Comparative Study of Effects of Metformin and Donepezil in $AlCl_3$ Induced Oxidative Stress in the Brain of AD Mouse Models

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A thesis submitted in partial fulfillment of the requirements for degree of

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DEDICATED TO

My beloved parents and my lovely sister!

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ABBREVATIONS

AD	Alzheimer's Disease
Aβ	Amyloid Beta
NFTs	Neurofibrillary Tangles
T2DM	Diabetes mellitus type 2
$AlCl_3$	Aluminium chloride
APP	Amyloid Precursor Protein
ROS	Reactive Oxygen Species
IPG	Immobilized pH-gradient
PMSF	Phenylmethylsulfonyl fluoride
PTMs	Post translational modification
DDT	Dithiothreitol
SDS	Sodium dodecyl sulphate
2DE-GE	Two-dimensional electrophoresis
AMPK	AMP kinase
DCX	Doublecortin
PBS	Phosphate buffer saline
T2DM	Diabetes mellitus type 2
$AlCl_3$	Aluminium chloride
PSEN1	Presinilin
BACE1	β-site amyloid precursor protein cleaving enzyme 1
CA	Cornu Ammonis
kDa	Kilo Daltons
DG	Dentate gyrus
MCI	Mild cognitive impairment

ABSTRACT

Alzheimer's Disease (AD) is a polygenic progressive neurological disorder which effect about 45 million people worldwide. It is characterized by the deposition of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs) in the brain. The FDA approved drugs for AD temporarily slower the progression of the disease however there are no therapeutic drugs that permanently inhibit progression or reverse the neural degradation in AD. Emerging evidence indicated molecular association between diabetes mellitus type 2 (T2DM) and AD that led to examine the effects of several anti-diabetic medications against AD. In this study, we performed a comparative assessment of therapeutic effects of Metformin, a widely prescribed anti diabetic drug and Donepezil, a classical drug for AD that inhibit cholinesterase and in turn up-regulate acetylcholine thus enhancing cholinergic transmission. The aluminium chloride ($AlCl_3$)-induced neurotoxicity mouse models were used to study AD associated aberrations and were placed into two groups treated with Donepezil and Metformin (n=8, each group), while the control group was administered distilled water only. A 2-Dimensional Gel electrophoresis and SDS-PAGE analysis was performed to evaluate the differentially expressed proteins in the treated groups. $AlCl_3$ induced the formation of Aβ plaques in hippocampus, whereas significant differences were observed in Donepezil and Metformin-treated groups evident through histological assessment of the treated tissue sections. Interestingly, Metformin-treatment reduced the Aβ plaques formation and normalized the protein expression pattern. Moreover, molecular docking analysis was also performed to compare the binding potential of Donepezil and Metformin with pathologically significant proteins involved in AD associated molecular dysregulation. Interestingly, Metformin showed strong binding affinity with

the target proteins. Doublecortin (DCX), Presinilin (PSEN1), and Ki-67 showed the most stable conformations in comparison to Donepezil. In conclusion, these findings reemphasize proteomic alterations in AD and suggested a potential effect of antidiabetic drug on aberrant protein expression which provides basis for future drug repurposing for better therapeutic interventions for AD.

Chapter 1

INTRODUCTION:

Alzheimer's disease is a polygenic and multifactorial disorder, which is characterized by neurodegeneration. Globally, it affects approximately 45 million people. (Prince et al., 2016). The main characteristics of AD are persistent memory loss and cognitive decline. AD is largely sporadic, and ageing was found to be the most potent risk factor at late onset, whereas early onset familial AD was determined to be associated with APP or Presenilin mutation (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). Genetic risk factors i.e. APOE- ε4 allele carrier, TREM2 variants, brain damage, cardiovascular risk factors, different GWAS loci and numerous environmental factors are considered to be the main causes of AD AD formation and progression (Baumgart et al., 2015; Coon et al., 2007; Killin et al., 2016). Genetic analysis also confirms the role of microglia (Efthymiou & Goate, 2017; Hardy et al., 2014a; K.-l. Huang et al., 2017).

The development of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs) in the brain is a defining feature of AD (Brion et al., 1985; Grundke-Iqbal et al., 1986; Prince et al., 2016). The NFTs are consist of aberrant tau protein, while amyloid plaques are made up of extracellular amyloid aggregation. They are also linked to impaired glucose metabolism and inflammation induced through microglia. Association of synaptic loss and tau pathology with impaired cognition and β-secretase BACE1 and the γ-secretase induced lysis of Amyloid precursor protein's derived aggregates of amyloid-β is also reported (Sun et al., 2012). The soluble fragment (sAPP) produced via proteolytic cleavage inhibit Aβ formation which are considered to be toxic, and its

altered medication and aggregation in brain is determined to be related with the onset of AD pathological cascade (Cole & Vassar, 2007; Selkoe, 1998; Vetrivel et al., 2006).

The hippocampus is a primary target of neurofibrillary tangles (NFT), Aβ, and neuronal loss, all of which are hallmarks of AD. Their number and spatial distribution are linked to cognitive loss in the course of AD (H Braak et al., 1998). The NFT first affects the brain in the entorhinal and perirhinal cortex, which targets hippocampal cornu Ammonis (CA) subfields, linked cortex, lastly the primary neocortex to disrupt the genesis of the perforant route projection to the hippocampus. Extracellular amyloid plaques on the other hand are made up of aggregated Aβ peptides that have a spatiotemporal distribution less predictable as compared to NFT. They mostly build up in the isocortex, and their presence is unrelated to dementia (Heiko Braak & Braak, 1991; Giannakopoulos et al., 2003; Thal et al., 2002).

