrier (BBB) which affect microglia. This process lead to the worsening of the neuroinflammation, which in the end take part in neuronal degeneration and damage [27].
- vii. The oxidative stress hypothesis postulates that oxidative stress plays an important role in the pathogenesis of Alzheimer's disease. It suggests that any imbalance between the production of reactive oxygen species (ROS)

and the ability of body to detoxify these dangerous compounds can lead to damage to the component of the neuronal membrane like; lipids, proteins, and nucleic acids, which cause the neuronal cell death. The mitochondrial dysfunction is key contributors to oxidative stress in Alzheimer's disease. This includes the reduced the activities of essential enzymes and disrupts the dynamics of the mitochondrion. This oxidative stress is interrelated with other pathological processes in Alzheimer's disease, for example the modulation of APP processing and tau phosphorylation, which leads to the formation of neurofibrillary tangles. Moreover, if free metals and complexes of  $A\beta$  are present, they can catalyze ROS production, which than cause further worsening neuronal damage [28, 29].

- viii. According to the metal ion hypothesis, the abnormal regulation of metal ions, mainly copper, zinc, and iron, which have an important role in the pathogenesis of Alzheimer's disease. This hypothesis proposes that any disturbance in the physiological homeostasis of these metals can cause oxidative stress which is associated to various pathological events in Alzheimer's disease, for example amyloidosis and tauopathy. Iron related oxidative stress can result in the release of iron from iron-containing proteins which may lead to neuronal death through mechanisms like Ferro ptosis and lipid peroxidation. In the same way, copper can prompt toxicity by binding to specific proteins which result in cellular damage. Zinc deficiency is also emphasized as a factor that may contribute to excitotoxicity of glutamate and synaptic dysfunction in Alzheimer's disease. In conclusion, the accumulation or any mismanagement of metal ions are strictly related with the neurodegenerative processes detected in Alzheimer's disease [30].
- ix. The abnormal autophagy concept states that disruption of autophagy may cause the development of Alzheimer's disease. The process of autophagy plays an important role in the maintenance of cellular homeostasis due to the degrading of protein aggregates and damaged cellular organelles. The abnormality of autophagy can lead to the accumulation of amyloid protein and amyloidosis in Alzheimer's disease. The functioning of protein homeostasis can be hampered because of the abnormalities in the autophagy process.

The abnormalities may occur because of genetically determined problems of expression of proteins and availability of vesicles [16].

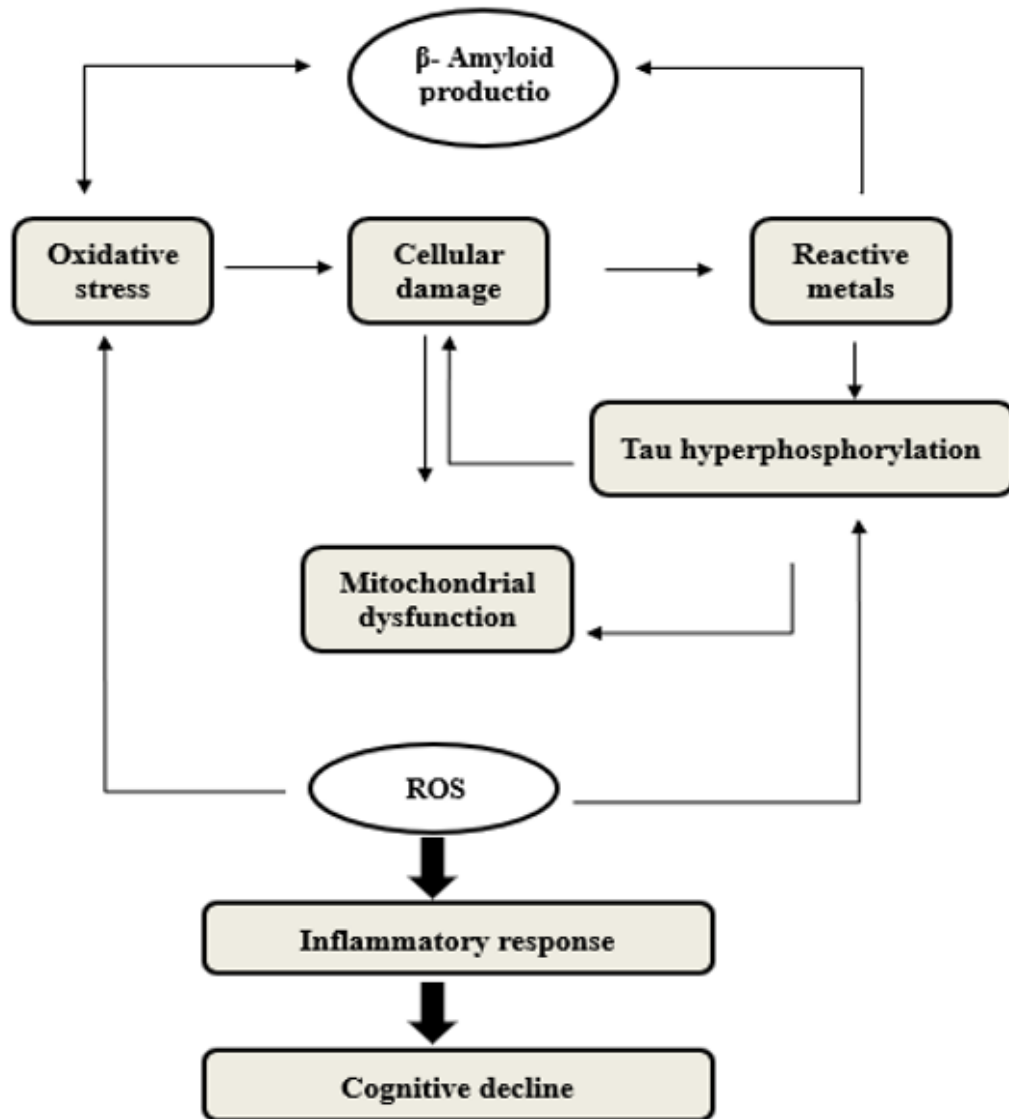


FIGURE 1.3: Worsening of Alzheimer's due to oxidative stress.

### 1.3.7 Symptoms and Stages of Alzheimer's Disease

The clinical course of is divided in multiple phases on the basis of degree of symptom's severity and the causing factor of the disease. AD is presented with behavioral abnormalities, decline in cognitive abilities, and impairment of memory as early symptoms in AD patients [31]. Initially, the preclinical phase occurs, which can last over a few years, and during this phase, symptoms are very minute. This

phase can be identified by memory lapses and minimal cognitive gain, while the person does not suffer from any problem in functionally executing his/her duties. Pathologists identify this stage through aggregation of amyloid plaques and NFTs.

As the disease moves into the early symptomatic or mild phase, the person starts having symptoms that limit his/her daily functioning. Despite the fact that there is still no full scale impairment of functions, some disturbances may appear in memory, attention span, etc [32].

As the condition moves into the moderate phase, pathological alterations involve wider regions of the cerebral cortex, leading to more serious impairments related to cognition and functional abilities. At this moment the patient suffers from severe memory dysfunction, having problems in recognizing various well-known individuals; for example, he/she may not recognize even immediate family members and old friends.

Also, patients suffer from several problems in language development such as reading, writing, and speaking. Patients are prone to irritability, emotional instability, and inability to make proper judgments [33].

At the last stage of Alzheimer's disease called severe stage, the cortex of the brain is seriously damaged by a vast number of neuritic plaques that contribute to the appearance of NFTs. Patients in this phase experience significant cognitive decline. They cannot recognize even the closest relatives of theirs (for instance, mother, father, siblings, friends) and cannot communicate with other people.

Patients at this stage are unable to walk, eat food, or take care of themselves in any way. Complications that may develop at this stage include dysphagia, incontinence, immobility, etc., which can threaten the life of the person at this stage [34].

### 1.3.8 Regions of Brain Affected

Following areas of the brain that are affected in Alzheimer's as the disease progresses [35].

#### **1.3.8.1 Hippocampus**

The hippocampus is the first region to be affected in Alzheimer's. It is responsible for making new memories and spatial navigation. Any damage to the hippocampus can lead to difficulty in recalling new events [35].

#### **1.3.8.2 Entorhinal Cortex**

Entorhinal cortex is a gateway from the hippocampus to the neocortex. Any disturbance in the function of the entorhinal cortex disturbed the signals that is needed for memory storage [35].

#### **1.3.8.3 Temporal Lobes**

The temporal lobes of brain are liable for language comprehension and are involved in storage of memory. Any damage or disturbance in the temporal lobe's function can lead to anomia aphasia which is characterized by difficulty in finding words, understanding speech, and problems in naming things [35].

#### **1.3.8.4 Parietal Lobes**

The parietal lobes of the brain are responsible for spatial awareness and is also involved in navigation. When the parietal lobes are affected in the course of AD, the patients find difficulty in locating places and objects. They often get lost or misjudge distances [35].

#### **1.3.8.5 Frontal Lobes**

The frontal lobes of the brain are very important in decision-making, making judgements, and in developing personality. It is affected in later stages of Alzheimer's disease. The diseased frontal lobe causes poor reasoning, impulsivity in the patient. It also changes their social behavior [35].

#### 1.3.8.6 Cerebral Cortex

AD spreads throughout the cortex in the advance stage. Cortex gets thin and shrinks, which leads to global cognitive decline. The shrinking of the cortex is the reason of loss of long term memories and overall loss of intellectual abilities of the patients [35].

#### 1.3.9 How Alzheimer's Disease Affects Brain and its Treatment

AD disrupts the brain areas like hippocampus and entorhinal cortex along with neocortex as all these areas have a central role in cognitive performance and the storage of memory. Hippocampus is present bilaterally in medial temporal lobe and shows an important part in converting short-term into long-term memories and by facilitating longitudinal knowledge [13].

In AD, the hippocampus is the first affected brain region as it experiences progressive neuronal degeneration as well as atrophy. This degeneration is attributed to the buildup of plaques of amyloid-beta ( $A\beta$ ) and NFTs consisting of hyperphosphorylated tau protein. These two irrational developments are greatly intervened in hippocampal region and their excessive accumulation causes interruption in signaling between neurons, alteration of normal mitochondrial functions, oxidative stress and neuroinflammation. This means that memory loss and decline of cognitive functions represent only a part of the clinical manifestations of AD [34].

A modest degree of shrinkage occurs in brain during normal aging; but, there is no significant loss of neuronal is typically detected. By contrast, in Alzheimer's disease, in which widespread neurodegeneration occurs. In this condition, many neurons become damaged, they also lose their connections, then ultimately expire. The ailment limits with crucial neuronal functions, including energy metabolism, communication, and restoration of cellular mechanisms [36]. Strategies targeting tau are aimed at preventing aggregation of the tau protein. Combination therapies incorporating multiple approaches have been used to treat the symptoms and

pathology of Alzheimer disease and offer synergistic benefits for treatment. These drugs increase the level of ACh in the brain. but they have severe adverse effects like hepatotoxicity which limit their effectiveness [37].

Monoclonal antibodies an advanced treatments which shows promising effects but also face challenges which may include great costs and they also need more extensive clinical validation for their effects [38].

These results show possible opportunities for preventative interventions that may lessen the danger of decline in cognition related Alzheimer's disease, and may have comprehensive suggestions for neurodegenerative diseases in general. The main problem in the pitch is the introduction of improvement of timely revealing methods of the disease in the pre-clinical stage [39].

A major challenge for the development of practical therapeutics for Alzheimer's disease is the efficient delivery of therapeutic drugs and their transport to the targeted brain area. This is mainly due to the protective barriers of central nervous system (CNS).

The main challenges are the brain barrier which is an extremely careful and firmly controlled boundary that protects the brain from destructive materials such as neurotoxins, circulating chemicals and disease agents. The barrier plays a very important role in maintaining CNS homeostasis, but its selectivity also represents a main restriction, limiting the entry of many therapeutic particles of interest, thus limiting the effectiveness of drug distribution to the target neural tissue [40].

The current therapeutic modalities are insufficient because they provide only symptomatic relief which is for a limited duration and they are not efficient in stopping the progression of disease. Researches are successively being directed to find the effective cholinesterase inhibitors that are proficient of improving the action of acetylcholine, anti-amyloid drugs, antioxidant drugs, and anti-inflammatory treatment. The direction of new researches for disease-modifying treatments are the complications of timely analysis, effective drug distribution through the BBB, and the multi-factorial etiology of Alzheimer's disease pathology.

The limitations of the recent treatments and the increasing prevalence of Alzheimer's disease continues, there is a serious need to determine new beneficial drugs which can target the cause that is responsible for pathogenesis of disease at early stages of Alzheimer's disease.

## 1.4 Scopolamine-Induced Memory Deficit Model

### 1.4.1 Mechanism of Muscarinic Receptor Blockade

It is the most commonly used experimental paradigm in neuropharmacological studies of cognitive impairment and potential medicines. Scopolamine is a non-specific muscarinic acetylcholine receptor antagonist and it readily penetrates the blood–brain barrier. Scopolamine binds to muscarinic receptors in the hippocampus, cortex and other cholinergic-rich brain areas and blocks acetylcholine transmission effectively. The cholinergic system includes many brain regions such as the cholinergic neurons in basal forebrain projecting to the hippocampus and neocortex. These cholinergic neurons are involved in attention, encoding and retrieval of memory. When scopolamine damages this system, marked deficits of spatial memory, working memory and associative learning are induced in animals. The mechanism of scopolamine-induced amnesia is highly conserved with the cholinergic hypothesis of AD, which suggests that degeneration of cholinergic neurons and decrease of acetylcholine levels are major pathogenic factors for cognitive impairment. Therefore, scopolamine-induced amnesia can be used as a pharmacological model to mimic Alzheimer's pathology *in vivo*. Scopolamine does not induce neurodegeneration *per se*, but induces a pharmacological blockade of cholinergic transmission in the brain. Therefore, scopolamine is an ideal *in vivo* model for screening medicines that can enhance cholinergic signaling or prevent cholinergic blockage [22].

A scholar named Chen concluded a study in 2022, in his study he worked to see the interaction of cholinergic signaling with amyloid and tau pathology at an early

stage of Alzheimer's-like diseases, to know how they are related to cause dysfunction. *Frontiers in Aging Neuroscience* published an animal study in 2023 which discussed about how scopolamine damages hippocampal long-term potentiation (LTP) which is the cellular basis of learning due to which it is closing involved in human cognitive decline [22].

Different behavioral paradigms were used to characterize the scopolamine-induced cognitive deficits. Because these paradigms are associated with specific cognitive processes, such as memory and learning, different aspects of cognitive function can be assessed. Pharmacological test agents can be evaluated with regard to their efficacy.

### 1.4.2 Morris Water Maze

The most widely used paradigm to assess spatial learning and memory in rodents is Morris Water Maze. Rodents are placed into a circular pool of water and must use spatial cues to find a hidden platform. Normal rodents learn to find the platform position over successive trials and consequently, demonstrate a reduction in escape latency.

In contrast, scopolamine-treated rodents display an increase in escape latency, swim randomly within the pool and fail to remember the platform location during a probe trial. Deficits in the Morris water maze are associated with impaired hippocampal-dependent spatial memory. The MWM has been extensively validated as a sensitive measure of cholinergic dysfunction [41].

### 1.4.3 T Maze Test

The T-maze test is a simple and widely used tool to assess learning, memory, and spatial navigation in mice. It consists of a start arm and two goal arms forming a "T" shape. The spontaneous alternation method was used, where rodents were expected to choose a different arm than the one previously visited, indicating working memory. The test was conducted over multiple sessions, and correct alternations were recorded and analyzed to evaluate cognitive performance [42].

#### 1.4.4 Y-Maze

The Y-maze tests working memory based on the working memory dependent behavior spontaneous alternation. Rodents explore the arms of the Y-maze and was prefer to alternate between novel and previously visited arms of the maze. This alternation behavior is dependent on working memory and executive function. Scopolamine hormone therapy results in a large decrease in the percentage of alternations made. The Y-maze is recommended for detecting subtle deficits and for drug screening that focuses on effects on short-term memory. *Behavioral Brain Research* 2023. In press. Scopolamine treated mice have low alternation scores and cholinesterase inhibitors reverse scopolamine effect [43].

The scopolamine-induced memory deficit model has several strengths that make it a valuable tool in Alzheimer's research. First, it provides a rapid and reproducible induction of cognitive deficits, allowing researchers to evaluate drug effects within hours or days. Second, the deficits are reversible, meaning that animals can recover once scopolamine is metabolized, which reduces ethical concerns and allows repeated testing.

Third, the model is well-established and widely used, with decades of research validating its reliability. Finally, scopolamine-induced deficits closely mimic the cholinergic dysfunction observed in Alzheimer's disease, making it particularly relevant for testing cholinergic enhancers [44].

However, the model also has important limitations. Unlike Alzheimer's disease, which involves progressive neurodegeneration, amyloid plaque deposition, tau tangles, and chronic inflammation, scopolamine produces only acute and transient deficits. This means that while the model is excellent for studying cholinergic dysfunction, it does not capture the full complexity of Alzheimer's pathology. Moreover, drugs that show efficacy in scopolamine models often fail in human clinical trials, highlighting the translational gap between animal models and human disease. Another limitation is that scopolamine may produce off-target effects, including sedation and motor impairment, which can confound behavioral outcomes [44].

However the cholinergic hypothesis was acceptable for pathology of Alzheimer's disease but scholar named Francis in 2022 noted that multiple pathological pathways were involved in Alzheimer's disease, like scopolamine models should be added with amyloid and tau-based models [45]. The scopolamine models are best suited for screening cholinergic enhancers this was stated by the Gurrieri in 2025, he also stated that we should not rely on it for drug development [46].

Scopolamine model irrespective of its limitations it is still very important in the pre-clinical trials. It is used for assessing the drugs which target cholinergic signaling pathway, oxidative stress and which are involved in neuroprotection. Drugs like cholinesterase inhibitors such as donepezil and rivastigmine were seen to be potent in reversing the deficits which are induced by scopolamine. Recent researches worked on the natural compound named curcumin and *ginkgo biloba*, showed protective effects on scopolamine model, these compound have a broader therapeutic spectrum [35].

Scopolamine model has the extension of its significance to new compounds named INCA-6, which is supposed to produce neuroprotective effects by modulating the cholinergic pathways and also have antioxidant properties. On researching more on this new compound on animals treated with scopolamine, it showed improvement in spatial learning, working memory, and associative learning. These evidence provided a strong evidence for cognitive enhancement in patient with complex Alzheimer's models.