To date there are no therapeutic drugs developed for the inhibition of AD progression or reversal of neural degradation caused by AD, however four FDA approved drugs were found to temporarily slower or inhibit the progression. Donepezil, Rivastigmine, and Galantamine were found to inhibit cholinesterase and in turn up-regulates acetylcholine and are prescribed in mild to moderate stages (Association, 2013; Herrmann et al., 2011). Donepezil (Sugimoto et al., 2002), which has selective reversible activity in Central nervous system (CNS) and other tissues is an acetylcholinesterase (AChE) inhibitor. Tacrine another AChE inhibitor, is not as effective as Donepezil but shows more selectivity than butyrylcholinesterase (BuChE) for AChE (Giacobini et al., 1996; Nochi et al., 1995; Sugimoto et al., 2002). Another compound Memantine dosage was determined to up-regulate another neurotransmitter, glutamate, and is prescribed in moderate to severe stages.

A strong association between Diabetes Mellitus Type 2 (T2DM) and AD is established (Baker et al., 2011; Profenno et al., 2010). This relation between T2DM and AD as well as high prevalence of these disorder among the elderly has led to the use of many anti-diabetic drugs and some of them have been found effective against AD (Gold et al., 2010; Harrington et al., 2011). Clinical trials have demonstrated that insulin improve cognition in dementia patients (Reger et al., 2006; Reger et al., 2008). In addition, clinical trials on one of the FDA-approved anti-diabetic drugs called thiazolidinediones which is a PPAR agonists has shown better cognition and memory in AD patients (Jiang et al., 2008; Risner et al., 2006; Ryan et al., 2006; Watson et al., 2005). The effects of another FDA-approved drug metformin is likely to be investigated. Metformin (GlucophageR, 1, 2-dimethylbiguanide hydrochloride) an insulin sensitizing drug is a biguanide with pleiotropic metabolic effects such as insulin sensitization, decreased liver glucose synthesis, increased absorption of glucose, AMPK activation and mitochondrial inhibition (Hundal et al., 2000; Jiang et al., 2008). It is the world's most regularly prescribed oral T2DM treatment. It increases insulin signalling by activating AMPK, which then inhibits the negative feedback loop to insulin receptor substrate 1 mediated by mTOR/p70S6K (Boura-Halfon & Zick, 2009). In T2DM, the activation of AMPK has positive effects on insulin sensitivity, it lowers hyperglycaemia and contribute to decreasing gluconeogenesis in the liver. Another intriguing function of AMP is that it regulates tau phosphorylation, Aβ synthesis, and autophagy, all of which are important events in AD. (Salminen et al., 2011).

1.1 RESEARCH OBJECTIVE:

- To look into the effect of metformin on protein expression of $AlCl_3$- induced mouse models of AD.
- To compare the efficacy of metformin with a choline acetyltransferase inhibitor Donepezil in AD mouse models.
- To study the morphological changes and plaque formation through histopathological examination of treated brain regions as compared to control.
- Mapping differential proteome profile of hippocampus by performing SDS-PAGE and 2DE.
- To compare the interaction of donepezil and metformin against AD proteins through molecular docking.

Chapter 2

LITERATURE REVIEW

2.1 Alzheimer's Disease

Alzheimer's disease was named after Alois Alzheimer, a German psychiatrist. It is a slowly progressing neurodegenerative condition characterised by the accumulation of neuritic plaques and neurofibrillary tangles (NFTs) in brain, particularly in the medial temporal lobe and neocortical area, due to the formation of amyloid beta peptides (Correia et al., 2012). The NFTs consist of unhealthy tau protein and amyloid plaques contain extracellular aggregation of Aβ. The main characteristics of AD is continuous loss of memory and decline of cognitive function. There are about 50 million patients of AD in the world right now, after five years the no is expected double and by 2050 it is estimated to reach to 152 million. The estimated annual global cost of AD US$1 trillion which badly effects people, their family and the overall economy (Livingston et al., 2020; Yiannopoulou & Papageorgiou, 2020).

2.2 Alzheimer's Disease Neuropathology:

On a macroscopic level, Alzheimer's disease patients have mild cortical demage , particularly in the frontotemporal association cortex (Frisoni et al., 2010; Gearing et al., 1995; Tarawneh et al., 2015). After microscopic examination diagnosis is made based on the post-mortem brain. At this point, amyloid plaques, neurofibrillary tangles, and neuronal death were the pathological risk for AD. These cause severe brain injury, as a result of which the structural and functional brain circuit disrupt (Perl et al., 2010).

2.2.1 Neurofibrillary Tangles:

NFTs are fibrous intracellular aggregations of hyperphosphorylated tau, the main component of which is a microtubule linked protein. (Iqbal et al., 1986; Lee et al., 1989). Microtubules being a potent protein structures found in the cytoskeleton of cells. They are involved in cell shape maintenance, organelle trafficking in the cytoplasm, and chromosome separation during mitosis. (Song & Brady, 2015). phosphorylation state of tau governs function of tau to bind and stabilise microtubules. Non-phosphorylated tau binds to microtubules, whereas phosphorylation causes tau to dissociate, causing microtubules to dismantle (Biernat et al., 1993). When tau is hyperphosphorylated in pathological situations like Alzheimer's disease, so the equilibrium in tau binding to microtubules is disrupted (Goedert et al., 1992; Grundke-Iqbal et al., 1986; Lee et al., 1989), There are two significant ramifications. First, microtubules become unstable, causing a disruption in neuronal architecture and cellular component trafficking along axons. This has a significant impact on the capability of neurons which is extremely long procedure (Ballatore et al., 2007). Secondly, free unbound hyperphosphorylated tau levels rise in the cytoplasm, resulting in aberrant tau aggregation in fibrillary formations known as paired helical filaments (PHFs) (Iqbal et al., 1986). These aggregates are produced by pairs of fibrils joined in a helical way, as revealed by ultrastructural investigation, hence the name. PHFs subsequently self-assemble to generate NFTs, which are filamentous inclusions (Alonso et al., 2001).