## 1.5 Introduction to INCA-6

### 1.5.1 Chemical Nature of INCA-6

A small synthetic molecule named INCA-6, which is chemically known as Triptycene - 1, 4 - quinone, it is a selective inhibitor of the calcineurin–NFAT (nuclear factor of activated T-cells) signaling pathway. The chemical structure of INCA-6 is triptycene scaffold, a rigid polycyclic aromatic hydrocarbon that provides stereois

stability and this compound is also resistant to metabolic degradation. Chemical Properties of the compound include its physical appearance; the compound is yellow solid of 284.31 molecular weight.

The chemical formula is  $C_{20}H_{12}O_2$ , chemical name of (9s,10s)-9, 10-[1,2] benzenoanthracene - 13, 16(9H,10H)-dione [47]. The INCA-6 is able to interact with protein molecules by transfer of electrons and hydrogen bonding. This occurs by addition of Quinone groups which starts this redox activity.

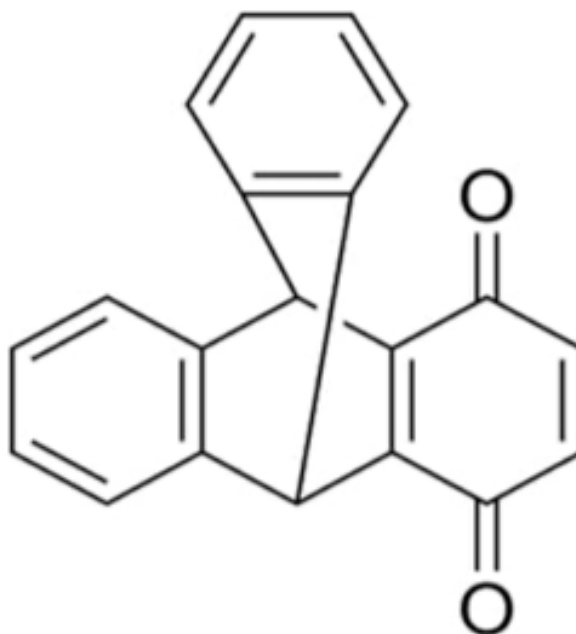


FIGURE 1.4: Structure of INCA-6

The triptycene derivatives molecule are popular for their unique 3D structure due to this it has been the part of most researches now. This three dimensional structure allows INCA-6 molecule to perfectly fit into binding sites of protein with a high specificity. This geometry of INCA-6 is misused to block the attachment site of NFAT on calcineurin.

INCA-6 not like the known calcineurin inhibitors for example; cyclosporine A and tacrolimus, which act to cause a systemic immunosuppression, it aimed to selectively inhibit the NFAT docking site without affecting other calcineurin substrates. The selectivity of this molecule is important as the calcineurin is involved in regulating multiple cellular processes which include synaptic plasticity, immune activation, and calcium signaling [48].

Pharmacologically, INCA-6 is a selective inhibitor of calcineurin–NFAT signaling. The calcineurin is a molecule which is calcium/calmodulin-dependent, serine/threonine phosphatase who dephosphorylates the NFAT. This dephosphorylates enable the translocation of nucleus and starts process of transcription. The genes like IL-2, TNF- $\alpha$ , and IFN- $\gamma$  which are involved in mediating immune responses are regulated by NFAT, they also contribute to neuronal signaling and synaptic plasticity [47, 49].

### 1.5.2 Mechanism of Action

The NFAT is a universal family name of transcription factors essential in immune response. These factors are involved in immune system response in most cells. The important factor for NFAT activation is Calcium signaling. The calcineurin (CN) which is a serine/threonine phosphatase is activated Calmodulin, which act on the amino termini of NFAT proteins by dephosphorylating them resulting a conformational change. NFAT was collaborate with the resident transcription factors because they have weak capacity to bind with DNA. INCA-6 is a selective inhibitor of transcription factor NFAT who have  $K_d$  value of 0.8 mM *in vitro* [47]. It prevents dephosphorylation and nuclear translocation by blocking NFAT docking site. This blocking reduces transcription of NFAT-dependent genes. There are two major variations of its mechanism:

- i. Immune modulation: INCA-6 work as immune modulator. For this it suppresses T-cell activation and releases cytokine. This offers a potential in autoimmune and inflammatory conditions.
- ii. Neuroprotection: The NFAT signaling pathway is involved in synaptic dysfunction and neurodegeneration. If it inhibits NFAT signaling pathway, it may protect neurons from toxicity caused due to calcium and prevent damage due to inflammation.

### 1.5.3 Pharmacological Profile

INCA-6 shows following pharmacological effects:

- i. It reduces NFAT-dependent transcription *in vitro* [49].
- ii. Its tolerability has been demonstrated in animal models of neuropathic pain [50].
- iii. It also exhibits a potential neuroprotective effects by stabilizing calcium signaling.

INCA-6 has greater selectivity towards NFAT docking and shows fewer systemic side effects as compared to cyclosporine A and tacrolimus. It acts as a classical calcineurin inhibitors which comprehensively block all calcineurin substrates which lead to extensive immunosuppression and toxicity. INCA-6 is also known to preserve other calcineurin functions other than NFAT docking. This special feature makes it more highlighted and good candidate to be used as a treatment for neurological condition in which large immune suppression is not require [50].

Safiri, a scholar published her article in *Frontiers in Medicine* journal in 2024. In this article they have discussed the role of neuroinflammation in Alzheimer's disease. This research has worked to treat the condition by targeting the NFAT signaling. INCA-6 has the same therapeutic effects which makes it more relevant and potential treatment for cognitive disorders [51].

#### 1.5.4 Hypothesized Mechanism of Action in Memory Deficit Models

Due to the role of INCA-6 in calcineurin–NFAT signaling in synaptic plasticity, its importance has been raised in memory deficits. The calcineurin plays a role in maintaining balance between strengthening and weakening of synapsis. It is also regulating long-term depression (LTD). The increased activity of calcineurin hinders the stability of synaptic connections thus impairs the long-term potentiation (LTP) which is the cellular basis of learning and memory.

The muscarinic receptors are blocked which disrupts the cholinergic signaling, impairs cellular basis of learning and memory coding in scopolamine-induced memory

deficit models. This dysfunction is a combination of impaired regulation of calcium and neuroinflammation. The selective inhibition of calcineurin–NFAT signaling by INCA-6, may respond these effects by several mechanisms:

- i. Preservation of synaptic plasticity: INCA-6 is known to stabilize the synapsis, thus involved in supporting LTP and prevent excessive LTD.
- ii. Reduction of neuroinflammation: INCA-6 is involved in inhibition of NFAT pathway, this decreases cytokine transcription and limits the damage to neurons due to inflammation.
- iii. Stabilization of calcium signaling: INCA-6 restores the calcium balance, when the calcium homeostasis is disrupted by the over activation of calcineurin.
- iv. Indirect support of cholinergic function: INCA-6 enhances the effect of acetylcholine in memory processes.

This hypothesized mechanism of INCA-6 lead to the improved cognitive performance in scopolamine models by the process of neuroprotection by modulating the calcineurin–NFAT signaling.

## 1.6 Aim and Objectives

In this investigation, we were pharmacologically explore INCA-6 in an animal model of scopolamine-induced cognitive impairment. The main objective of the study was be to assess the cognitive impairment effects due to scopolamine by using established behavioral tests including Morris water maze, Y-maze, and T-maze to measure spatial learning, working memory, and recognition memory. As an endpoint, we also determined the neuroprotective and nootropic properties of INCA-6.

# Chapter 2

## Literature Review

### 2.1 Introduction

In 1906, Alois Alzheimer first reported a neurodegenerative disease and named it after his name AD, which is described by memory impairment, reduction in cognitive task, and major neuropathological deviations in the brain. AD is a prominent reason of dementia which advances a significant and increasing public health issue in geriatric population across global. The chronic, low-grade inflammation related with aging is the phenomenon of inflammation in AD which connects with neuroinflammation. The main risk factor for AD is aging, and augmented levels of reactive oxygen and nitrogen species adds to the neuroinflammation in the course of the disease. These free radicals interrupt the process of intracellular signaling, induce cellular aging, and become reason of pyroptosis. These molecular events collectively compromise the functional and structural reliability of the Blood Brain Barrier, they also enable the immune component from peripheries to interfere with CNS and increase inflammation [52]. In 1960, Sir Peter Medawar and Sir Frank Macfarlane Burnet were first to introduce the idea of the central nervous system as an “immune-privileged”, this concept was based on the grounds that the BBB allows very selective molecules to enter the primacies of the brain from the peripheral immune system [53]. On the base of this concept in the early hypothesis for pathophysiology of neurodegenerative disease, inflammation was

not considered as a main factor in causing the condition. Researches from the past decade has viewed that the blood brain barrier is not a rigid wall but a selectively permeable, dynamic membrane. It can only be breached in case of any disease and while aging. This barrier can also be breached by the cytokines and other inflammatory mediators; these can disturb its permeability. This increase in permeability promote the immune cells to infiltrate, creating a link between peripheral inflammation and CNS degeneration [54].

This increasing alertness is chiefly vital in the case of Alzheimer's disease where neuroinflammation is being recognized as a main part of pathogenesis with acknowledgment. Inflammation in Alzheimer's is not because of neuronal loss but act as a precursor of the pathogenesis and a promote the pathological cascade which include the phosphorylation and deposition of tau protein, synaptic impairment, and loss of neurons. The microglia cells which are brain's resident immune cell, they have two major roles. Out of which one is to eliminate deposits of  $A\beta$  and reestablish it homeostasis, but constant initiation can consequences in the discharge of pro-inflammatory cytokines and neurotoxic products that can promote the process of neurodegeneration [55].

The diminished function of Blood Brain Barrier occur mainly in hippocampus no matter it is in aging or in AD, this permits deposition of other complement components with tau tangles as plaques [56]. The inhibition of C3aR1 was found to be effective in decreasing the activation of microglial cell and it is also good at normalizing the cortical and hippocampal volume. It shows the potential to modulate the complement signaling pathways in order to reduce neuroinflammation and neurodegeneration [36].

However, the part of inflammation is evident in case of AD, still the anti - inflammatory therapeutic effects of non-steroidal anti-inflammatory drugs (NSAIDs) in order to reduce the inflammation were not favorable. The evidence from epidemiological studies suggests the long-term use of NSAID as a drug lowering risk for AD, but the clinical trials on the symptomatic patients were unsuccessful to prove its efficiency in this matter. The reason for the failure is due the multiple factors, such as late treatment, low drug medicating routines, and inadequate

knowledge of the role of inflammation. Most of the anti-inflammatory agents act on pro-inflammatory processes, which prevent the inflammation but do not trigger anti-inflammatory or reparative mechanisms [57]. This propose that that new therapeutic approaches should focus on the reduction of specific inflammatory process rather than reducing the general inflammation.

The challenges of early detection and monitoring the neuroinflammation has equal importance as of the other factors. As the central nervous system do not have its own inflammatory biomarkers, lead to the delayed analysis and treatment of the condition. However, the biomarkers in the outer body, which include serum and plasma cytokines, do indicate the about the inflammation this is still under investigation. These biomarkers are easily accessible and function as non-invasive markers of onset of the disease and tell about its progression, particularly combined with cognitive functions and neuroimaging. Advancement in these biomarkers will be beneficial for early diagnosis of the disease and it also facilitates the distribution of the patient for their personalized immunotherapeutic interventions [58].

The key factor of AD pathology is the multifarious interaction between immune functions of the CNS and peripheral inflammatory particles. Neurodegeneration is increased due the neuroinflammation caused by disturbance in blood brain barrier and abnormal activation of microglial cell due to its abnormal interaction to complement activation and immune system infiltration. Identifying the most effective intervention periods and therapeutic targets requires a comprehensive understanding of how this process develops over time and across different regions. With ongoing advances towards treatment development, focusing on immune modulation and biomarker-based approaches has the potential to improve the effectiveness of therapeutic interventions for Alzheimer's disease [54].

Microglia and astrocytes are the primary mediators of neuroinflammation in Alzheimer's disease (AD). Microglia, the resident immune cells of the central nervous system, become activated and migrate to sites of tissue injury in response to AD-related pathological changes, where they help remove cellular debris and protein aggregates, including amyloid- $\beta$  ( $A\beta$ ). While acute microglial activation plays a protective role by facilitating  $A\beta$  clearance, persistent or chronic activation triggers

a sustained inflammatory response that contributes to neuronal damage, synaptic dysfunction, and enhanced  $A\beta$  production. This prolonged neuroinflammatory state accelerates disease progression and exacerbates cognitive decline.  $A\beta$  oligomers activate microglia through Toll-like receptors (TLRs) and inflammasome pathways. Activated microglia cells induce activation of astrocytes which also contribute to progression of Alzheimer's disease. Astrocytes play a crucial role in the neuroinflammatory response associated with Alzheimer's disease (AD). Two major types of astrocytic changes have been identified during disease progression: reactive astrocytes and atrophic astrocytes. Reactive astrocytes are commonly found surrounding amyloid- $\beta$  ( $A\beta$ ) plaques and other areas of neuronal injury, where they contribute to the inflammatory response by releasing pro-inflammatory mediators. In contrast, atrophic astrocytes appear during the early stages of AD and are characterized by the loss of their normal supportive functions. Reduced structural and metabolic support from these cells can disrupt synaptic connectivity, ultimately contributing to synaptic dysfunction and cognitive decline [59].

## 2.2 Neuroinflammation in AD

Many studies show that the anti- $A\beta$  and anti-tau strategies were not effective in case of Alzheimer's disease and were extremely expensive, these raised a need for the invention and the search of more new approaches. Although, the US FDA recently approved a drug named aducanumab for the treatment of AD. This works on the neuroinflammation which is thought to play main role in the AD. NSAIDs which are used as anti-inflammatory drugs, have shown very mixed consequences in clinical trials. Which may be influenced by their broad mechanisms and late-stage administration. Pharmacological and genetic approaches are being used to reduce the microglia activation which is thought to be the cause of the worsening the course of AD. This technique has been applied on the mouse models with the Alzheimer's disease mouse. These models have produced positive outcomes to the intervention. The CSF1R is an essential microglial surface receptor. Studies using CSF1R inhibitors in various mouse models of Alzheimer's

disease have demonstrated a reduction in neuroinflammation, neuritic plaque formation, and dendritic spine loss, while also improving cognitive function. These findings suggest that targeting CSF1R-mediated microglial activity may represent a promising therapeutic strategy for slowing disease progression [60].

## 2.3 Immune Signaling in AD

Many studies show that the anti- $A\beta$  and anti-tau strategies were not effective in case of Alzheimer's disease and were extremely expensive, these raised a need for the invention and the search of more new approaches. Although, the US FDA recently approved a drug named aducanumab for the treatment of AD. This works on the neuroinflammation which is thought to play main role in the AD. NSAIDs which are used as an old anti-inflammatory drugs, have shown very mixed consequences in clinical trials. Which may be influenced by their broad mechanisms and late-stage administration. Pharmacological and genetic approaches are being used to reduce the microglia activation which is thought to be the cause of the worsening the course of AD. This technique has been applied on the mouse models with the Alzheimer's disease mouse. These models have produced positive outcomes to the intervention. The CSF1R is an essential microglial surface receptor. Studies using CSF1R inhibitors in various mouse models of Alzheimer's disease have demonstrated a reduction in neuroinflammation, neuritic plaque formation, and dendritic spine loss, while also improving cognitive function. These findings suggest that targeting CSF1R-mediated microglial activity may represent a promising therapeutic strategy for slowing disease progression [60].

## 2.4 Neuroinflammation Targeting Therapeutic Strategies for AD

The continuous rise in the prevalence of Alzheimer's disease (AD) has created an urgent need for more effective treatment strategies. Every year, pharmaceutical

companies and research institutions invest billions of dollars in developing therapies aimed at amyloid- $\beta$  ( $A\beta$ ) and tau proteins, which are considered the major pathological hallmarks of AD. Despite these extensive efforts, most anti- $A\beta$  and anti-tau drugs have failed to demonstrate satisfactory clinical benefits.

Although the U.S. Food and Drug Administration (FDA) has approved aducanumab, a monoclonal antibody targeting amyloid- $\beta$ , concerns regarding its clinical effectiveness and long-term therapeutic value remain [60].

In recent years, neuroinflammation has gained considerable attention as a major contributor to the development and progression of Alzheimer's disease. This has shifted research toward identifying anti-inflammatory approaches that specifically target the inflammatory processes involved in AD.

However, conventional anti-inflammatory drugs, particularly nonsteroidal anti-inflammatory drugs (NSAIDs), have produced inconsistent results in clinical studies. These disappointing findings may be explained by their non-specific mechanisms of action and the fact that treatment is often initiated after significant neurodegeneration has already occurred [60].

Microglia, the resident immune cells of the central nervous system, play a crucial role in regulating neuroinflammation. Under pathological conditions, prolonged microglial activation can exacerbate neuronal damage by releasing pro-inflammatory mediators, thereby accelerating disease progression.

Based on this understanding, researchers have explored both genetic and pharmacological strategies to regulate or temporarily deplete microglia. Studies conducted in experimental models of Alzheimer's disease have demonstrated that controlled microglial depletion can reduce neuroinflammation and improve several pathological and behavioral outcomes [60].

Therapeutic treatment is the colony-stimulating factor-1 receptor (CSF1R), which is essential for the survival and maintenance of microglia. Inhibition of CSF1R has been shown to reduce microglial numbers, suppress neuroinflammatory responses, decrease neuritic plaque formation, preserve dendritic spine integrity, and improve

cognitive function in several preclinical models of Alzheimer’s disease. These findings suggest that modulation of microglial activity through CSF1R inhibition may represent a promising therapeutic strategy for slowing the progression of AD [61].

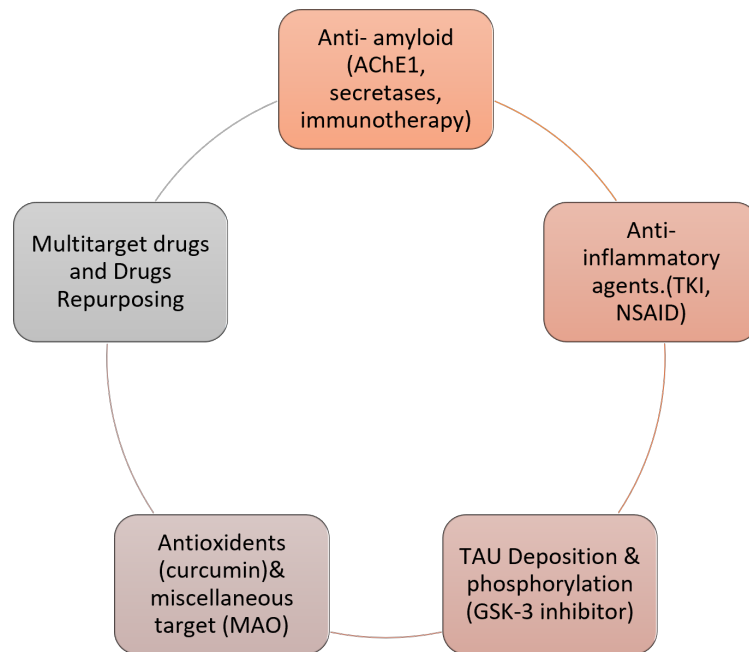


FIGURE 2.1: Therapeutic strategies of Alzheimer’s disease

The figure above summarizes the complex and multifactorial pathogenesis of Alzheimer’s disease (AD) and outlines the major therapeutic strategies currently under investigation. Since AD develops through multiple interconnected pathological processes, effective treatment is likely to require interventions that target more than one disease mechanism[61].

One of the primary therapeutic approaches focuses on reducing amyloid- $\beta$  ( $A\beta$ ) accumulation and plaque formation. This strategy includes the use of acetylcholinesterase inhibitors (AChEIs), secretase inhibitors, and immunotherapeutic agents designed to decrease  $A\beta$  production, enhance its clearance, or limit its aggregation. In parallel, considerable attention has been directed toward therapies targeting tau pathology. Agents such as glycogen synthase kinase-3 (GSK-3) inhibitors are being investigated to reduce tau hyperphosphorylation, prevent neurofibrillary tangle formation, and ultimately slow neuronal degeneration[61].

Oxidative stress is another key factor contributing to neuronal injury in AD. Consequently, naturally occurring antioxidants, including curcumin, have been explored for their neuroprotective properties. These compounds may help neutralize reactive oxygen species, reduce oxidative damage, and preserve neuronal function.

Chronic neuroinflammation also plays a central role in the progression of Alzheimer's disease. Therefore, anti-inflammatory therapies, including nonsteroidal anti-inflammatory drugs (NSAIDs) and tyrosine kinase inhibitors (TKIs), have been evaluated for their ability to suppress inflammatory signaling and reduce microglial activation. Although the clinical benefits of these agents remain under investigation, they continue to represent an important area of therapeutic research [61].

In recent years, drug repurposing and the development of multitarget-directed ligands (MTDLs) have emerged as promising strategies for AD treatment. Unlike conventional therapies that focus on a single pathological process, multitarget compounds are designed to simultaneously modulate several disease mechanisms. Among these, MBT derivatives have attracted considerable interest because of their combined anti-inflammatory and antioxidant activities.

By targeting neuroinflammation, oxidative stress, and other pathological pathways simultaneously, these compounds may provide greater therapeutic efficacy than single-target agents. Current preclinical studies continue to evaluate their potential as novel therapeutic candidates for slowing disease progression and improving cognitive outcomes in Alzheimer's disease [61].

## 2.5 Interleukin-1 Beta and Memory

One of the most important family of pro-inflammatory cytokine is IL-1, and the cytokine that is most extensively used is the IL-18 and it is prominently used in the pathophysiology of the AD. This cytokine is primarily formed by the a-microglia as a result of the reaction of pathogenic stimuli just like the buildup of the  $A\beta$ . The neurons damage and the progression of the disease is fastening by the cytokine IL-13 and this cytokine is the primary cause of the persistent neuro-inflammation. The most common cause of its production is the microglial activation, and this

activation of microglia is produced in reaction to the pathological stimuli such as  $A\beta$  accumulation. One of the major driver of the chronic inflammation is IL-18 and it is the one that adds to the neuronal damage and accelerates the advancement of the disease.

A critical process involved in the formation of NFTs, reduction in the tau hyperphosphorylation and also improve cognitive function while modestly influencing  $A\beta$  levels, are shown by the studies that have inhibition of the IL-1R in Alzheimer's disease mouse models and these model have significant role in the following critical processes listed above. An interesting fact is that the paradoxical effects on the amyloid pathology is effectively demonstrated by the cytokine IL-13.

Marked reduction has been seen in the  $A\beta$  plaque burden by the over expression of the IL-13, which is shown by different experimental studies and this was possibly observed due to the increased microglial activation and  $A\beta$  clearance, whereas in other contexts the increased production through upregulation of  $\beta$ -secretase activity also been associated with it [62].

Disturbance in the long term potentiation (LTP) which serves as the key mechanism for underlying the learning and memory and contributes to the cognitive decline in Alzheimer's disease occurs due to the impairment of the synaptic plasticity by the cytokine IL-18. The thing that makes IL-18 a complex yet potential cytokine is its ability to promotes AD clearance under desired conditions. Despite its detrimental inflammatory effects is the potential but compelling therapeutic target.

Thus, as by sustaining the neuroinflammation and promotion of tau pathology a very harmful role is being played by the IL-18 in Alzheimer's disease. If we carefully modulates the signaling pathways of it, it may offers significant therapeutic benefits which may provide a path in altering disease progression [63].

Activation of microglia and astrocytes is a characteristic feature of neuroinflammation in Alzheimer's disease. Once activated, these glial cells release a variety of pro-inflammatory mediators, including interleukin- $1\beta$  (IL- $1\beta$ ), tumor necrosis

factor- $\alpha$  (TNF- $\alpha$ ), and several chemokines, which further intensify the inflammatory response. Sustained production of these molecules disrupts the integrity of the blood–brain barrier (BBB), impairs cerebral blood circulation, and promotes synaptic dysfunction and neuronal injury. In addition, continuous communication among activated glial cells, endothelial cells, and neurons creates a self-perpetuating inflammatory environment that exacerbates neuronal damage and contributes to the progressive neurodegeneration observed in Alzheimer’s disease [63].

# Chapter 3

## Material and Methods

### 3.1 Animals

The Guidelines of the US-based Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) were followed during the handling and care of the laboratory animals used in the present studies. The mice (25-30 grams in weight) were randomly assigned to one of four group treatments (the control, scopolamine, Donepezil treated and INCA-6 treated). All of the mice included in the present studies were produced from the CUST animal house in Islamabad, Pakistan. The mice were retained together in groups of three in cages and were maintained in a temperature-controlled environment under a 12-hour light/dark cycle (lighting from 8:00 am to 8:00 pm), with access to water and food ad libitum.

### 3.2 In-silico Studies

In-silico studies were performed to evaluate the effectiveness of INCA-6 against Alzheimer's disease by assessing their binding affinity to key neuroinflammatory targets. Molecular docking and molecular dynamics simulations were utilized to predict interaction stability and inhibitory potential. Moreover, computational

pharmacokinetic and toxicity analyses were conducted to determine the therapeutic suitability of the INCA-6.



FIGURE 3.1: Mice in the study

### 3.3 Food Composition

In most tests, the mice are provided a balanced diet of pellets that have been chemically prepared to give them all the nutrients they need (18–24% protein, 4–6% fat, and 40–60% carbs; in addition to fiber, vitamins, and vital minerals) [Table 3.1]. Fish, flour, bran, and powdered milk are a few common ingredient examples. Mice raised on a regular diet were be healthy and generate accurate experimental results for studies.

TABLE 3.1: Animal food composition

| Food item         | Quantity (5.0 kg) |
|-------------------|-------------------|
| Fish              | 750 g             |
| Wheat Flour       | 3.25 Kg           |
| Vitamins/Minerals | 75 g              |
| Dry Skimmed Milk  | 200 g             |
| Chokar            | 750 g             |

### 3.4 Mice Handling

All experimental procedures involving animals were performed in accordance with internationally accepted guidelines for the care and use of laboratory animals and were approved by the Ethics Committee CUST, Islamabad. Every effort was made to ensure the welfare of the animals throughout the study ethical approval# **CUST/DERC/PH/ERC/PG/2026/013**.

Before the start of the experiment, all mice were acclimatized to the animal facility for seven days to minimize environmental stress and allow physiological adaptation. During this period, the animals were observed daily for any signs of illness, stress, or abnormal behavior. They were also handled gently on a regular basis to familiarize them with human contact and reduce anxiety during subsequent experimental procedures.

Each cage was properly labeled for identification, and individual mice were marked using a non-invasive tail-marking method to facilitate accurate identification throughout the study.

Following acclimatization, the animals were randomly allocated to experimental and control groups to minimize selection bias and ensure the reliability of the results.

Standard hygienic practices were followed during all handling procedures. Disposable gloves were worn before handling the animals to maintain cleanliness and reduce the risk of contamination. Mice were initially lifted by gently holding the base of the tail and placed on the wire cage lid to allow secure restraint.

They were then restrained by gently grasping the loose skin over the back of the neck (scruff), while the tail was supported along the wrist and secured with the little finger. This technique provided adequate control of the animal while minimizing stress and allowing normal respiration.

Throughout the experimental period, the mice were monitored daily for their general health and well-being. Parameters such as body weight, food and water intake,



FIGURE 3.2: Mice Handling in laboratory

grooming behavior, posture, mobility, and overall activity were carefully recorded. Animals showing signs of severe distress, illness, or pain that met the predetermined humane endpoints were euthanized by cervical dislocation in accordance with institutional ethical guidelines to minimize unnecessary suffering.

The accompanying figure illustrates the proper technique for the safe and humane handling of laboratory mice while wearing protective gloves.

### 3.5 Mice Bedding

Mice were given a place to live with bedding that's comfortable and helps to keep them dry and smell nice. The common kinds of bedding are wood shavings, like aspen. The people in charge thought about how the bedding would affect the mice's health and how they behave, and whether it would change the results of the experiments. The bedding was changed every day so the mice would have a safe place to live without bad things in the air that can hurt them.

The mice that were used in the lab were given a kind of bedding made from corn cobs. This is a popular choice for labs because it is very good at soaking up moisture, keeping smells down, and giving the mice a clean and comfortable place to live. This is very important for keeping the mice healthy and happy. The corn

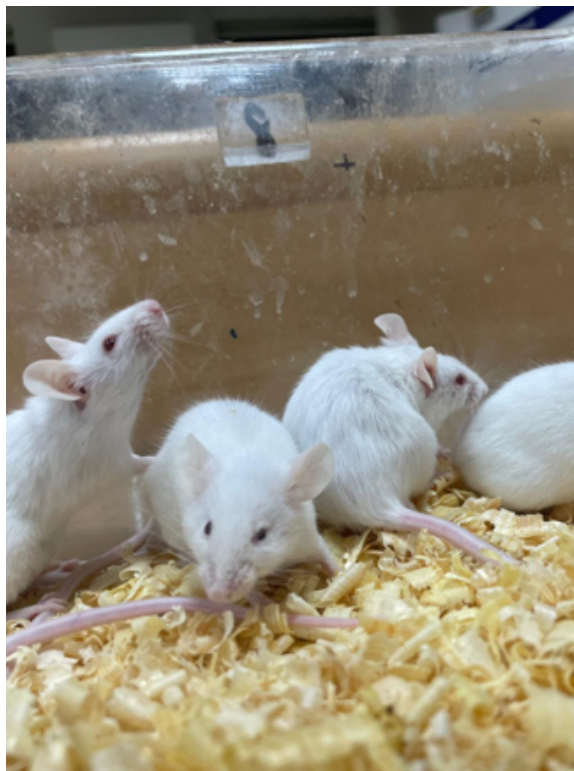


FIGURE 3.3: Normal housing environments for laboratory mice.

cob bedding was changed every day to keep everything clean, stop bad smells from building up, and prevent the growth of bad germs.

The cages that the mice lived in were made of clear plastic and they could be cleaned in a special machine to kill all the germs. The cages were also very strong and did not block the view. The people taking care of the mice could watch them without bothering them. This way of keeping the mice supported their behavior like exploring and making nests and helped to keep them calm and not stressed. All of this helped to make sure that the results of the experiments were consistent and accurate and that the mice were healthy and happy.

These practices contributed to improved animal welfare and enhanced the reliability of experimental outcomes. The photograph shown was taken at the Faculty of Pharmacy, CUST, Islamabad.

## 3.6 Animal Gender

The sex determination in mice and rats is commonly performed by examining the anogenital distance, which is the distance between the genital and anal openings. This distance is significantly greater in males than in females, making it a reliable method for distinguishing between the two sexes.

## 3.7 Randomization

We wanted to make sure everything was fair and that the groups were even so we mixed up the animals before we started the study. To tell the animals apart we marked their tails with ink that does not hurt them. We put circles on their tails in different patterns so we could tell them apart easily.

For example, Animal #1 got one circle on its tail. Animal #2 got two circles that were spaced out evenly. Animal #3 got three circles and Animal #4 got four little circles. Animal #5 got one circle on its tail. Animal #6 got one circle and then one little circle with a little space between them. This way we could watch each animal and get the right information during the study.

The distance between the genital orifices of the mouse and rat is an important thing to look at. We looked at this distance in female mouse and rat to see the difference.

## 3.8 Euthanizing Procedures

### 3.8.1 Cervical Dislocation Technique

According to the recommendations of the AAALAC Panel on Euthanasia (1972), cervical dislocation is an acceptable method of euthanasia for laboratory mice when performed correctly. The procedure should induce rapid unconsciousness, typically within approximately 15 seconds, while minimizing pain and distress. To ensure humane euthanasia, cervical dislocation should only be performed by

trained personnel and is recommended for small rodents weighing less than 200 g. Since then people have used different methods to do this. Each method has its way of making sure the mouse dies quickly and humanely. One way is the hemostat-assisted cervical dislocation method. This is when the person doing it pulls the mouse's tail back fast to dislocate the cervical spine. At the same time they use a big hemostat to press on the skull.

Another commonly used method of euthanasia is cervical spine dislocation, which involves rapid dislocation of the cervical vertebrae to induce immediate unconsciousness and death when performed correctly by trained personnel. This is similar. The person uses their thumb and forefinger to press on the skull instead of a tool. They also pull the tail fast to dislocate it.

There is also the cervical dislocation method. This is when the person holds the mouse tail with one hand and uses the other hand to press the top of the cervical spine down and forward. Sometimes they even twist it a little. This dislocates the skull from the vertebrae without hurting the skin. Then there is the thoracic dislocation method. This is when the person grabs the mouse chest at the bottom of the ribcage with their thumb and forefinger.

They then pull the tail fast to make the thoracic vertebrae come apart. When the cervical spine is dislocated it stops the brain from working with the spinal cord. This means the mouse loses consciousness right away and does not feel any pain. The mouse then dies because its diaphragm stops working, which means it cannot breathe. The blood vessels also stop working, which means the mouse's heart stops beating.

After the procedure the person has to make sure the mouse is really dead. They do this by making sure the mouse's lungs are collapsed, which is called pneumothorax. Studies have shown that these methods work. The mouse's brain stops working in 0.5–2 seconds. It does not show any signs of pain. The AAALAC guidelines are also followed. All of these methods are meant to kill the mouse humanely with as little pain as possible. This is in line with the way to treat animals.



FIGURE 3.4: The technique for Cervical dislocation.

The AAALAC guidelines are important to follow when it comes to euthanizing mice. The methods used are all designed to minimize pain and distress for the mice [64]. It helps kill lab mice in a humane way. A wooden spatula is used for dislocation. The mouse is held gently. The spatula is pressed firmly on the base of the skull. A quick pull is made on the tail. This separates the vertebrae.

### 3.8.2 Mild Head Injury

A concussion is a method used to kill small laboratory animals by hitting their skull with a lot of force. This hit should be strong enough to cause bleeding in the brain right away and stop the central nervous system from working so the animal loses consciousness instantly. The way this method works is by moving the head fast and then stopping it suddenly, which makes the brain hit the hard inside of the skull. This quick movement causes alteration in the pressure inside the skull and hurts the delicate parts of the brain and its blood vessels, which stops the brain from working properly. The hit is meant to be so strong that it overwhelms the brain and makes the animal unconscious or even kills it right away.

When a concussion happens it interferes with the way the brain works by damaging the parts of the brain that control being awake, breathing and heart function. The sudden injury can hurt the blood vessels, cause swelling in the brain, and stop the brain's signals from working right. All of this happens so fast that it knocks out the animal's ability to feel and move in just a few milliseconds. Even though it might seem simple, this method requires a lot of skill and accuracy.

The person doing the procedure needs to know a lot about the animal's body, especially where the brain is and where to hit the skull. The hit needs to be strong enough to cause damage but not so strong that it hurts the animal's body too much, especially if the body needs to be kept intact for later study. If the hit is not done right it can be very bad for the animal. If the animal is not hit enough or if the hit is in the wrong place the animal might stay awake or wake up again and feel a lot of pain, fear and stress, which is not good for the animal's welfare.

What is most important is that just using a concussion is not considered a way to kill an animal by the groups that make the rules for using animals in laboratories, like the AAALAC guidelines. This is because the effects of a concussion can be temporary and the animal can wake up again. So to be sure the animal is killed humanely, a concussion must be followed by another method that cannot be reversed, like breaking the neck, bleeding out, or cutting off the head. This two-step method makes sure the animal does not wake up again and dies right away. In science and veterinary medicine a concussion is usually only allowed when other methods, like using chemicals to kill the animal, would interfere with the results of the study. In these cases the use of a concussion must be carefully considered and the people doing it must be well trained and qualified.

Finally, although a concussion is a way to make an animal unconscious, its use must be carefully thought out and considered against what is ethical, what is good for the animal's body, and what is technically possible. It must be part of a plan for killing animals that respects their welfare and follows international standards. A concussion is a way to kill laboratory animals by hitting them on the head with a lot of force. This hit is meant to be so strong that it kills the animal right away. The person doing the procedure needs to be very careful and know a lot about

the animal's body. The use of a concussion is not considered humane and must be followed by another method to be sure the animal is killed quickly and does not wake up again. This is very important for the animal's welfare.

The use of a concussion is only allowed in situations where other methods would interfere with the results of a study. In these cases the people performing it must be well trained and qualified. They must also be careful to follow the rules and guidelines for using animals in laboratories, such as the AAALAC guidelines. The goal is to kill the animal humanely without causing it any pain or stress. A concussion is a method that should only be used when necessary. It is not a simple procedure and requires a lot of skill and accuracy.

The person performing it must be very careful and know what they are doing. The animal's welfare is the most important thing and the use of a concussion must be carefully thought out and considered against what is ethical and what is good for the animal.

In the end a concussion is a way to kill laboratory animals but it is not a humane method on its own. It must be followed by another method to be sure the animal is killed quickly and does not wake up again. The use of a concussion must be carefully thought out, considered against what is ethical, what is good for the animal's body, and what is technically possible. It must be part of a plan for killing animals that respects their welfare and follows international standards [64].