NFTs are a major neuropathological characteristic of Alzheimer's, although they also appear in a variety of other disorders. Tauopathies are a group of illnesses describe as the aggregation of insoluble tau inside the brain, which usually induce dementia and motor system degeneration (Williams, 2006). As most frequent of these is Alzheimer's

disease, followed by type C Niemann-Pick disease, corticobasal degeneration, post-encephalitic parkinsonism, and the Parkinson's disease complicated of Guam (Williams, 2006). All of them share a problematic intracellular build-up of tau, which leads to neurodegeneration and cell death, at least in part (Regan et al., 2017). All of them share a problematic intracellular build-up of tau, which leads to neurodegeneration and cell death, at least in part (Regan et al., 2017). As a result, medications aimed at reducing tau aggregation or enhancing microtubule stability is being studied as possible treatments tauopathies (Anand et al., 2014; Ballatore et al., 2007).

2.2.2 Amyloid Plaques:

The Aβ peptide, which is caused by a transmembrane cleft glycoprotein known as the APP protein, is the main component of amyloid plaques, known as "senile" and "neuritic" plaques (Kang et al., 1987). Although evidence suggests that the APP protein is involved in cell proliferation, motility, and neurite outgrowth, the detailed function of the APP protein in neuron physiology is uncertain (Oh et al., 2009; Young-Pearse et al., 2007). However, A refining progression that this protein can go through are well-known. The non-amyloidogenic process undergo enzymatic cleavages by both α and γ-secretases, as a result no Aβ peptides are produced; however, amyloidogenic process, the cleavage site converts to α and then γ-secretases, resulting in the formation of 4 kDa fragment of Aβ peptide (O'brien & Wong, 2011). In the extracellular region Aβ peptide is let out, as a result it forms amyloid plaques with other proteins by aggregating into a β-sheet structure (Castillo et al., 1997; Dickson et al., 1997; Palop & Mucke, 2010).

The pathogenic Aβ aggregates builds up in the brain, in combination with the hereditary component APO-E4, increasing the likelihood of developing AD and loss in cognition

(Farrer et al., 1997; Hardy et al., 2014b). The Synaptic plasticity and signalling are negatively impacted by Aβ are one popular theory for how Aβ can impair cognition. The idea that Aβ decreases glutamatergic synaptic transmission is backed up by a significant amount of evidence from studies of Alzheimer's disease mouse models (Chapman et al., 1999; Hsia et al., 1999), that Aβ reduces excitatory synaptic transmission by reducing the no of surface α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs) (Hsieh et al., 2006; Shankar et al., 2007). Evidence indicates Aβ reduces long-term potentiation (Cleary et al., 2005; Walsh et al., 2002) and increases long term depression (Hsieh et al., 2006; Li et al., 2009) of synaptic transmission underpins the current model of Synaptic dysfunction caused by Aβ. In animal models, LTP and LTD are two important chemical processes that regulate memory function, so their disturbance has been linked to synapse loss and memory issues (Martin et al., 2000). Significantly, there is mounting evidence that soluble Aβ oligomers are more essential in causing these effects than Aβ fibrils and amyloid plaques (Cheng et al., 2007; Shankar et al., 2008; Tomiyama et al., 2010), as connect more closely to cognitive deterioration (Mucke et al., 2000; Näslund et al., 2000). As a result, attempts are being made to better understand the pathogenic mechanisms.

2.3 Alzheimer's Disease and Oxidative Stress:

Oxidative stress is a process characterised by a disturbed redox balance, which occurs either due to ROS production or reduce in efficiency of antioxidant system of the body to get rid of the stress. It may have a vital part in age related neurodegeneration and decline in cognition (Christen, 2000; Retz et al., 1998). The glutathione is responsible for the antioxidant defence mechanism in the body (Thornalley, 1998). The main job of glutathione is to scavenge ROS by donating electrons to them. It has been found that

the concentration of glutathione decreases with increasing age (T. S. Chen et al., 1989).This decrease in the concentration of intracellular glutathione disturbs the equilibrium between ROS production and capability of the antioxidant system of the body thus resulting in increased oxidative stress. Research shows that oxidative stress that is caused due to mitochondrial damage also contributes to early phases of AD before the manifestation of Aβ pathology (Uttara et al., 2009). The higher level of oxidative stress in Alzheimer patients is related to abnormally more production of Aβ and neurofibrillary tangles (Christen, 2000). The oxidative stress markers were found inside the post-mortem brain tissues of people with early stages of AD (Sultana et al., 2011). Different discovers showed that besides mitochondrial damage associated ROS production the abnormal homeostasis of some metals i.e. iron (Fe), zicopper (Cu), magnesium (Mg), nc (Zn), and Aluminium (Al) may also contribute to free radicals production leading to Aβ and tau aggregation (Beal, 2005). During an inflammatory response to Aβ plaques the microglia is activated which is also involved in ROS production (Nakajima & Kohsaka, 2001). With the increase in Ab production ROS levels are also increased which binds to mitochondrial membranes thus altering the function of mitochondria which contributes to disturbed energy metabolism and synaptic function loss (Beal, 2005). The membrane associated oxidative stress that is caused as a result of Aβ plaques formation disturbs the metabolism of ceramide and cholesterol which then starts a neurodegenerative cascade resulting in further accumulation of Aβ (Mattson et al., 2005).