### 3.9 Chemicals and Drugs

TABLE 3.2: Ligand Profile

| Sr. No | Chemical / Substance     | Source                         |
|--------|--------------------------|--------------------------------|
| 1      | Scopolamine hydrobromide | Macklin (macklin.cn)           |
| 2      | Donepezil tablet         | Global Pharmaceutical Pvt. Ltd |
| 3      | INCA-6                   | Synthesized in lab             |
| 4.     | DMSO                     | Sigma-Aldrich                  |
| 5.     | Chloroacetyl chloride    | same as above                  |

Table 3.2 continued from previous page

| Sr. No | Chemical / Substance           | Source        |
|--------|--------------------------------|---------------|
| 6.     | Dichloromethane                | same as above |
| 7.     | Trimethylamine                 | same as above |
| 8      | Benzylamine                    | same as above |
| 9.     | Acetonitrile                   | same as above |
| 10     | K <sub>2</sub> CO <sub>3</sub> | same as above |
| 11.    | KI                             | same as above |
| 12.    | Sodium sulphate anhydrous      | same as above |

### 3.10 Acclimatization of Animals

The healthy adult Swiss albino mice of both gender were obtained from the Animal House facility (AA&M) and housed in standard laboratory housing conditions prior to the experiment for acclimatization. Mice were housed under well-ordered conditions (temperature =  $22 \pm 2^\circ\text{C}$ ; relative humidity =  $55 \pm 10\%$ ; lighting = 12 hr. light/12 hr. dark cycle) during a 7-day acclimatization period prior to the start of experimental procedures. Mice were given free access to standard rodent food and water throughout this entire period.

The acclimation period allowed for an adjustment to their environment and a decrease in the level of environmental stressors, therefore decreasing the amount of physiological variability between mice which may affect both behavioral and biochemical assessments of the animal.

In addition, to decrease stress associated with handling the animals, mice were handled daily throughout the acclimation period to familiarize them with the experimental procedures.

#### 3.10.1 Allocation of Experimental Animals

Following acclimatization, animals were randomly allocated into five experimental groups ( $n = 6$  per group) to certify uniform distribution of body weight and reduce

selection bias.

#### **3.10.1.1 Group I - Normal Control**

Animals received normal saline (10 mL/kg, i.p.) and served as the control group.

#### **3.10.1.2 Group II - Negative Control**

Animals received scopolamine hydrobromide (1 mg/kg, i.p.) to prompt cognitive impairment and memory deficit.

#### **3.10.1.3 Group III - Positive Control**

Animals received donepezil hydrochloride (5 mg/kg, p.o.) trailed by scopolamine (1 mg/kg, i.p.).

#### **3.10.1.4 Group IV - INCA-6 Low Dose**

Animals received INCA-6 (5 mg/kg, i.p.) followed by scopolamine (1 mg/kg, i.p.).

#### **3.10.1.5 Group V - INCA-6 High Dose**

Animals received INCA-6 (10 mg/kg, i.p.) followed by scopolamine (1 mg/kg, i.p.).

The treatment schedule was continued daily throughout the experimental period according to the study protocol.

### **3.10.2 Drug Administration**

Body weight of all study animals was recorded prior to each administration to allow for correct dosing calculations.

Scopolamine hydrobromide, INCA-6, and donepezil hydrochloride were prepared each day, fresh, using the proper vehicle to preserve the chemical and pharmacological stability of the drug.

Scopolamine hydrobromide (1 mg/kg; i.p.) produced an impairment of memory by blocking the central muscarinic cholinergic receptors, thereby producing cognitive deficits that mimic the type of cognitive deficits seen in Alzheimer's disease. Donepezil hydrochloride served as the standard reference drug for cognitive enhancement and for its known effects of AChE inhibition.

Donepezil hydrochloride served as the standard reference drug for cognitive enhancement and for its known effects of AChE inhibition.

The INCA-6 doses (5 and 10 mg/kg; i.p.) were given to the subjects 30 minutes prior to administering scopolamine. The doses were chosen according to past studies and clinically determined dosing prior to starting.

Each type of INCA-6 was given at the same time each day to decrease the variability as a result of being given a single drug. The control group received the same volume of vehicle as the experimental groups under the same experimental conditions.

Intraperitoneal injections were made using disposable sterile insulin syringes (1.0 mL, 26–30 gauge needles). The site of injection was located at the right-lower portion of the abdomen to avoid potential injury to any organs that could be located just beneath this location (e.g., bladder, intestines, etc.), by inserting the needle into the abdominal wall at an angle of 15–30° while holding the animal.

Prior to administering the drug, an aspiration of the needle was conducted to ensure that the needle was free of blood and accessible during the experiment. Donepezil hydrochloride was given orally by means of a properly sized orally administered gavage needle which provided a procedure as close to the "clinical" administration route as possible.

All procedures were conducted under sterile conditions and met the institutional guidelines for the care and use of laboratory animals.

The purpose of this administration protocol is to determine if the drug INCA-6 protects against cognitive dysfunction due to scopolamine administration as measured by deficits in memory impairment due to scopolamine.

### 3.10.3 Drug Solubility

The amount of solubility of the test compound (test compound) in water is minimal (very poor). Therefore, trying to dissolve the test compound in normal saline first by vortexing was not successful for the reason of low solubility. Consequently, a co-solvent method was chosen for this initial preparation of drug for use in animals. In this regard, the test compound was dissolved in DMSO (40  $\mu$ L of DMSO was added to an empty Eppendorf tube).

The tube containing the DMSO–test compound solution was then vortexed to create a homogeneous mixture of both liquids. Once the test compound was dissolved in DMSO, normal saline (950  $\mu$ L of NS) was added to the Eppendorf containing the homogeneous solution. The NS was mixed by vortexing with the DMSO–test compound solution, and the final volume of the combined solutions (1 mL) was created. The resulting preparation was a homogeneous and stable drug solution that could be injected intraperitoneally.

### 3.10.4 Limitations of Dimethyl Sulfoxide

- i. Not inert: toxic even at low doses ( $>1\%$  v/v *in vitro*; 1-8% v/v retinal apoptosis *in vivo*).
- ii. Systemic effects: enhances penetration of co-administered agents and alters coagulation and histamine release .
- iii. Assay interference: confounds solvent-sensitive assays, so keep it low ( $<1\%$  v/v *in vivo*) with untreated and vehicle controls.

## 3.11 Dose Preparation

A drug solution with concentration 5 mg/Kg, was prepared by dissolving 0.5 mg of drug into 40  $\mu$ L DMSO in sterile Eppendorf tube. It was then added to 950  $\mu$ L of normal saline and vortex mixed until dissolved completely. The drug appeared to be completely dissolved in DMSO and was used for dilution. The final drug

solution concentration was 10 mg/Kg. A total 1 mg of drug was weighed out and added to 40  $\mu$ L of DMSO to create a separate solution.

After this solution was mixed with 950  $\mu$ L of normal saline and again vortex mixed until created a clear homogeneous solution indicating drug was completely dissolved in DMSO before dilution. Scopolamine was prepared by weighing out 0.1 mg of the drug and dissolving it in 1 mL distilled water before administering to animal using 1 mL syringe (distilled water is preferred solvent). The solution had to be thoroughly stirred to dissolve completely.

### 3.12 Scopolamine Induced Memory Impairment

Memory impairment were induced in mice by administration of 1mg/kg (i.p.) of Scopolamine for 7 days.

#### 3.12.1 MWM Test

The MWM behavioral test was conducted using a circular stainless-steel tank, selected for its durability, chemical resistance, and ease of cleaning between trials. The tank measured 135 cm in diameter and 42 cm in height and was filled with water to a depth of approximately 40 cm. Water temperature was maintained at  $22 \pm 2^\circ\text{C}$ , which is within the optimal range for behavioral testing in mice to minimize stress and prevent hypothermia. To render the water opaque and eliminate visual cues beneath the surface, non-toxic white powdered milk was added. This prevented the animals from using proximal visual cues to locate the hidden escape platform, ensuring that navigation relied primarily on spatial learning and memory.

Geometrically marked divisions on the interior wall of the tank created four equal virtual quadrants. Each quadrant included unique extramaze visual cues such as distinct patterned symbols or color-coded flags on and above the rim of the tank, and these stimuli remained constant throughout the experiment to allow the mice to develop spatial associations with the cues.

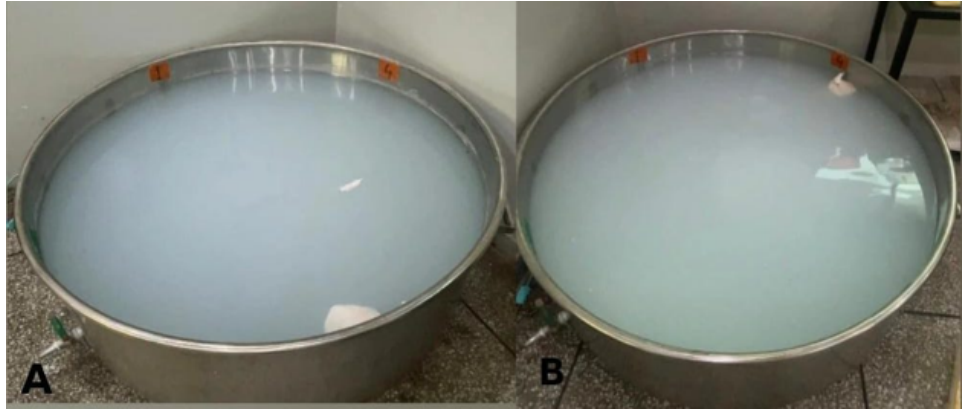


FIGURE 3.5: Apparatus for Morris Water Maze

The tank was furnished with a circular transparent acrylic escape platform (10 cm diameter, 25 cm height), which was located in one of the four quadrants (the target quadrant) and submerged 1 cm below the water surface, thus rendering it invisible to the mice throughout the study.

Pre-conditioning acclimatization involved exposing the mice to the testing apparatus over a 2-day acclimatization period before the training trials to reduce novel stimulus-induced stress and allow the animals to become accustomed to the testing environment. During this acclimatization period, the animals were not rewarded and were allowed to swim freely in the tank for approximately 30–60 seconds each day in the target quadrant, thereby becoming familiar with the water and the spatial cues.

The training trials were conducted over six consecutive days, with one training trial for each mouse per day. The escape platform remained at the same location (target quadrant) throughout the training period; however, each mouse was released into the pool from a different starting quadrant each day to prevent the use of non-spatial search strategies (i.e., “learn how to island hop”).

Mice were allowed a maximum of 60 seconds after being released into the pool to locate the submerged platform by using distal visual cues. Animals that successfully located the platform were allowed to remain on it for 15 seconds to reinforce spatial learning. Animals that failed to locate the platform within the allotted time were gently guided to the platform and were also allowed to remain there for 15 seconds.

This ensured that all animals received equal exposure to the learning process regardless of their initial performance. Escape latency (time required to locate the hidden platform), path length, and swimming behavior during each trial were recorded using computerized video-tracking software connected to an overhead camera.

On Day 7, a probe trial was conducted to evaluate spatial memory retention and memory consolidation. During the probe trial, the escape platform was removed from the tank before the mouse was introduced into the water. Each mouse was released from the side opposite the former platform location to avoid positional bias. The animals were allowed to swim freely for 60 seconds while their behavior was recorded. The primary parameter measured was the amount of time spent in the target quadrant where the platform had previously been located. A greater amount of time spent in the target quadrant indicated better spatial memory retention. Additional parameters, including the number of platform-site crossings, search strategy, and quadrant preference, were also recorded.

All behavioral testing was carried out under standardized lighting and sound conditions to minimize environmental variability. The apparatus consisted of a circular pool filled with opaque water that concealed the submerged escape platform just beneath the water surface. During each trial, mice were released from different starting locations and were required to use external visual cues to locate the hidden platform.

When an animal encounters a platform it has previously learned, the objective is to determine whether it can retain and recall the platform location. This is assessed by repeating the test over multiple trials and measuring both the time required to locate the platform and the amount of time spent in the target quadrant during the probe trial.

The Morris Water Maze is a well-established method for evaluating hippocampus-dependent spatial learning and memory deficits. The photograph of the experimental setup was taken at the Faculty of Pharmacy, CUST Islamabad. This

behavioral test was used to evaluate cognitive impairment and memory performance by assessing the animal's ability to learn and remember the location of the hidden platform.

### 3.12.2 T Maze Test

The T-maze test is a simple and well-validated method of measuring cognitive functions such as learning, memory, and spatial navigation in mice. The T-maze consists of a start arm and two goal arms that form a "T"-shaped configuration. Mice were expected to choose a different goal arm than the one that they had just been in during each trial; this is representative of the animals use of working memory. The test consisted of several sessions where the data from the number of correct alternations (i.e., alternating from arm to arm) were recorded and analyzed statistically to measure cognition [44].

A T-maze test was conducted to measure cognition, specifically learning, memory, and spatial navigation, by using a test apparatus composed of one start arm and two goal arms configured as a "T" shape. The animal is placed in the start arm and allowed to select a goal arm. In order to measure working memory, rodents randomly chose the arm of their selection; if the animal was exposed to an arm in an earlier trial, it was expected that it would alternate its choice of arms for the next trial. The measurements were taken over several sessions to allow time for learning. In addition, the number of correct alternations was recorded and analyzed statistically to determine the rodent's cognitive ability.

### 3.12.3 Y Maze Test

The Y-maze apparatus consisted of three identical white arms positioned at 120° angles to one another. Each arm measured 20 cm in length, 6 cm in width, and 10 cm in height. Before the initiation of treatment, the mice were acclimatized to the Y-maze environment to reduce novelty-induced stress and ensure consistent behavioral performance during testing. Each trial began with the mice at an initial position on one of the designated arms. All three arms (A, B and C) had

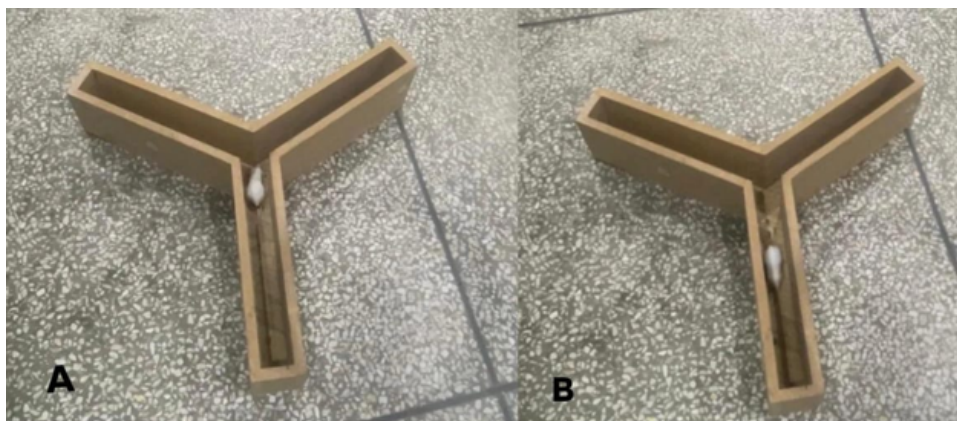


FIGURE 3.6: Apparatus for Y-maze

identifying marks, and entries into each arm were recorded using a video camera based on the mice's entries. An entry recorded for the mice into an arm was based upon the entire body of the mouse having entered into the arm (the tail was included in this determination). Spontaneous alternation had been defined as the number of times that the mice entered into each of the arms without entering into the same arm twice in a row cumulatively (i.e. ABC, BCA or CAB). The proportion of spontaneous alternation was calculated according to the formula:

$$\% \text{ Alternation} = \frac{\text{Number of actual spontaneous alternations}}{2[\text{Total number of entries into all arms}]}$$

In this study, the results demonstrated that the administration of scopolamine significantly decreased the spontaneous alternation rate, indicating impairment of spatial working memory. However, administration of donepezil (5 mg/Kg and 10 mg/Kg) in conjunction with MBT caused a important rise in spontaneous alternation, indicating enhancement in cognitive function [65, 66].

The Y-maze was made of stainless steel, it was cast-off to assess impulsive fluctuating behaviors of rodents as it indicates spatial working memory. Each of the 3 arms were equal in length and positioned at a 120° angle from each other at the center point. Each mouse was placed at the end of one arm and allowed to freely explore the Y-maze for the designated test period. The sequence and number of arm entries were recorded for subsequent analysis to assess spatial working memory and cognitive performance in experimental models of Alzheimer's disease. The photographs shown were taken at the Faculty of Pharmacy, Capital University of

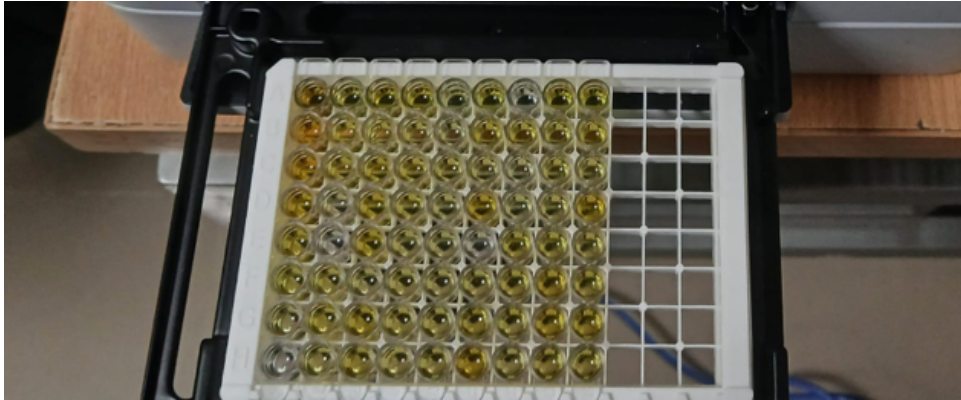


FIGURE 3.7: ELISA

Science and Technology (CUST).

#### 3.12.4 Enzyme-Linked Immunosorbent Assay

To govern  $\text{TNF-}\alpha$  levels (a principal neuroinflammatory marker associated with cognitive deficits in neurodegeneration), ELISA assays were performed on hippocampal extracts from mice. The cytokine levels measured by the concentration of absorbance (an assay of several variables) of the immune-enzyme  $\text{TNF-}\alpha$  antibodies, through the use of an enzyme-linked detection system, used to generate the absorbance readings, could be correlatively interpreted relative to the effects of  $\text{TNF-}\alpha$  levels on cognitive impairment [67].

The quantitative measure of  $\text{TNF-}\alpha$ , Amyloid beta and Tau protein levels in homogenized hippocampal tissue by ELISA will be evaluated by means of the use of specific antibodies for each of the target proteins and subsequently linked to accurate measurement of protein concentrations and evaluating their correlations with cognitive impairment in order to examine how neuroinflammation contributes to cognitive impairment at the molecular level.

Quantified levels of  $\text{IL-1}\beta$ - $\text{TNF}\alpha$  (by ELISA) were used to evaluate the degree of neuroinflammatory condition in model mice. The measurements of these two cytokines represent two of the most commonly used biomarkers of neuroinflammation, as the expression of both of these cytokines has been shown to be considerably elevated in the hippocampus of animals with neurodegenerative disorders (such as AD).

The concentrations of interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were quantified using commercially available mouse ELISA kits according to the manufacturer's instructions. The assays were performed in 96-well microplates pre-coated with monoclonal antibodies specific for mouse IL-1 $\beta$  or TNF- $\alpha$ . Cytokine standards were serially diluted to generate standard curves for quantitative analysis. Hippocampal supernatants were diluted at a ratio of 1:5 by mixing 10  $\mu$ L of sample with 40  $\mu$ L of sample diluent. Subsequently, 50  $\mu$ L of each standard or diluted sample was added to the designated wells. All standards and samples were analyzed in duplicate, while blank wells containing only sample diluent were included to determine background absorbance.

The ELISA microplates were sealed and incubated at 37°C for 30 minutes to facilitate the binding of cytokines present in the samples to the immobilized capture antibodies. After incubation, the wells were washed five times with wash buffer prepared by diluting the concentrated wash solution (20–30 $\times$ ) with distilled water to remove any unbound substances.

Subsequently, 50  $\mu$ L of horseradish peroxidase (HRP)-conjugated detection antibody was added to each well, except the blank wells, followed by a second incubation at 37°C for 30 minutes. After incubation, the plates were washed again to eliminate any excess detection antibody. Thereafter, 50  $\mu$ L of Chromogen Solution A and 50  $\mu$ L of Chromogen Solution B were added to each well to initiate the colorimetric reaction.

The plates were then incubated in the dark at 37°C for 15 minutes, allowing the HRP enzyme to catalyze the formation of a blue-colored product. The enzymatic reaction was stopped by adding 50  $\mu$ L of stop solution to each well, which changed the color from blue to yellow. The absorbance of each well was measured at 450 nm using a microplate reader within 15 minutes after the addition of the stop solution.

The concentrations of IL-1 $\beta$  and TNF- $\alpha$  in hippocampal tissue samples were determined by comparing the optical density (OD) values of the samples with the corresponding standard calibration curves. Cytokine levels were expressed as

picograms per milliliter (pg/mL). The obtained data were statistically analyzed and compared among the different experimental groups.

The ELISA assay provided reliable and reproducible quantification of hippocampal IL-1 $\beta$  and TNF- $\alpha$  levels, enabling the assessment of neuroinflammatory status and the evaluation of the anti-inflammatory effects of the tested treatment [68].

### 3.13 Molecular Docking Studies

To study how INCA-6 interacts with potential molecular targets related to memory dysfunction and neurodegeneration, molecular docking studies were performed. ChemDraw Professional, PyMOL (V3.0.4), AutoDock Tools (V1.5.7) and AutoDock Vina were used for all computational analyses, while Discovery Studio Visualizer (V2017) was used for visualization.

Using ChemDraw Professional, the chemical structure of INCA-6 was created and later converted into a 3D structure. The positive control drug chosen was donepezil hydrochloride, which is an acetylcholinesterase inhibitor that has been approved for clinical use.

The structural files for the selected protein molecular targets associated with cognitive impairment and AD (acetylcholinesterase [AChE], butyryl cholinesterase [BuChE], TNF- $\alpha$  and IL-1 $\beta$ ) were retrieved from the Protein Data Bank (PDB) and molecular docking simulations were conducted to evaluate the binding affinity, binding orientation and molecular interactions of INCA-6 within the active site of each target protein.

To identify the presence of hydrogen bonds, hydrophobic interactions,  $\pi$ - $\pi$  stacking interaction and specific amino acids involved in the binding event of the ligand, the docking results were analyzed using Discovery Studio Visualizer as well as PyMol. Binding energies were measured and then compared to those obtained for the positive control drug.

The results of these computational studies gave insight into possible molecular mechanisms responsible for the cognitive enhancement and neuroprotective actions of INCA-6 using the scopolamine-induced memory deficit model.

### 3.13.1 Ligand Preparation

ChemDraw Professional was used to create chemical structures for INCA-6 and reference compound donepezil hydrochloride, which were then saved as MOL files. Ligands were brought into the Discovery Studio Visualizer, where they underwent energy minimization using the CHARMM force field to determine stable conformations.

Minimized structures were translated into PDB format and imported into AutoDock Tools (v1.5.7) for docking studies. During preparation of ligands for docking studies, polar hydrogen atoms were added and Gasteiger charges assigned and rotatable bonds defined. Finally, ligands were saved in PDBQT format for further use in docking experiments.

### 3.13.2 Protein Preparation

Cryogenically determined three-dimensional structure information for the target proteins analyzed were recovered from the Protein Data Bank (PDB). Water molecules, non-co-crystallized ligands and heteroatoms that are not involved in ligand-receptor binding were eliminated from the structures using Apollo Visualiser.

All missing hydrogen atoms and Kollman charge assignment to protein structures were accomplished using AutoDock Tools, as well as saving each structure as a PDBQT file. The grids or mesh boxes have been generated around the active sites were used to provide a visual reference for both active site identification, based on the co-crystallized ligand and compound with literature-documented active-site residue information and the generation of the appropriate docking simulation.

The prepared protein structure files underwent visual examination via PyMOL to confirm the fidelity and orientation of the putative active site residues prior to docking.

### 3.13.3 Molecular Docking Procedure

Molecular docking simulations have been approved using AutoDock Vina and AutoDock Tools. The INCA-6 and receptor proteins were docked to allow flexibility of the ligand while maintaining a fixed position of the receptor. All proteins had their respective active sites covered with a grid box; all docking parameters were at default values unless noted otherwise. Each ligand-receptor combination produced multiple binding poses and the one selected for each was based on lowest binding energy score (-kcal/mol). The precision of the simulation was verified by calculating the root mean square deviation (RMSD) for the docked conformations, using a cut-off of  $\leq 2.0$  Å to signify acceptable conformations. Discovery Studio Visualizer was used to analyze binding interactions for all residues in the active site with reference to INCA-6.

Two-dimensional (2D) interaction maps and three-dimensional (3D) binding models were made to identify hydrogen bond, van der Waals, hydrophobic,  $\pi$ -alkyl and  $\pi$ - $\pi$  stacking interactions contributing to the binding of ligands. The docking scores and interaction profiles of INCA-6 were compared with donepezil hydrochloride to assess the ability of INCA-6 to function as a cognitive enhancer.

## 3.14 Toxicity Assessment

The online chemical toxicity prediction systems ToxTree and ProTox-II were used to forecast the toxicological profile of INCA-6. Before beginning any experimental work, these instruments enable the identification of safety precautions for the medicine. ChemDraw Professional was used to create the INCA-6 chemical structure, which was then transformed into a SMILES string. The two toxicity prediction systems were then used to process the SMILES string.

### 3.15 Statistical Analysis

Statistical analysis of all behavioral, biochemical, and histological data was performed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA), a widely validated statistical software package commonly used for biomedical research. Data were analyzed to compare the effects of different treatment groups, including the control, scopolamine, donepezil, INCA-6 low-dose, and INCA-6 high-dose groups.

For biochemical parameters (cytokine levels), histological findings, and behavioral outcomes measured at a single time point, statistical comparisons among groups were performed using one-way analysis of variance (ANOVA). Tukey's multiple-comparison post hoc test was applied to identify differences between individual groups. Tukey's test was selected because it effectively controls the family-wise error rate while allowing multiple pairwise comparisons.

A  $P$ -value  $< 0.05$  was considered statistically significant for all analyses. Where appropriate, exact  $P$ -values (e.g.,  $P = 0.032$ ) were reported to improve transparency. Levels of statistical significance were denoted as  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ .

All quantitative data are presented as mean  $\pm$  standard error of the mean (SEM). GraphPad Prism was also used to generate bar graphs and time-course plots, including escape latency curves from the Morris Water Maze, to illustrate the effects of INCA-6 treatment compared with the control and scopolamine-treated groups. To minimize experimental bias and ensure data integrity, all analyses were performed under blinded conditions, whereby the investigator responsible for data analysis remained unaware of the treatment group allocations throughout data processing and interpretation.

# Chapter 4

## Results

### 4.1 Acute Toxicity

To obtain an initial indication of safety, INCA-6 was tested in a single-dose acute toxicity trial using four adult male mice. Each animal received 100 mg/kg body weight by intraperitoneal injection. Following dosing, the mice were watched without interruption for 4 hours and then checked twice a day over the next 3 days for any sign of toxicity or abnormal behavior. None of the animals showed behavioral disturbances or changes in motility, suggesting that this dose of INCA-6 was tolerated under the conditions of the study. Because the trial was brief and involved few animals, toxic effects emerging later cannot be excluded. A longer follow-up of up to 14 days, in line with OECD guidance, would be required to establish the safety profile more firmly.

### 4.2 Behavioral Assessment

#### 4.2.1 Effect of INCA-6 Compound on Spatial Memory Deficit in MWM Test

Testing the Morris Water Maze (MWM) assessed the degree of cognitive learning and memory retrieval for both baseline measurement and spatial memory retention

following 7 days of treatment.

#### 4.2.1.1 Baseline Latency Measurement

Testing for each experimental group indicated similar escape latencies for both groups (mean of  $59.8 \pm 1.64$  for experimental non-cognitive and  $60.0 \pm 1.58$  for experimental cognitive), indicating that none of the experimental groups had any initial differences in physiology, vision or locomotion when assessed before any cognitive impairment was introduced.

TABLE 4.1: MWM test (Latency)

| Experimental Group                   | M1   | M2 | M3 | M4 | M5 | M6   | Mean $\pm$<br>(mean) SD |
|--------------------------------------|------|----|----|----|----|------|-------------------------|
| Control                              | 58   | 59 | 60 | 61 | 62 | 60.0 | $60.0 \pm 1.58$         |
| Scopolamine<br>(1 mg/kg)             | 58   | 59 | 59 | 61 | 62 | 59.8 | $59.8 \pm 1.64$         |
| Scopolamine + Donepezil<br>(3 mg/kg) | + 58 | 59 | 60 | 61 | 62 | 60.0 | $60.0 \pm 1.58$         |
| Scopolamine + INCA-6<br>(0.1 mg/kg)  | 58   | 58 | 60 | 61 | 62 | 59.8 | $59.8 \pm 1.79$         |
| Scopolamine + INCA-6<br>(0.5 mg/kg)  | 58   | 59 | 60 | 59 | 61 | 59.4 | $59.4 \pm 1.14$         |
| Scopolamine + INCA-6<br>(1 mg/kg)    | 58   | 59 | 60 | 61 | 62 | 60.0 | $60.0 \pm 1.58$         |

#### 4.2.1.2 7-Day Post-treatment Latency

The healthy, non-treated group demonstrated appropriate spatial learning as evidenced by their average escape latency of  $18.6 \pm 0.89$  when finding the platform in MWM after 7 days of no treatment. Compared to this healthy group, the scopolamine-treated group was unable to effectively recall where they had previously found the platform. This was evidenced by their significantly shorter average escape latency of  $9.0 \pm 0.63$  in finding the platform in the MWM test. Thus, this significantly lower latency escape time is likely indicative of a profound disturbance

of the normal exploratory behaviors and classical deficits in spatial memory exhibited by the scopolamine-treated animals relative to the healthy, control group.

TABLE 4.2: MWM test (after 7 days)

| Experimental Group                | M1 | M2 | M3 | M4 | M5 | M6   | Mean $\pm$ SD<br>(mean) SD |
|-----------------------------------|----|----|----|----|----|------|----------------------------|
| Control                           | 18 | 18 | 20 | 18 | 19 | 18.6 | $18.6 \pm 0.89$            |
| Scopolamine (1 mg/kg)             | 8  | 10 | 9  | 9  | 9  | 9.0  | $9.0 \pm 0.63$             |
| Scopolamine + Donepezil (3 mg/kg) | 17 | 19 | 18 | 18 | 17 | 17.8 | $17.8 \pm 0.75$            |
| Scopolamine + INCA-6 (0.1 mg/kg)  | 12 | 11 | 13 | 14 | 11 | 12.2 | $12.2 \pm 1.17$            |
| Scopolamine + INCA-6 (0.5 mg/kg)  | 15 | 14 | 17 | 14 | 15 | 15.0 | $15.0 \pm 1.10$            |
| Scopolamine + INCA-6 (1 mg/kg)    | 17 | 17 | 19 | 17 | 17 | 17.4 | $17.4 \pm 0.89$            |

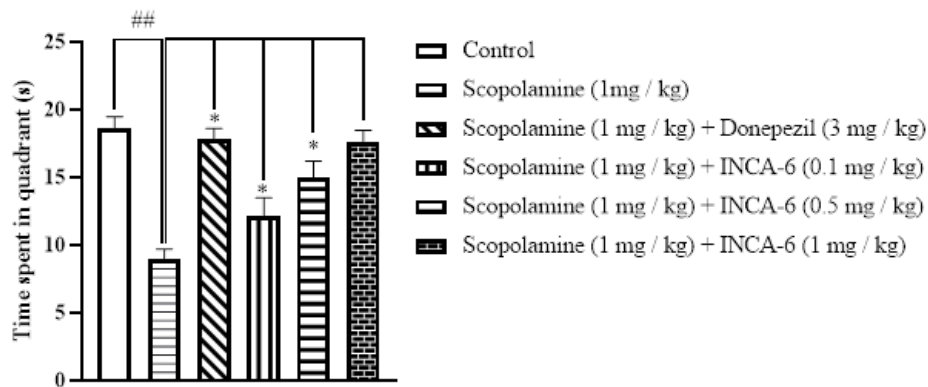


FIGURE 4.1: Impact of INCA-6 on POST-TREATMENT LATENCY in MWM test (after 7 days)

Pre-treatment with the reference drug, Donepezil (3mg/kg) was shown to be effective in reversing scopolamine-induced deficits, and escape latency was restored to  $17.8 \pm 0.75$  seconds. INCA-6 produced a clear and dose-dependent therapeutic effect in ameliorating scopolamine-induced cognitive deficits (see figure 4.1).

The lowest dose of INCA-6 (0.1mg/kg) produced a modest improvement in escape latency ( $12.2 \pm 1.17$  seconds), while the intermediate dose of INCA-6 (0.5mg/kg) normalized retrieval times further ( $15.0 \pm 1.10$  seconds). Pre-treatment with the

highest dose of INCA-6 (1mg/kg) almost completely ameliorated the scopolamine-induced cognitive deficit, with escape latency of  $17.4 \pm 0.89$  seconds, which is comparable to the escape latency displayed by the Donepezil pre-treated group.

4.2.2 Evaluation of the Effect of the INCA-6 on Short-Term Spatial Working Memory Using the Y-Maze Test

Short-term working memory (spatial memory) was evaluated in the Y-maze test by measuring the percentage of correct spontaneous alterations.

Control & Negative Control Comparison: Healthy control animals demonstrated a high level of exploratory behavior and had a high level of intact overall spatial memory, achieving a correct alteration rate of  $58.0 \pm 1.8\%$ . The administration of scopolamine (1mg/kg) produced a significant impairment of short-term working memory which was evidenced by a dramatic decline in the alteration rate to  $28.0 \pm 1.6\%$ .

TABLE 4.3: Y maze data (%correct alterations)

| Group |                                   | Treatment M2 |    | M3 | M4 | M5 | M6 | Mean   | ±     |
|-------|-----------------------------------|--------------|----|----|----|----|----|--------|-------|
|       |                                   | M1           |    |    |    |    |    | (mean) | SD    |
| 1     | Control                           | 60           | 58 | 57 | 59 | 56 | 57 | 58.0   | ± 1.8 |
| 2     | Scopolamine (1 mg/kg)             | 27           | 30 | 26 | 29 | 28 | 26 | 28.0   | ± 1.6 |
| 3     | Scopolamine + Donepezil (3 mg/kg) | 53           | 51 | 54 | 52 | 50 | 54 | 52.0   | ± 1.6 |
| 4     | Scopolamine + INCA-6 (0.1 mg/kg)  | 34           | 36 | 35 | 37 | 33 | 35 | 35.0   | ± 1.6 |
| 5     | Scopolamine + INCA-6 (0.5 mg/kg)  | 47           | 45 | 48 | 46 | 47 | 48 | 46.6   | ± 1.1 |
| 6     | Scopolamine + INCA-6 (1 mg/kg)    | 55           | 54 | 56 | 53 | 55 | 56 | 54.6   | ± 1.3 |

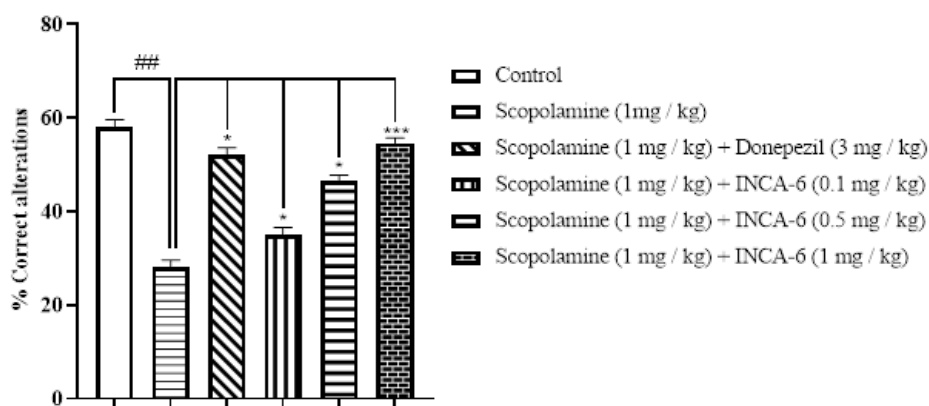


FIGURE 4.2: Impact of INCA-6 on Spatial learning in Y-Maze Test

**Effect of Interventions:** In contrast, pretreatment with Donepezil (3mg/kg) antagonized these deficits and restored the correct alteration rate to  $52.0 \pm 1.6$ . Treatment with INCA-6 resulted in a highly significant and dose-dependent recovery of working memory. At a dose of 0.1mg/kg, the percentage of alternation was increased to  $35.0 \pm 1.6$ . At the intermediate dose of 0.5mg/kg, this performance was improved to  $46.6 \pm 1.1$ . The highest cognitive recovery was observed with the maximum dose of INCA-6 (1mg/kg) ( $54.6 \pm 1.3$  correct alterations). This result was superior to the Donepezil reference group and very close to healthy control values, remarkably improving the spontaneous alternation behavior.

### 4.2.3 Effect of INCA-6 Compound on Spatial Memory Deficit in T-Maze Test

Another measure of spatial working memory was the T-maze test, in which the percentage of correct alterations between experimental cohorts was measured.

**Control and Negative Control Performance:** The control cohort produced a healthy baseline of  $64.0 \pm 1.4$  correct alterations. Administration of scopolamine (1mg/kg) produced a severe memory impairment as it significantly reduced the correct alternation behavior to  $30.8 \pm 1.5$ .