2.4 Hippocampus and Alzheimer's Disease:

The hippocampus is the most studied part in brain and emaciation of this part is associated with clinical consequences. It is an important structure of the brain that is critically affected in many neuropsychiatric disorders like AD (Stone et al., 2011). The

hippocampus head, the anterior component, body, and tail are the primary divisions (Duvernoy, 2005). The hippocampus is classified as the cornu ammonis subfields (CA1-4), the dentate gyrus (DG), and the subiculum (Duvernoy, 2005) based on its cytoarchitecture. In the majority of AD patients Hippocampal emaciation is one of the earliest signs of continuous neurodegeneration (Jack et al., 2002). In clinical trials for AD, it has been used as diagnostic and prognostic marker (Mueller et al., 2010). During the shift from MCI to AD dementia, hippocampal shrinkage progresses nonlinearly, exhibiting sigmoidal patterns as it accelerates (Jack et al., 2002).. Patients having mild cognitive impairment (MCI) show a hippocampal volume loss of 10-15%, but those early Alzheimer's disease patients suffer a loss of 15-30%. (Frisoni et al., 2010) It can reach 50% in people with moderate Alzheimer's disease. (Dhikav et al., 2011).

There is growing evidence that suggests encouragement of adult hippocampus neurogenesis improve pattern separation and spatial memory (Dhikav et al., 2011; Frisoni et al., 2010). A loss in neurogenesis, on the other hand, could be at the basis of cognitive impairments linked with ageing and illnesses as AD. Surprisingly, mounting data propose that central molecular players in AD control the creation of new hippocampus neurons, and that significant changes in neurogenesis occur prior to the start of hall- mark lesions or neuronal loss (Lazarov & Marr, 2010). The preservation or enhancement of new neuron Development has been proposed as a viable therapeutic method. technique for postponing or preventing the cognitive loss linked with AD. Furthermore, knowing the processes of alterations in neurogenesis in the early and late stages of AD can aid in the development of early AD biomarkers and give light on the aetiology of AD.

2.5 Symptoms:

The inability to remember new knowledge is the initial indication of AD, which is caused by the failure of neurons in memory-related parts of the brain, for instance the hippocampus. Multiple cognitive impairments emerge as the disease advances and other brain areas degrade, however the rate at which it evolves differs from person to person (Y. Huang & Mucke, 2012). Problems with daily tasks are one of them, problem solving, speaking and writing, mood swings, and time and place confusion (Alzheimer's & Dementia, 2018). These symptoms are mostly common in AD patients, however, other form of dementias can also show these symptoms.

2.6 Diagnosis:

AD Patients are typically diagnosed in their later instant, When the primary diagnosis is clinical, cognitive testing and neuropsychological examination are used. (McKhann et al., 2011). The development of new biomarkers which identify the preclinical stage of Alzheimer's disease that can be employed in the clinic is one promising approach. A biomarker strategy based on the identification of two pathologically significant proteins in Alzheimer's disease, amyloid β42 (Aβ 42) and tau protein, in cerebrospinal fluid, for example, has been proposed. (Jack Jr et al., 2010). Furthermore, Early AD has been detected using various imaging methods including positron emission tomography (PET) for tracking the Aβ levels, and magnetic resonance imaging (MRI) that detect brain atrophy which corresponds to impairment in cognition in clinical trials (Mufson et al., 2008; Onor et al., 2007).

2.7 Risk Factors:

Alzheimer's disease, like many other complicated disorders, has no one cause, but rather the product of complicated interactions between multiple sources. These elements include both hereditary and environmental variables., increasing age, vascular diseases, head injury, and some infections. Some of the main risk factors are briefly discussed below.

2.7.1 Genetics:

Over the years, genetic factors have been discovered to have a significant part in the development of AD. It has been discovered that Around 70% of AD cases were shown to be linked to hereditary factors. The majority of EOAD cases are autosomal dominant, Amyloid precursor protein, The presinilins (PSEN-1 &2) and apolipoprotein E mutations have all been linked to Alzheimer's disease. (Khanahmadi et al., 2015; Van Cauwenberghe et al., 2016). Some of the most potent genetic risk factors are discussed below.

2.7.1.1 Amyloid Precursor Protein:

The gene of APP lies on chromosome 21 coding for a type l transmembrane protein, cut by α, β and γ-secretase releasing Aβ. There are about 30 mutations reported in APP gene out of which 25 are associated with AD and results in the build-up of Aβ in large amount. Some of the mutations are protective in nature which decreases the Aβ deposition while other results in increasing the accumulation of Aβ. A protective mutation (A673) was reported in APP which causes a decrease in Aβ deposition. A mutation in proximity to the secretase cleavage site such as KM670/671NL has shown increased Aβ plaques in hippocampus and cortex however no NFTs were observed. A

deletion mutation E693delta causes the increase in deposition of synaptotoxic Aβ (Dai et al., 2018; J. Zhao et al., 2020).