**Effect of Interventions:** Pre-treatment with Donepezil (3mg/kg) abolished the alteration rate to  $57.0 \pm 1.4$ . INCA-6 treatment revealed a clear dose-response

profile in all testing groups. The low dose (0.1mg/kg) showed minimal protection  $39.0 \pm 1.4$  while the 0.5mg/kg dose showed moderate protection  $51.5 \pm 1.0$ .

However, the INCA-6 at a dose of 1mg/kg with a correct alteration rate of  $59.5 \pm 1.0$  showed the highest neuroprotective effects, which was significantly different from the Donepezil standard group, indicating a strong neuroprotective efficacy.

TABLE 4.4: T maze (% correct alterations)

| Experimental Group                | M1 | M2 | M3 | M4 | M5 | M6 | Mean $\pm$ (mean) SD |
|-----------------------------------|----|----|----|----|----|----|----------------------|
| Control                           | 65 | 63 | 66 | 64 | 62 | 64 | $64.0 \pm 1.4$       |
| Scopolamine (1 mg/kg)             | 30 | 33 | 29 | 31 | 32 | 30 | $30.8 \pm 1.5$       |
| Scopolamine + Donepezil (3 mg/kg) | 58 | 56 | 59 | 57 | 55 | 57 | $57.0 \pm 1.4$       |
| Scopolamine + INCA-6 (0.1 mg/kg)  | 38 | 40 | 39 | 41 | 37 | 39 | $39.0 \pm 1.4$       |
| Scopolamine + INCA-6 (0.5 mg/kg)  | 52 | 50 | 53 | 51 | 52 | 51 | $51.5 \pm 1.0$       |
| Scopolamine + INCA-6 (1 mg/kg)    | 60 | 59 | 61 | 58 | 60 | 59 | $59.5 \pm 1.0$       |

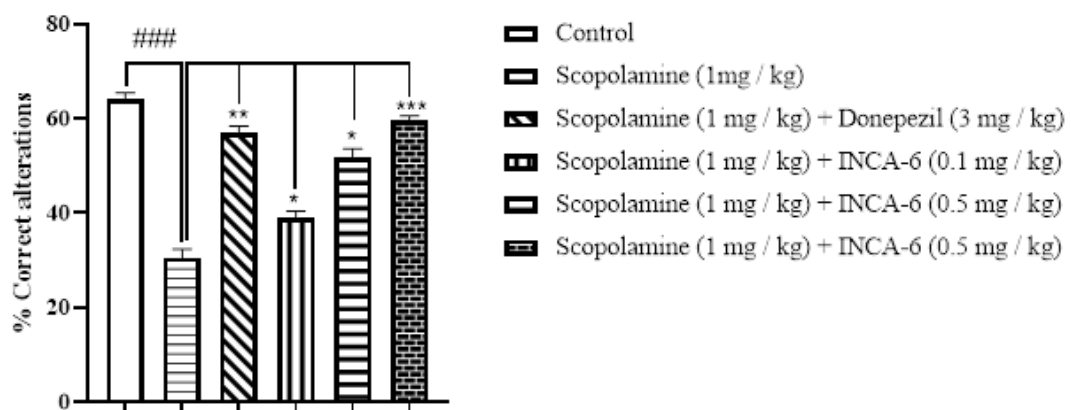


FIGURE 4.3: Impact of INCA-6 on spatial learning in T-Maze test

### 4.3 Assessment of the Effects of the INCA - 6 Compound on Hippocampal Pro - Inflammatory Cytokine Levels Using ELISA

#### 4.3.1 Tumor Necrosis Factor

Using enzyme-linked immunosorbent assay (ELISA), the quantitative analysis of proinflammatory cytokine TNF- $\alpha$  in hippocampal tissue was performed with a method that provides validity to the qualitative estimation of BCA total protein values to determine appropriate sample normalizations.

Control and Negative Control Levels: Healthy control tissues showed low baseline levels of TNF- $\alpha$  to be  $6.33 \pm 0.09$  pg/mg, which reflects the normal physiological baseline of TNF- $\alpha$  in the hippocampus. Following scopolamine administration, an acute neuro-inflammatory response was triggered, resulting in the peak level of  $36.67 \pm 0.77$  pg/mg TNF- $\alpha$  expression, signifying that the TNF- $\alpha$  expression was representative of an increased level of neuro-inflammation in the hippocampus that are consistent with the mechanisms of neuroinflammation previously characterized in human disease mechanisms associated with neurodegenerative conditions.

Effect of Interventions: The acute neuroinflammatory response seen with scopolamine was suppressed upon treatment with Donepezil (3mg/kg), thereby resulting in reduced levels of TNF- $\alpha$  to  $13.59 \pm 0.23$  pg/mg providing partial protection against scopolamine-induced neuroinflammation. INCA-6 produced a dose-responsive anti-inflammatory result showing lower TNF- $\alpha$  levels at 0.1 mg/kg ( $29.51 \pm 0.55$  pg/mg) than at 0.5 mg/kg ( $19.42 \pm 0.48$  pg/mg), thus illustrating a decreased degree of neuroinflammation with increasing doses of INCA-6.

Noteworthy, the most robust reduction of TNF- $\alpha$  levels was seen after administration of INCA-6 at 1 mg/kg, resulting in a final expression of  $11.04 \pm 0.24$  pg/mg TNF- $\alpha$ . This final level of expression is significantly less than the reference standard of Donepezil indicating a robust, greater than unit (i.e.,  $> 1$ mg/kg)

dose-dependent mechanism of action for attenuating scopolamine-mediated neuroinflammation in the hippocampus.

TABLE 4.5: TNF- $\alpha$  (pg/mg)

| Experimental Group                | M1    | M2    | M3    | M4    | M5    | M6    | Mean $\pm$ SD<br>(mean) |
|-----------------------------------|-------|-------|-------|-------|-------|-------|-------------------------|
| Control                           | 6.33  | 6.23  | 6.470 | 6.39  | 6.25  | 6.30  | 6.33 $\pm$ 0.09         |
| Scopolamine (1 mg/kg)             | 36.14 | 37.29 | 35.63 | 36.76 | 37.67 | 36.50 | 36.67 $\pm$ 0.77        |
| Scopolamine + Donepezil (3 mg/kg) | 13.41 | 13.93 | 13.56 | 13.72 | 13.29 | 13.60 | 13.59 $\pm$ 0.23        |
| Scopolamine + INCA-6 (0.1 mg/kg)  | 29.86 | 29.02 | 30.37 | 29.50 | 28.93 | 29.40 | 29.51 $\pm$ 0.55        |
| Scopolamine + INCA-6 (0.5 mg/kg)  | 19.06 | 19.80 | 18.75 | 19.47 | 20.02 | 19.40 | 19.42 $\pm$ 0.48        |
| Scopolamine + INCA-6 (1 mg/kg)    | 10.82 | 11.37 | 11.10 | 11.23 | 10.74 | 11.00 | 11.04 $\pm$ 0.24        |

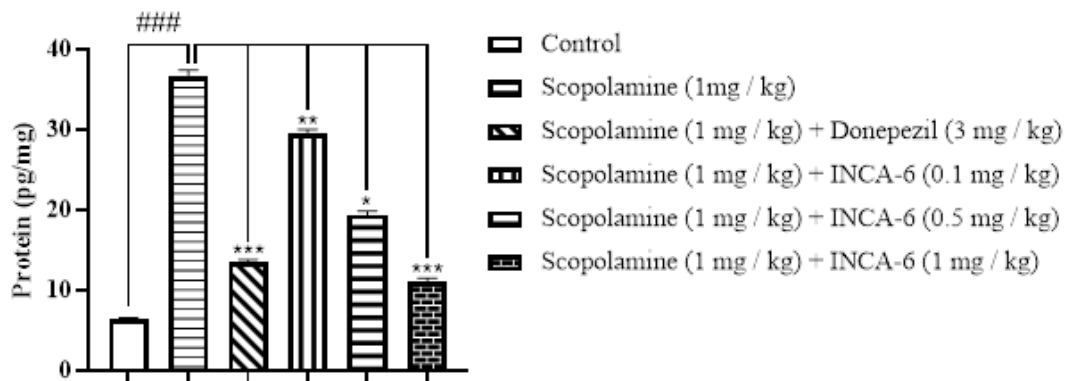


FIGURE 4.4: Impact of INCA-6 on TNF- $\alpha$

### 4.3.2 Interleukin-1 beta IL-1 $\beta$

Quantitative analysis of interleukin-1 beta (IL-1beta) was accomplished via an ELISA on samples taken from the hippocampus to assess the anti-inflammatory mechanisms of the treatments included in this study.

Control and Negative Control Levels: In the healthy control group, IL-1beta levels were at a physiologically normal level of  $5.06 \pm 0.06$  pg/mg in the tissue. However, the administration of scopolamine (1 mg/kg) caused a marked increase in

IL-1beta levels to  $31.25 \pm 0.74$  pg/mg. This increase in IL-1beta supports that neuroinflammatory pathways are activated following the administration of scopolamine and are indicative of the development of neurotoxic effects associated with scopolamine.

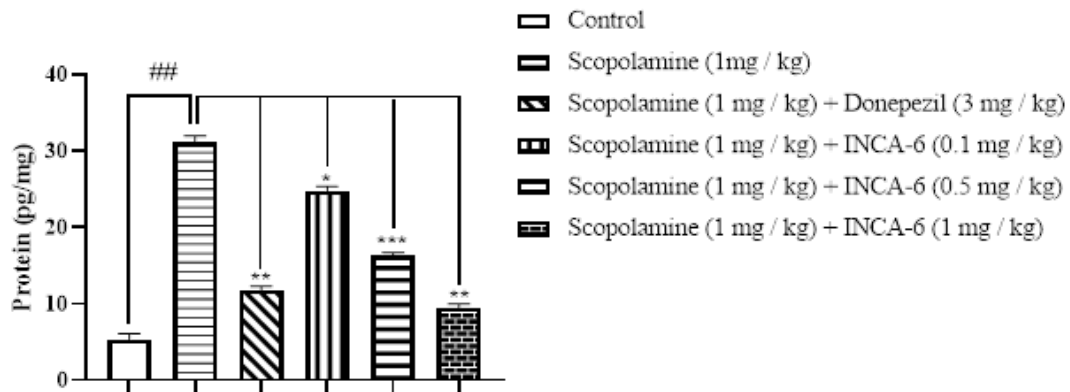
Effect of Interventions: Following treatment with Donepezil (3mg/kg) the levels of IL-1beta began to be partially normalized with a level of  $11.62 \pm 0.24$  pg/mg; IL-1beta concentration levels with chronic INCA-6 treatments at a dosage of 0.1mg/kg were found to be  $24.81 \pm 0.58$  pg/mg.

Thus suggesting a limited anti-inflammatory effect at this lower dose. A treatment dosage of 0.5mg/kg further down-regulated expression to an IL-1beta concentration of  $16.27 \pm 0.45$  pg/mg.

Conversely, INCA-6 at the highest treatment dosage of 1mg/kg resulted in a significant reduction in IL-1beta with average concentrations of  $9.31 \pm 0.24$  pg/mg. This is significantly lower than any of the other two groups receiving lower treatment dosage and the Donepezil reference standard doses; thus demonstrating the treatment dosage dependent improvement of inflammation in the hippocampus beyond standard acetylcholine esterase inhibition effects on reducing inflammation in this region of the brain.

TABLE 4.6: Interleukin-1 beta IL-1  $\beta$  (pg/mg)

| Experimental Group                | M1    | M2    | M3    | M4    | M5    | M6    | Mean $\pm$<br>(mean) SD |
|-----------------------------------|-------|-------|-------|-------|-------|-------|-------------------------|
| Control                           | 5.02  | 5.14  | 5.06  | 5.10  | 4.98  | 5.05  | $5.06 \pm 0.06$         |
| Scopolamine (1 mg/kg)             | 30.63 | 31.92 | 30.35 | 31.29 | 32.22 | 31.10 | $31.25 \pm 0.74$        |
| Scopolamine + Donepezil (3 mg/kg) | 11.42 | 11.94 | 11.67 | 11.78 | 11.30 | 11.60 | $11.62 \pm 0.24$        |
| Scopolamine + INCA-6 (0.1 mg/kg)  | 25.15 | 24.33 | 25.64 | 24.80 | 24.06 | 24.90 | $24.81 \pm 0.58$        |
| Scopolamine + INCA-6 (0.5 mg/kg)  | 15.97 | 16.66 | 15.63 | 16.30 | 16.84 | 16.20 | $16.27 \pm 0.45$        |
| Scopolamine + INCA-6 (1 mg/kg)    | 9.16  | 9.63  | 9.29  | 9.51  | 8.98  | 9.30  | $9.31 \pm 0.24$         |

FIGURE 4.5: Impact of INCA-6 on IL-1 $\beta$ 

#### 4.4 Effect of INCA-6 Treatment on Total Protein Concentration in the Hippocampal Tissue of Mice

In order to evaluate total protein content across each experimental group via BCA assay, we also monitored the integrity of the cells that were maintained as the standard baseline for normalization. Mean values of total protein content across all experimental conditions were extremely consistent, with means remaining between  $5.10 \pm 0.02$  mg and  $5.12 \pm 0.03$  mg. Both the absence of substantial variance and loss of protein across experiment cohorts indicated that scopolamine induced no neurotoxic effects and the doses of INCA-6 produced no systemic protein degradation, disruption of cellular mass or confounding effects on tissue integrity. Therefore, structural stability demonstrates the accuracy of utilizing biochemical normalization as well as confirms the safeness of INCA-6 at a cellular level.

TABLE 4.7: BCA Data

| Experimental Group                | M1   | M2   | M3   | M4   | M5   | M6   | Mean $\pm$ SD   |
|-----------------------------------|------|------|------|------|------|------|-----------------|
| Control                           | 5.12 | 5.08 | 5.15 | 5.10 | 5.13 | 5.11 | $5.12 \pm 0.03$ |
| Scopolamine (1 mg/kg)             | 5.09 | 5.11 | 5.07 | 5.13 | 5.10 | 5.10 | $5.10 \pm 0.02$ |
| Scopolamine + Donepezil (3 mg/kg) | 5.11 | 5.14 | 5.08 | 5.12 | 5.09 | 5.10 | $5.11 \pm 0.02$ |

Table 4.7 continued from previous page

| Experimental Group                  | M1   | M2   | M3   | M4   | M5   | M6   | Mean $\pm$<br>(mean) SD |
|-------------------------------------|------|------|------|------|------|------|-------------------------|
| Scopolamine + INCA-6<br>(0.1 mg/kg) | 5.10 | 5.12 | 5.09 | 5.13 | 5.11 | 5.11 | 5.11 $\pm$ 0.02         |
| Scopolamine + INCA-6<br>(0.5 mg/kg) | 5.12 | 5.09 | 5.11 | 5.10 | 5.13 | 5.10 | 5.11 $\pm$ 0.02         |
| Scopolamine + INCA-6<br>(1 mg/kg)   | 5.10 | 5.13 | 5.11 | 5.09 | 5.12 | 5.11 | 5.11 $\pm$ 0.02         |

## 4.5 Molecular Docking Outcomes

### 4.5.1 Binding Affinities of INCA6 and Donepezil Against TNF- $\alpha$ and IL-1 $\beta$

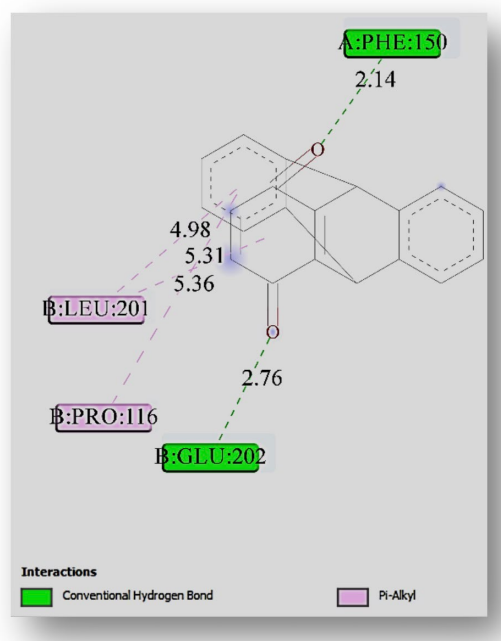
The molecular docking analysis produced binding free energy estimates for INCA6 and the reference compound donepezil against both neuroinflammatory targets. The best-ranked binding poses (mode 1, RMSD = 0.0 Å) for all four docking runs are summarized in 4.8.

TABLE 4.8: Summary of Molecular Docking Results for INCA6 and Donepezil Against TNF- $\alpha$  (1TNF) and IL-1 $\beta$  (8C3U)

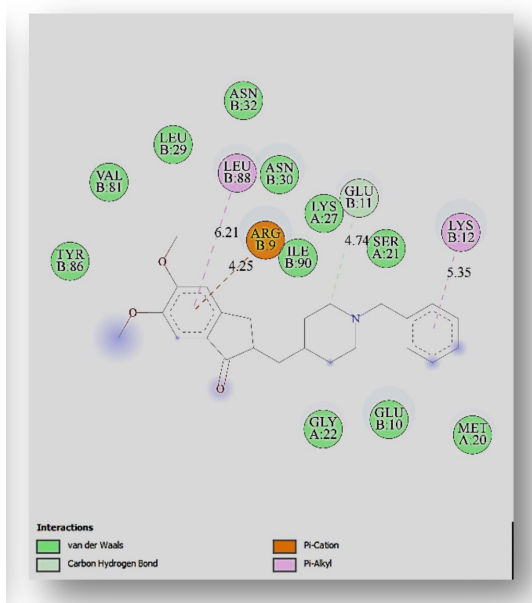
| Target Protein       | Ligand    | Binding Affinity (kcal/mol) |
|----------------------|-----------|-----------------------------|
| TNF- $\alpha$ (1TNF) | INCA6     | -6.9                        |
| TNF- $\alpha$ (1TNF) | Donepezil | -6.4                        |
| IL-1 $\beta$ (8C3U)  | INCA6     | -8.1                        |
| IL-1 $\beta$ (8C3U)  | Donepezil | -7.6                        |

Binding affinities are expressed in kcal/mol; lower (more negative) values indicate stronger predicted binding. All values represent the top-ranked mode 1 docking pose. INCA6 showed the highest affinity towards the IL-1 $\beta$  (−8.1 kcal/mol) among all docking results of this study. INCA6 bound to TNF- $\alpha$  with an affinity of −6.9 kcal/mol. In both, the top-ranked pose had RMSD lower and upper bound values of 0.0 Å, which would indicate that this pose is one of the global energy minima identified by the AutoDock Vina search algorithm and not a sampling artefact.

The reference compound donepezil turned out to be the most potent compound with a best binding affinity of  $-7.6$  kcal/mol to IL-1 $\beta$  and  $-6.4$  kcal/mol to TNF- $\alpha$ . Importantly, INCA6 was able to outperform donepezil in both of the two targets tested, with INCA6 being better than donepezil by 0.5 kcal/mol against TNF- $\alpha$  and much better with a difference of 0.5 kcal/mol against IL-1 $\beta$ .

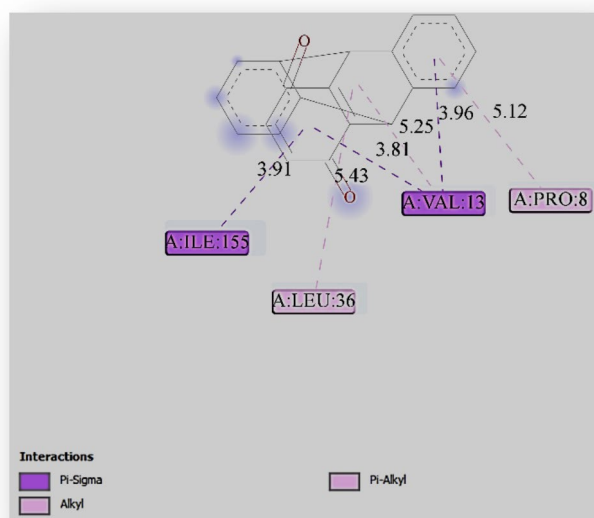


(a)

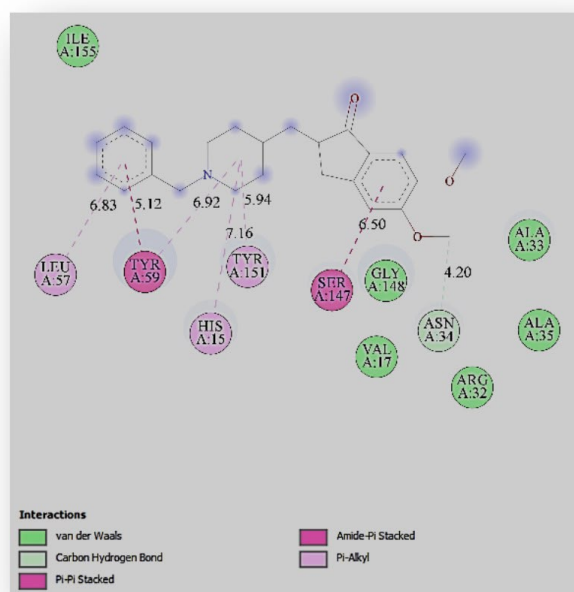


(b)

FIGURE 4.6: Two-dimensional ligand interaction diagram of INCA6 (L) and donepezil (R) docked into the binding interface of TNF- $\alpha$  (PDB: 1TNF)



(a)



(b)

FIGURE 4.7: Two-dimensional ligand interaction diagram of INCA6 (a) and Donepezil (b) docked at the receptor-binding surface of IL-1 $\beta$  (PDB: 8C3U)

The multiple low energy poses of INCA6 for  $-6.5 < -6.9$  kcal/mol, a few alternative poses had RMSD values greater than 20 Å from the top ranked pose against TNF- $\alpha$ . This RMSD distribution suggests that there are multiple pockets or binding modes that exist on the surface of TNF- $\alpha$ , which agrees with the

surface structural plasticity documented for the TNF- $\alpha$  homotrimer interface to accommodate various small molecule binders (He et al., 2005).

Despite the apparent dominance of the global minimum at  $-6.9$  kcal/mol, however, INCA6 is an acceptable binder in the functionally important interface of the TNFR interaction.

In all the data, INCA6 showed the best binding affinity in the presence of IL-1 $\beta$  ( $-8.1$  kcal/mol). A few other poses were also found, in the range of  $-7.1$  to  $-7.6$  kcal/mol, but with significantly higher RMSD values to the reference pose (up to  $46$  Å), which correspond to alternative binding sites that are spatially further away from the reference pose than from the native pose.

The high RMSD differences between the different poses along with the high energetic preference of the global minimum pose ( $-8.1$  kcal/mol) compared to all other poses indicate that the docking search is very focused on a single preferred binding cavity on the surface of IL-1 $\beta$ , which is a good indication of binding specificity.

## 4.5.2 Normal Mode Analysis of Protein–Ligand Complexes

### 4.5.2.1 TNF- $\alpha$ and INCA6 Complex

The iMOD normal mode analysis of the complex of TNF- $\alpha$  / INCA presented the dynamics of the bound protein system. The deformability plot (Figure 3.1) showed that the N-terminus (approx. 1–10) and the loop region in the proximity of atom index 28–32 had noticeably high deformability values with values close to 1.0 and 0.8 respectively. The central deformability profile (between the indices of the core atoms, approximately 40–120) was relatively stable and low, being consistently below 0.4, which is the region of structural rigidity.

A moderate deformability peak was also found at loop-containing areas which were scattered throughout the C terminal half of the structure (atom indices 120–155), similar to the loop-free architecture of TNF- $\alpha$ .

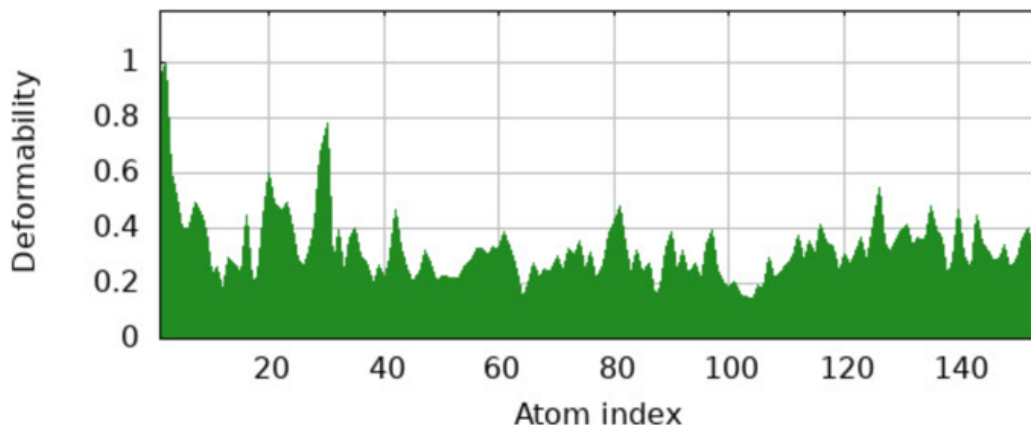


FIGURE 4.8: Deformability profile of the TNF- $\alpha$  / INCA-6 complex

The mode 1's eigenvalue for the TNF- $\alpha$  / INCA6 complex was calculated to be  $6.088 \times 10^{-4}$ . For the NMA, the first (lowest-frequency) normal mode has an eigenvalue that is inversely proportional to the amplitude of the large-scale motion with the highest amount of energy. The smaller the eigenvalue, the more flexible the system was on a global scale. The value of  $6.088 \times 10^{-4}$ , which is close to the  $6.15 \times 10^{-4}$  previously observed for the TNF- $\alpha$  homotrimer in its ligand bound form, is a measure of collective deformability of the TNF- $\alpha$  / INCA6 complex.

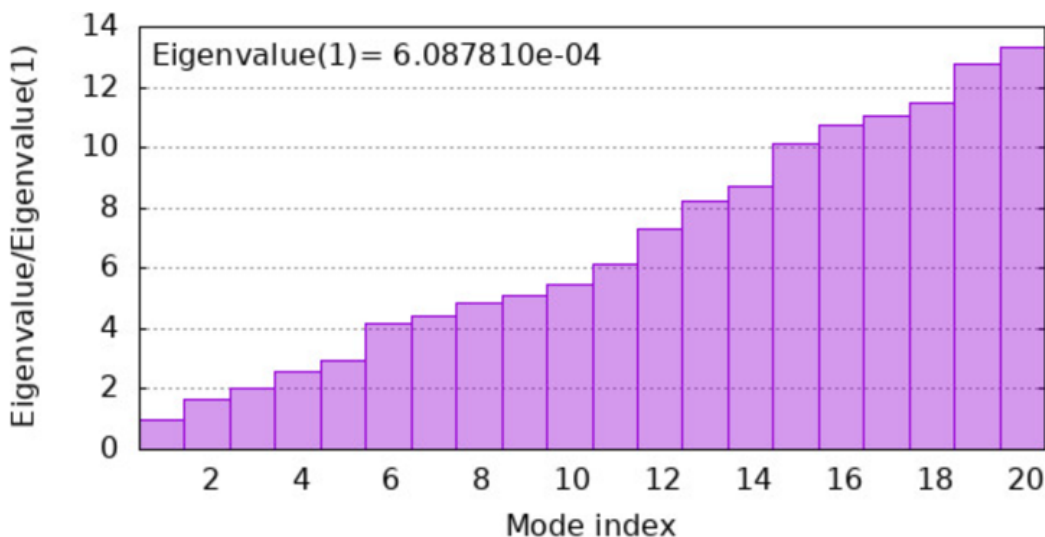


FIGURE 4.9: Eigenvalue spectrum of the TNF- $\alpha$  / INCA6 complex

The variance plot (Figure 4.9) indicated that the variance accounted for by mode 1 was about 20 percent of the total variance and dropped off very quickly thereafter, so that by mode 6 about 60 percent of the variance was accounted for.

The important presence of mode 1 suggests that a high energy low frequency conformational dynamics dominates the principal conformational dynamics of the complex, typical of biologically meaningful, large-scale hinge or twisting motions of the TNF- $\alpha$  trimeric assembly.

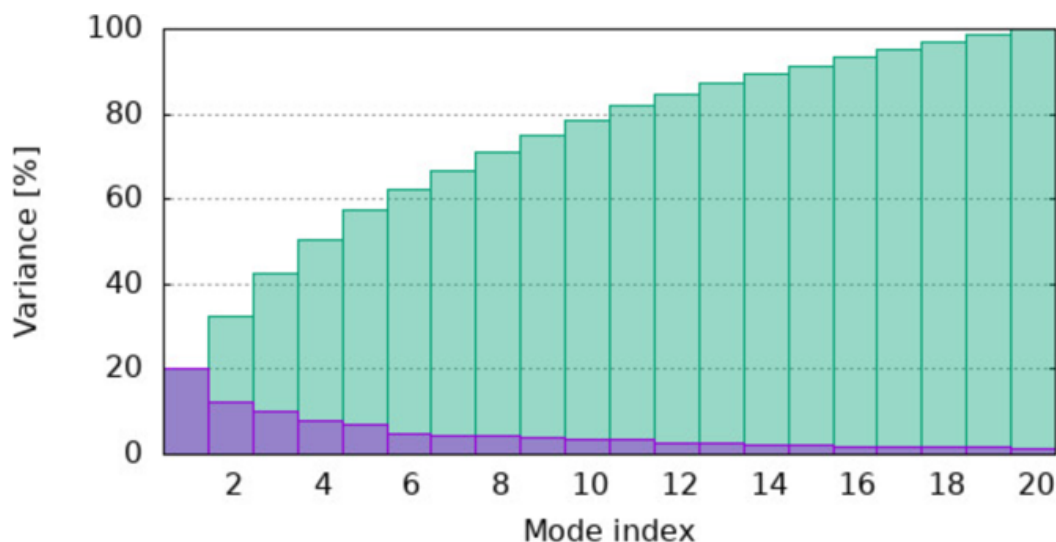


FIGURE 4.10: Variance plot of the TNF- $\alpha$  / INCA6 complex from iMOD normal mode analysis

The covariance map (Figure 4.10) showed a very clear pattern of correlated (red) and anti-correlated (blue) residue-motions. The positive correlations were clustered along the principal diagonal and a striking off-diagonal band representing the motions of long-range correlated parts of the protein (roughly N-terminal and C-terminal).

The anti-correlated regions (blue) were widely scattered all over the map, suggesting that the movement of certain domain pairs in the principal vibrational modes are in opposite directions, a characteristic of allosteric communication networks for TNF- $\alpha$  (Bai and Bhattacharya, 2018).

The elastic network model (Figure 4.11) showed the network of spring contacts (grey lines) among the 155-residue chain. The network was dense at the core and gradually sparse at the end of long-range connections, which is characteristic of the beta-jelly-roll fold of TNF- $\alpha$ . The arrow field representation showed the direction of the atomic shifts within the lowest frequency mode and indicated large scale

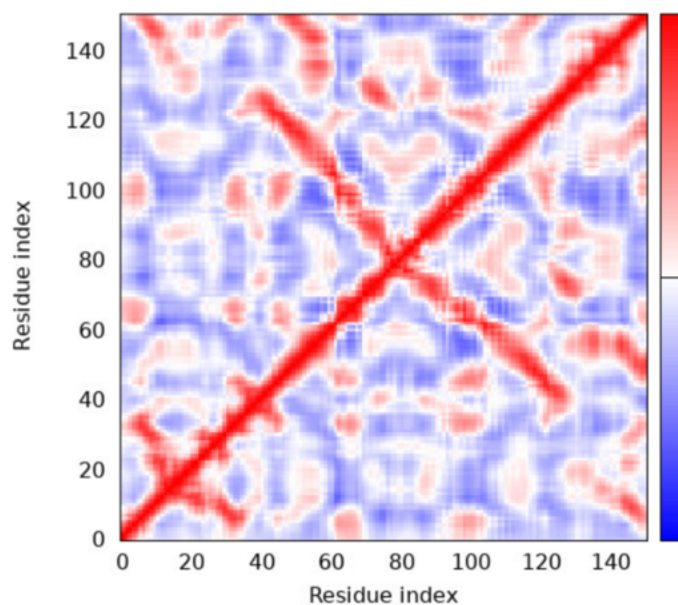


FIGURE 4.11: Covariance (co-evolution) map of the TNF- $\alpha$  / INCA6 complex generated by iMOD.

rotation/twisting of the C terminal sheet elements with respect to the N terminal loop region, the largest vectors being found in the N terminal beta strands.

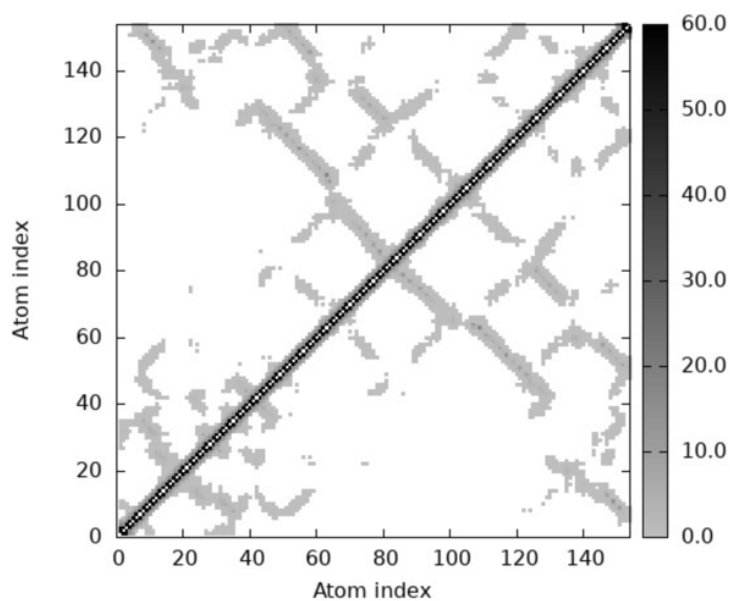


FIGURE 4.12: Elastic network contact map of the TNF- $\alpha$  / INCA6 complex from iMOD.

#### 4.5.2.2 IL-1 $\beta$ and INCA6 Complex

The normal mode analysis of the IL-1 $\beta$  / INCA6 complex showed that its dynamics were different from that of the TNF- $\alpha$  complex. A more spread-out flexibility

pattern along the protein chain was exhibited by the deformability plot (Figure 3.6). The initial deformability of the N-terminal region (atoms 1–10) was moderate ( $\sim 0.7$ ), and a sharp peak at high deformability ( $\sim 1.0$ ) was observed at the region of atom index 18–22, which is the flexible surface loop near the IL-1 $\beta$  receptor-binding interface.

The deformability is much more variable, ranging from 0.2 to 0.65 across the entire chain (atoms 30–160) and did not have a single structurally rigid core domain that was as extensive as that of the TNF- $\alpha$ . This is a more uniform flexibility because of the topology of IL-1 $\beta$  which is beta-trefoil, with several surface loops having functional significance distributed throughout the fold.

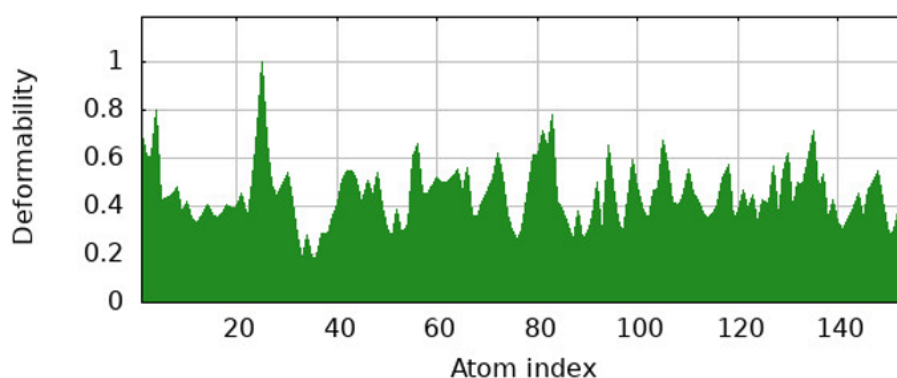


FIGURE 4.13: Deformability profile of the IL-1 $\beta$  / INCA6 complex

The eigenvalue of mode 1 for the IL-1 $\beta$  / INCA6 complex was  $1.254 \times 10^{-3}$ , which is approximately twice the eigenvalue determined for the TNF- $\alpha$  / INCA6 complex ( $6.088 \times 10^{-4}$ ). This higher eigenvalue indicates that the IL-1 $\beta$  / INCA6 complex is structurally stiffer in its lowest-frequency collective motion relative to the TNF- $\alpha$  complex, implying that INCA6 binding may impose a more pronounced conformational restraint on IL-1 $\beta$ .

A higher eigenvalue in the context of drug–target binding is generally interpreted as indicative of a more stable, energetically well-defined binding interaction (Dror et al., 2012), an observation that is consistent with the superior binding affinity of INCA6 for IL-1 $\beta$  observed in the docking experiments.