2.7.1.2 Apolipoprotein E:

The ApoE, belongs to glycoproteins that is abundantly synthesised in the liver and astrocytes of the brain, as well as certain microglia. This protein play as ligand for the receptor mediated endocytosis of lipoprotein particles like cholesterol, that is necessary for build-up of myelin and proper function of the brain. ApoE2, ApoE3, and ApoE4 are the three isoforms of ApoE. SNPs on the coding area. In comparison to ApoE2 and ApoE3, ApoE4 is highly linked to both EOAD and LOAD. The ApoEε2 allele is associated with lower risk while the ApoEε3 causes protective effect (Kim et al., 2009). ApoE4 is implicated in vascular injury in the brain, which contributes to AD pathogenesis (Van Giau et al., 2015), as well as Aβ deposition, which results in senile plaque and produces cerebral amyloid angiopathy. (Liu et al., 2013)

2.7.1.3 Presenilins:

PSEN1 and PSEN2 genes lies on chromosome 14 and 1 respectively. PSEN1 gene mutations are more common, with over 200 identified, whereas PSEN2 gene mutations are rare, with less than 40 identified (Cai et al., 2015). PSEN1 a key protein which activates the γ-secretase complex which is complex in the synthesis of Aβ from APP. PSEN1 knockout mice demonstrated synaptic disruption and memory impairment, indicating that It has a significant impact on memory. and neuron maintenance (Dai et al., 2018). PSEN1 mutations can be simple, resulting in a single amino acid replacement, or severe, resulting in two amino acid alterations (De Strooper, 2007). PSEN-2 mutations, on the other hand, are uncommon and only play a minimal impact

in the generation of Aβ. PSEN-2 Mutation could show a major impact on A 42/40 ratio on the healthy PSEN-1 alleles., resulting in familial AD. Some PSEN-2 mutations, like N141I, T122P, M239V, and M239I, result in a remarkable expansion in γ-secretase activity as well as an increase in the A-42 and A 42/40 ratio levels (Kelleher & Shen, 2017).

2.7.1.4 Other Genetic risk factors:

Polymorphism in vitamin D receptor (VDR) gene changes vitamin D ability to bind to its receptor and may nerve damage (Khorshid et al., 2013), is another gene polymorphism associated which will be more prone to AD. Epigenetic factors such as DNA methylation, histone changes, and modification of chromatin are being associated to Alzheimer's disease (Armstrong, 2019).

2.7.2 Environmental Factors:

Alzheimer's disease a complex disorder, affected by both genetic as well as environmental factors. The environmental factors that increase the susceptibility to AD include air pollution, diet, infections, metals. Some of these factors may cause oxidative stress and inflammation which helps in AD development. Some of the main environmental factors associated with AD (Grant et al., 2002; Wainaina et al., 2014).

2.7.2.1 Increasing Age:

Aging is the most potent risk factor in AD. Younger people are frequently affected by this disease, and many of its cases begin after the age of 65 (Guerreiro & Bras, 2015). Aging irreversibly effect different organs and cell systems causing the decrease in weight and volume of the brain, it also contributes to synaptic loss ventricular

expansion and NFT. Furthermore, various disorders like glucose hypometabolism, mitochondria malfunction, cholesterol dyshomeostasis, depression, and cognitive decline may develop as people age. These alterations are also seen in normal ageing, making it hard to differentiate instances of early Alzheimer's (Hou et al., 2019). On the basis of age Alzheimer's disease is classified into two types: early onset AD and late onset AD. The EOAD is a rare type, having 1–6% of cases, the occurrence of more than one individual with Alzheimer's disease in more than one generation distinguishes this condition, which affects people aged 30 to 60 years. The other type is more prevalent in people above the age of 65. Both types can affect people having a positive family history of Alzheimer's disease also in families with a late-onset condition (Bekris et al., 2011).

2.7.2.2 DIET:

The importance of diet in AD has grown in recent years. Risk of AD being decreased by some of the dietary supplements including vitamins, fish, antioxidants. however high calorie intake and saturated fatty acids were involved with high risk of AD (Hu et al., 2013). Heat-sensitive micronutrients e.g., vitamin C and folates are destroyed, much water are lost, and harmful secondary products are created. By altering, structure and function of cell surface receptors and body proteins, AGEs can cause oxidative stress and inflammation. is referred to as their toxic effect. Several studies have found that more levels of AGEs in the blood are associated to decline in cognition and the progression of Alzheimer's disease. The AGE receptor (RAGE) is found in a number of tissues, including microglia and astrocytes which is overexposed in the AD patients brain, where it functions like a bearer and a cell surface receptor for Aβ (Abate et al., 2017). Some other threat for Alzheimer's disease is malnutrition, AD patient's disease

has difficulties in eating and swallowing, leading to malnutrition. The deficiency in nutrients like Vitamin B12, Vitamin D and folate may result in cognitive decline. (Koyama et al., 2016).