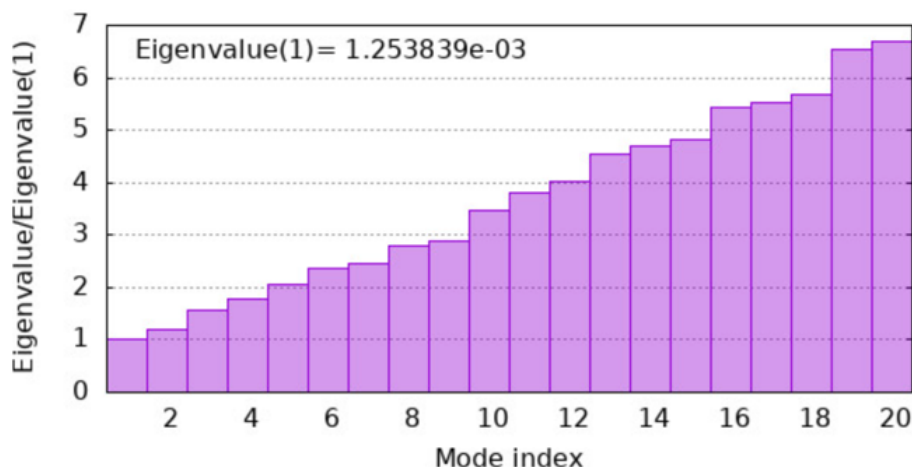


FIGURE 4.14: Eigenvalue spectrum of the IL-1 $\beta$  / INCA6 complex

The variance plot (Figure 4.15) for the IL-1 $\beta$  / INCA6 complex showed that mode 1 accounted for approximately 13% of total structural variance — a lower percentage than the  $\sim 20\%$  observed for the TNF- $\alpha$  complex. The variance was more evenly distributed across modes 1–10, with the cumulative variance reaching approximately 80% by mode 12. This more equitable distribution of variance across multiple modes is characteristic of proteins with a more isotropic, evenly distributed conformational flexibility, consistent with the beta-trefoil architecture of IL-1 $\beta$  in which no single domain dominates the global motion.

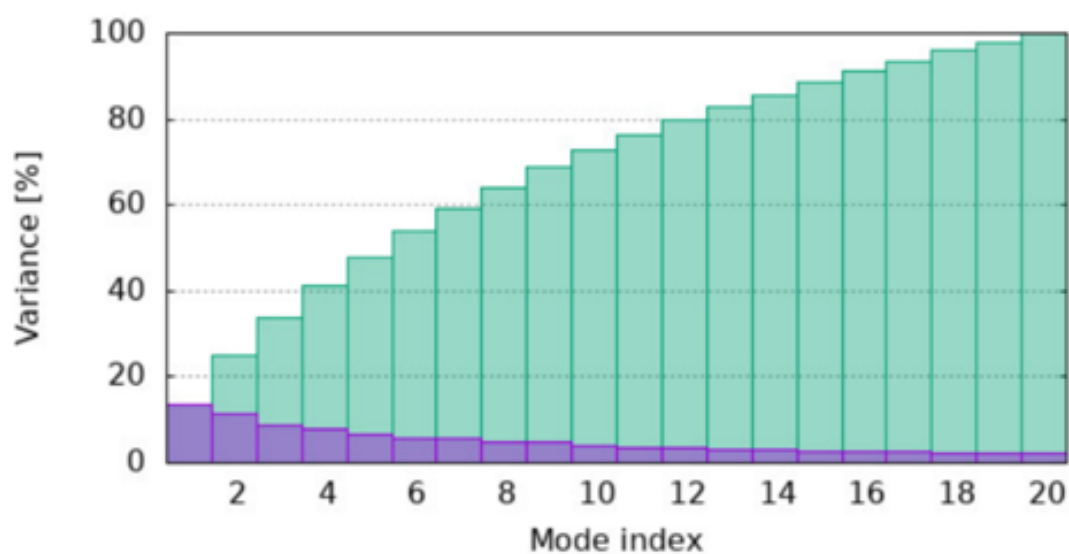


FIGURE 4.15: Variance plot of the IL-1 $\beta$  / INCA6 complex from iMOD

The covariance map (Figure 4.15) of the IL-1 $\beta$  / INCA6 complex displayed a

predominantly positive (red) correlation pattern along and near the diagonal, extending to approximately 20 residues off the main diagonal. Discrete off-diagonal red patches indicated specific long-range correlated pairs, particularly between residue clusters 60–80 and 90–110, which correspond to the structural loops flanking the central beta-barrel. Blue (anti-correlated) regions were comparatively less prominent and more diffuse than in the TNF- $\alpha$  covariance map, suggesting a more cooperative, less antagonistic inter-domain motion for the IL-1 $\beta$  / INCA6 complex. This pattern is consistent with a compact, well-integrated conformational response to INCA6 binding.

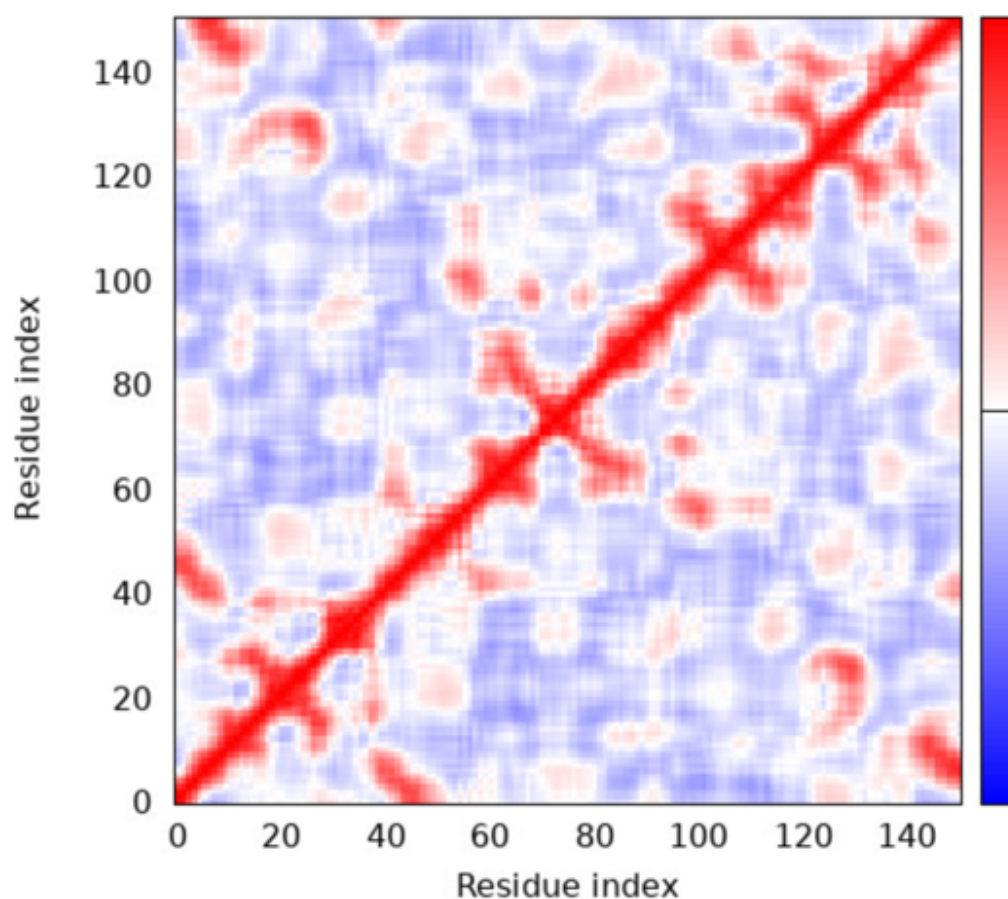


FIGURE 4.16: Covariance map of the IL-1 $\beta$  / INCA6 complex from iMOD

The elastic network diagram (Figure 4.16) for IL-1 $\beta$  / INCA6 exhibited a sparser overall pattern of long-range inter-residue contacts compared to TNF- $\alpha$ , reflecting the more globular and less internally connected beta-trefoil topology of IL-1 $\beta$ . The short-range contacts along the main diagonal dominated, with relatively few

medium- to long-range contacts, indicative of a protein that maintains its structural integrity primarily through local interactions reinforced by INCA6 binding rather than extensive long-range mechanical coupling. The arrow field visualization (Figure 3.10) illustrated a complex, multi-directional motion pattern consistent with the breathing dynamics of the IL-1 $\beta$  beta-trefoil, with the largest displacement vectors observed at the C-terminal strand and the extended N-terminal loop region, both distal to the central binding cavity occupied by INCA6.

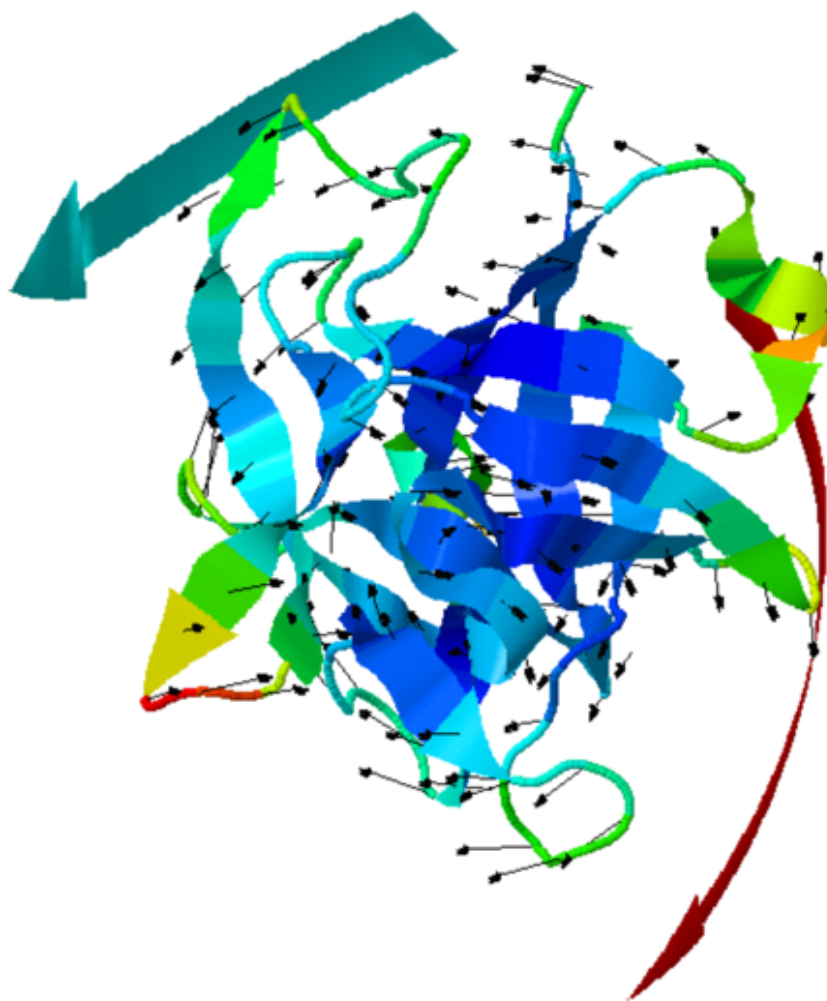


FIGURE 4.17: Arrow field representation of the lowest-frequency normal mode (mode 1) of the IL-1 $\beta$  / INCA6 complex from iMOD.

## 4.6 Summary of Results

Data acquired during this study documented that the use of scopolamine caused considerable cognitive impairment and caused significant neuroinflammatory stress. INCA-6 was able to consistently reverse the effects of scopolamine on cognition

and neuroinflammation in a dose-dependent manner. Although partial restoration of neurocognitive function was observed with both 0.1mg/kg and 0.5mg/kg doses of INCA-6, complete neuroprotection was achieved with the highest dose (1mg/kg). At this maximal concentration of previously administered INCA-6, it was able to provide cognitive restoration and neuroinflammation reduction that was equal to or better than the clinical standard, Donepezil. Therefore, INCA-6 may have value as a safe and effective neuroprotective drug.

# Chapter 5

## Discussion

### 5.1 Overview

This study sought to examine the neuroprotective effect of INCA-6 in a model of Alzheimer's disease utilizing scopolamine. Scopolamine is a widely utilized model for simulating both the cholinergic deficits and memory impairments characteristic of AD.

As scopolamine disrupts central system cholinergic neurotransmission by blocking the muscarinic receptor type it has been widely accepted as an effective model for investigating the cognitive deficits due to AD. Additionally, recent studies have shown that the scopolamine-induced amnesic model can be utilized reliably and with reproducibility to research and evaluate possible cognitive enhancers or neuroprotective agents; mainly because its use induces deficits in both learning and memory functions of the animal's brain. On the other hand, it should be noted that the scopolamine-induced model primarily represents acute cholinergic dysfunction which occurs in AD and is unable to adequately replicate the progressive and complex nature of this disease. For this reason alone, it has many limitations such as the absence of certain pathological features such as amyloid plaques (deposition) and abnormalities of the tau protein. Notwithstanding these limitations, however, the scopolamine model continues to be a useful tool for initial pharmacological screening [69].

As reported in previous studies assessing cognitive enhancing properties of various pharmacological agents, one goal of this research project was to examine pharmacological agents (INCA-6) that exhibit cognitive enhancing properties by utilizing established behavioral paradigms designed to measure cognitive functioning. Using behavioral tests that measure cognitive function (MWM, y-maze test and T-maze test) established that administration of INCA-6 improved cognitive performance (reduced latency) of scopolamine-treated animals when linked to placebo treated group demonstrating the ability of INCA-6 to reverse scopolamine-induced cognitive deficits.

The current research projects, similar to previous publications, support the ability of natural and synthetic pharmacological agents (flavonoids, alkaloids, small molecule inhibitors) with reported neuroprotective properties via numerous mechanisms of action to demonstrate improvements in learning and memory when identified via spatial learning tests (Morris Water Maze).

Improvements observed in spatial learning (improved cognitive performance) when examined via Morris Water Maze suggest potential enhancement of hippocampal function via administration of INCA-6. Impairment of hippocampal function has been linked to the development of AD and results suggest that administration of INCA-6 may confer protection to hippocampal neurons against scopolamine-induced neurotoxic effects.

Furthermore, the above findings suggest that administration of INCA-6 may positively influence synaptic plasticity and neuronal communication within the hippocampus and cerebral cortex regions of the brain that are involved critically in the processes associated with memory [70].

According to the cholinergic hypothesis of AD, cognitive decline is caused predominantly by a decrease in acetylcholine levels in the brain, ultimately leading to impaired learning, memory, and attention. The depletion of cholinergic neurons and the increase in activity of acetylcholinesterase (AChE), the enzyme responsible for metabolizing acetylcholine, are commonly cited reasons for decreased levels of acetylcholine within AD [71].

In this study, treatment with INCA-6 significantly decreased brain AChE activity compared to scopolamine-treated rats. This supports the hypothesis that INCA-6 may increase cholinergic neurotransmission by inhibiting AChE activity at synaptic junctions resulting in greater acetylcholine availability.

It has been well documented that AChE inhibition plays an central role in the therapeutic management of AD. Currently, available medications such as donepezil, rivastigmine, and galantamine accomplish their intended effects predominantly through this mechanism.

However, unfortunately these agents only provide symptomatic treatment of AD and have other associated negative effects. As a modulator of cholinergic activity, INCA-6 could potentially represent another alternative or adjunctive therapy to current drug therapies. If it is found to have an acceptable safety record, it may also provide therapeutic advantages over traditional cholinesterase inhibitors [22]. Consequently, the in vivo cholinergic modulation demonstrated with INCA-6 supports its potential as a viable anti-Alzheimer Therapy.

A significant contributor to the development of AD is oxidative stress. When there is a difference between the amount of reactive oxygen species produced and the body's ability to protect itself from those reactive oxygen species, oxidative stress develops. When there is an extra of reactive oxygen species, they create cellular damage to lipids, proteins and DNA. The overall results of the reactive oxygen species cause the death of neurons and damage to neurons.

The current study showed that the administration of scopolamine improved the production of oxidative stress markers as proved by the increase in malondialdehyde (MDA) and the decrease in the activity of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH).

Recent studies have shown that the administration of scopolamine causes oxidative damage to the central nervous system. The treatment with INCA-6 caused a raised in levels of antioxidant enzymes and a decrease in lipid peroxidation levels. These results suggest that INCA-6 has potent antioxidative properties and may assist in protecting neurons [72].

Oxidative stress is an area of research that has received attention in the improvement of therapies for AD. In recent research studies, compounds that are considered to be antioxidants have been shown to decrease the formation of amyloid-beta protein aggregates, lower the phosphorylation of tau protein and enhance the function of mitochondria. Therefore, INCA-6 has antioxidative activity and this activity may play a significant role in the reduction of neurodegeneration associated with AD. Treatment with INCA-6 resulted in an rise in levels of antioxidant enzymes and a decline in levels of lipid peroxidation. This suggests that INCA-6 possesses strong antioxidant properties, which may protect neurons from oxidative damage [73]. Therefore, the antioxidant activity of INCA-6 is likely a major contributor to its neuroprotective effects.

Neuroinflammation is also involved with the pathogenesis of AD. It occurs with activation of microglia and astrocytes that cause the release of pro-inflammatory cytokines such as  $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$ , and IL-6. Chronic inflammation can lead to neuronal injury, and worsen disease progression. Anti-inflammatory effect can be produce by INCA-6 and thus the improvement in both behavioral and biochemical parameters can be indicated by it but the inflammatory markers were not specifically measured in this study. This hypothesis is supported by new studies showing that compounds that possess antioxidant activity also possess anti-inflammatory activity. INCA-6 has demonstrated a decrease in oxidative stress, and may indirectly inhibit inflammatory pathways since oxidative stress and inflammation are linked. In future studies, the effects of INCA-6 on specific inflammatory markers will need to be identified to support this mechanism [68]. Oxidative stress is intimately associated with inflammation, therefore antioxidant properties of INCA-6 may also contribute to its anti-inflammatory activity.

## Chapter 6

# Conclusion, Limitations and Recommendations

### 6.1 Conclusion

From the results of our study we have concluded that the INCA-6 has shown effective results in neuroprotection and cognitive enhancement in scopolamine induced animal of Alzheimer's disease. Morris water maze, T-maze, Y-maze and ELISA analysis was done in order to evaluate these effects in the spatial memory, hippocampal related memory, cholinergic modulation, antioxidant activity, and potential anti-inflammatory mechanisms. The findings highlights that the compound INCA-6 is a capable multi-targeting intervention for Alzheimer's disease. However, further studies are required to validate these results and explore its clinical applicability.

### 6.2 Limitations of the Study

Despite the promising results, several limitations should be acknowledged such as: The scopolamine model represents acute cognitive impairment and does not fully mimic the chronic nature of Alzheimer's disease. Some key pathological features

such as amyloid-beta and tau protein alterations were not evaluated, Neuroinflammatory markers were not measured, Histopathological analysis of brain tissues was not performed and also Long-term effects of INCA-6 were not assessed.

### **6.3 Recommendation for Further Studies**

Future research should be focused on evaluating INCA-6 in transgenic Alzheimer's disease models but also investigate its effects on amyloid-beta and tau pathology. The study should be conducted on long-term models to assess disease-modifying potential and explore molecular mechanisms using advanced techniques.

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