2.7.2.3 Metals:

Metals being part of nature as well as biological system are classified as bio-metals. (Adlard & Bush, 2006). It has been found that certain metals play in the development and progression of AD (Huat et al., 2019). Aluminium is used in a variety of industries, including processed foods, cosmetics, medical treatments, and pharmaceuticals. Aluminium is connected to plasma transferrin and citrate molecules in the body, and these molecules can enable aluminium transfer to the brain. Al builds up in the cortex, hippocampus, and cerebellum, interacting with proteins leading to synthesis of misfolded and aggregated proteins, it also causes the increase in phosphorylation of different proteins such as tau which are highly phosphorylated which is one of the characteristics of AD. (Colomina & Peris-Sampedro, 2017). Lead competes with bio-metals such as calcium for binding sites which quickly pass the blood brain barrier, where it has the potential to disrupt neuronal development and synaptogenesis and cause serious damage. Acute lead exposure was linked to Alzheimer's disease, according to studies, and enhanced -secretase expression and Aβ build-up. Cadmium is also of the hazardous metals which can pass through BBB causing neurological illnesses. Cadmium ions have been linked to the deposition of Aβ plaques and the self-aggregation of tau in the brain of AD patients.

2.8 Treatment:

Despite the fact that AD is a health concern publicly, there are currently only two medication classes that have been approved to treat it: cholinesterase inhibitors and NMDA antagonists. AD reduce cholinergic transmission in the brain by damaging Ach producing cells. Ach is prevented from being broken down by cholinesterase enzymes which leads to high Ach levels in the synaptic cleft (Eldufani et al., 2019). NMDAR antagonists restore normal activity by preventing overactivation of NMDAR glutamate receptor and hence the influx of Ca^{2+} ions. These two classes, notwithstanding their therapeutic impact, are only beneficial in treating the symptoms of Alzheimer's disease; they do not cure or prevent the condition (Wang & Reddy, 2017). Different studies proposed for better understanding of AD pathophysiology and develop effective treatments, including altered metabolism of tau protein metabolism, β-amyloid, inflammatory response, and damage associated with free radicals. (Briggs et al., 2016). Most modifiable risk factors for AD, such as cardiovascular diseases or lifestyle, can on the other hand be avoided without medical intervention. In older people physical activities have shown positive effects on the health of brain, as it decreases AD by promoting neurogenesis, increasing vascularization in the brain and decreasing inflammation by reducing Aβ synthesis thus resulting in improved cognition. Furthermore, The Mediterranean diet, higher education and intellectual engagement all have the potential to temporarily inhibit the development of AD and loss of memory while enhancing the brain capacity and cognition.

2.8.1 Cholinesterase Inhibitors:

In AD, cholinergic neurotransmitter engagement is lower. Cholinesterase inhibitors are hypothesised to act by slowing the breakdown of acetylcholine. Tacrine was the first-

generation cholinesterase inhibitor, but it was associated with hepatotoxicity, which limited its use (Manning, 1994). Currently, donepezil, galantamine, and rivastigmine are approved for the treatment of AD, with the latter also accessible as a transdermal patch. Because all three are similarly effective and may temporarily improve cognition.

2.8.1.1 Donepezil:

Donepezil is started at a dose of 5 mg and can be increased to 10 mg after a month if necessary (Health & Excellence, 2011). The response of patient to the treatment is measured through rating improvement in memory and behaviour.

The patient's or caregiver's response is measured by a rating of improved memory, function, or behaviour. It is fruitless to employ fast mental status assessments like the short mental state examination, because they're not designed to discover clinically significant changes. After three months of treatment if no response is found, then quitting the medication at that point will be appropriate, some basic side effects include Gastrointestinal, exhaustion, and muscle cramps, so patients may have an electrocardiogram test before initiating a cholinesterase inhibitor because sick sinus syndrome and other abnormalities may take place. Starting a cholinesterase inhibitor in patients with a history of peptic or duodenal ulcer disease should be done with caution. After commencing the medication, small proportion of patients experience an immediate worsening of cognition or agitation; in this instance, the prescription should be withdrawn as quickly as possible. On average, the effects on cognition and function are mild (JS, 2003), and response rates vary, with almost one third of patients exhibiting no improvement and about a fifth showing a greater advantage. Because of the possibility of a negative effect, it's predicted that one-third of patients won't be able to take a cholinesterase inhibitor.

2.8.1.2 Memantine:

The NMDA receptor is inhibited by memantine (Robinson & Keating, 2006) in a non-competitive way, as a result it may be neuroprotective by avoiding neuronal loss and alleviating sign and symptoms via helping in the repair of effected neurons function. Initially Memantine started at a dose of 5 mg per day, with an increase of 5 mg up to a maximum dose of 20 mg weekly (Anjum et al., 2018). Although dizziness, headache, constipation, as well as hypertension are mostly the adverse effects, as compared to cholinesterase inhibitors It is well tolerated and has fewer adverse effects in general. Memantine has been considered to be less beneficial in moderate to severe AD, since There is minimal proof that it may be effective. in milder types of the condition (Areosa et al., 2005) Additionally, a combination of memantine and donepezil monotherapy is effective for persons with mid-stage AD with the one losing cognitive function (Atri et al., 2013), Both memantine and donepezil are ineffective. As such drugs are mostly available pharmacological treatments for AD, their total effect is small and they have no impact on the neurodegenerative process at all. Medications such as cholinesterase inhibitors are unlikely to have an effect since cholinergic transmission is downregulated too late in the process (Mufson et al., 2008).

2.8.1.3 Rivastigmine:

Rivastigmine is a derivative of carbamate that has a reversible inhibitory effect on acetyl (AChE) and butyryl (BuChE) cholinesterase (Onor et al., 2007). Which is only ChEI that inhibits BuChE in a meaningful way. As in the central nervous system Butyrylcholinesterase is present and is considered to be taking part in cholinergic function as well as neurodegeneration (Darvesh et al., 2003). It is still unknown that how selective BuChE inhibition relates to rivastigmine's clinical effects. It binds to proteins 40 percent of the time, which is being hydrolyzed through esterases including

cholinesterases, which eliminates in urine. As in metabolism of rivastigmine cytochrome P450 isoenzymes are not involved, drug to drug interactions are minimised. Rivastigmine's starting dose is 1.5 mg two times a day, which may be slowly increased to a maximum dose of 6 mg two times a day. 3mg two times a day is the minimum effective dose. Since 2008, a transdermal version of rivastigmine has been offered in most markets (Blesa et al., 2007; Winblad et al., 2007). The major goal of transdermal rivastigmine is to allow for titration to the medication's greatest and almost effective concentrations while minimising negative effects. This is accomplished through a gradual release of the medicine into the circulation. The effective and highest dose of transdermal rivastigmine is 10 cm2, while its starting dose is 5 cm^2.

2.8.1.4 Galantamine:

Galantamine is a tertiary alkaloid that suppresses AChE activity in a reversible manner(Robinson et al., 2006). That will increase cholinergic action by binding allosterically to nicotinic receptors. In humans, the therapeutic significance of allosteric binding to nicotinic receptors is unknown. After oral administration, galantamine is rapidly absorbed. The half-life of elimination is 7-8 hours. It attaches to plasma protein 18% of the time and is processed by cytochrome P450 isoenzymes 2D6 and 3A4. Galantamine is present in an extended release formulation that allows for daily use. The initial dose for galantamine is 8 mg once a day. The lowest effective dose is 16 mg per day, and the highest effective dose is 24 mg per day. Galantamine is recommended as a medication option for combined AD and cerebrovascular illness.

2.9 Drug Repurposing for Alzheimer’s:

Developing new drugs to fight a disease is highly time consuming and expensive process. It needs 10-15 years and about 2 billion dollars for a new drug to be approved

by FDA with a success rate of only 2%. Due to these reasons drugs approvals has been reducing since 1995. Moreover, investment in the development of new drugs has also been reducing which shows that the cost of new drug development will continue to increase (Dudley et al., 2011). This combined with the rapidly increasing population with AD creates a dire need for rapid new drugs development. This concept of repurposing already approved FDA drugs of one disease to combat another disease is not new. A great number of drugs have been repurposed in the past with success and in many cases a drug of one disease was accidently found to be effective for another disease. In Traditional drug discovery the drug has to pass through so many steps before its approval such as pre-clinical and clinical trials, safety review and post market safety and monitoring (Morgan et al., 2011). While repurposed drugs on the other hand are already approved by FDA and are ready to be used. There is a huge amount of pharmacological data available due to exponential increase in research which has vastly reduced the need to develop new drug from ground up. Moreover, various wet lab and *insilico* approaches have been developed recently with the help of which new drug-disease associations can be discovered.

2.9.1 Diabetes type 3 and Alzheimer's:

Type 1 type 2 diabetes are well familiar however there is another type of diabetes known as type 3 diabetes (T3DM). Such type of diabetes is lesser known and is linked with insulin resistance in brain which badly effects the neurocognition and contribute to the pathogenesis of AD. Both AD and Diabetes are complex and multifactorial diseases influenced by both environmental and genetic factors. A large amount of research shows that Insulin insensitivity is associated with cognitive decline, as well as many other signs and symptoms that are linked with AD. The failure of target tissues to respond appropriately to insulin is described as insulin resistance. It is a common

occurrence which has been associated with the pathogenesis of AD in both in vitro and animal studies (Nguyen et al., 2020). Studies have showed that the systemic infusion of insulin in healthy adults have improved verbal memory and selective attention (Reger et al., 2006). Insulin have been found to be neuroprotective and is involved in memory enhancement (Kang et al., 1987). Moreover, the memory of AD patients has been found to be improved after insulin administration (Oh et al., 2009).

Alzheimer's disease, which is also classified as a type of diabetes, is associated with endocrine abnormalities, particularly diabetes. Diabetes has a well-documented impact on memory processing (recognition and retrieval), brain morphology (brain atrophy), and synaptic communication, all of which influence AD pathology (Correia et al., 2012). In accordance with all these findings it has been hypothesized that AD associated cognitive deterioration can be reduced by using treatment strategies involving antidiabetic drugs.

2.10 Donepezil in Alzheimer's Disease:

Donepezil the acetylcholinesterase inhibitor hydrochloride is mostly used for the treatment of AD. It is a reversible piperidine derivative of AChEI is highly selective with an action of finest pharmacological profile in terms of cognitive enhancement, with a responder rate of 40–58%, dropout cases 5–13%, and side effects 6–13%. It is FDA approved medication for mild, moderate, and severe Alzheimer's disease. There is no evidence that donepezil affects the disease's progression. However, through enhancing cognitive and/or behaviour, it may be able to alleviate some symptoms (Kumar et al., 2021; Seltzer, 2005).

Donepezil is an acetylcholinesterase inhibitor that acts centrally, quickly, and reversibly. Acetylcholine is degraded by acetylcholinesterase when it is released from

the presynapse. Donepezil interacts with acetylcholinesterase reversibly and stops the hydrolysis of acetylcholine, leading to the increase in the availability of acetylcholine at synapses hence enhancing cholinergic transmission. Invitro studies showed that the anticholinesterase activity of donepezil is selective for acetylcholinesterase. Donepezil contributes to increasing nicotinic receptors activity thus making it more neuroprotective. This activity does not play any role in clinical outcomes however it decreases voltage-activated sodium currents and halts rectifier potassium and fast transient potassium currents (Seltzer, 2005).

Some general safety measures should be taken in event of donepezil intake, as cholinergic crisis can occur if you take too much donepezil. Severe nausea, vomiting, sweating, and salivation are all signs of an overdose. Bradycardia, hypotension, respiratory depression, collapse, and seizures are all possible side effects and if respiratory muscles are affected, increasing muscular weakening might occur, which can lead to death. Hepatotoxicity has been reported in a few cases of overdosing (Hefner et al., 2015).

2.11 Metformin in Alzheimer's Disease:

Metformin is a biguanide insulin sensitizing drug. It is most commonly prescribed medications for type-II diabetes. It reduces insulin resistance and lowers hepatic gluconeogenesis, resulting in decreased plasma glucose levels (Syal et al., 2020). It is assumed that the arousal of AMP-activated protein kinase (AMPK), that enhances insulin signaling by inhibiting the mTOR/p70S6K-mediated negative feedback loop to insulin receptor substrate-1(Boura-Halfon & Zick, 2009). Metformin passes the blood-brain barrier, compared to other anti-diabetic drugs which is being studied in AD e.g. insulin secretagogues, starch digesting inhibitors, incretin-based treatments, and

thiazolidinedione PPAR agonists (Łabuzek et al., 2010). Till now, there has been minimal published data on the effects of metformin on Alzheimer's disease. In a high insulin induced neuroblastoma cell line model of AD, metformin decreases AD like abnormalities in tau hyperphosphorylation and Aβ overproduction. (Gupta et al., 2011). Another study discovered that metformin inhibited tau phosphorylation in primary neuron cells derived from a tau transgenic mouse. (Kickstein et al., 2010). Moreover, the composition of the gut microbiota is being damaged by metformin, which may have an importance in pathogenesis of Alzheimer's illness (Markowicz-Piasecka et al., 2017)

Chapter 3

MATERIALS AND METHODS

3.1 *Insilico* Methodology:

3.1.1 Protein Preparation

The 3-dimensional structures of all target proteins (APOE, Presenilin, Ki67, DCX, AChE, BACE1) RCSB Protein Data Bank was used to obtain (PDB) https://www.rcsb.org/ , This is a large, freely accessible online resource of drug and drug target information. Before docking simulations all proteins were prepared using Biovia Discovery Studio by removing unnecessary water molecules and other ligands attached. Then structures of all the proteins were saved in pdb format.

3.1.2 Ligand Preparation

The 3D chemical structures of Ligand Metformin and donepezil were retrieved in sdf format from the PubChem database https://pubchem.ncbi.nlm.nih.gov/ and then converted to pdb format using Smiley.

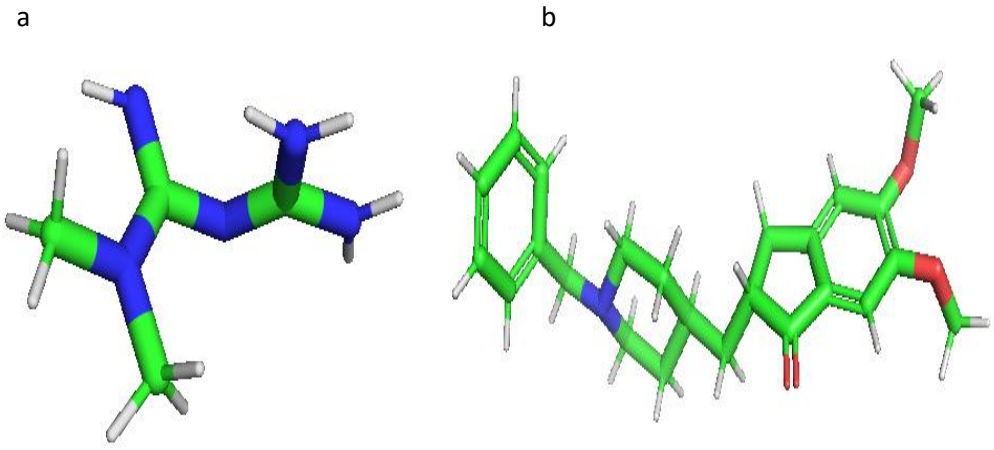

Figure 3.1: Chemical structure of (a) Metformin and (b) Donepezil

3.1.3 Molecular Docking Analysis:

For molecular docking simulations Patchdock server was used. It is a free online webserver which carries out the docking of the protein-small ligand molecule, available at https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php. The target protein and ligand pdb files were uploaded to the Patch dock server for docking analysis, with clustering RMSD set to 4.0 and protein small ligand complex type as the analysis settings.

3.2 WET LAB METHODOLOGY

3.2.1 Chemicals and Reagents:

Aluminium Chloride hexahydrate ($AlCl_3.6H_2O$) was obtained from Scharlau (Product catalogue# AL0770). Metformin Hydrochloride (Glucophage) and Donepezil Hydrochloride (Donecept) were synthesized by Merck Sereno and ATCO Laboratories respectively. Sigma-Aldrich (USA). provided all of the other compounds.

3.2.2 Animals:

BALB/c mice were kept and produced in the Atta ur Rahman School of Applied Biosciences (ASAB), National University of Science and Technology's lab animal house (NUST). All of the mice were housed in cages that were kept at temperature (25^oc) and on a natural light-dark cycle. (12-12 hours). The animals were given distilled water and fed with standard diet consisting of: crude protein 30%, crude fiber 4%, crude fat 9%and moisture 10.4%. Male mice (n=40) weighing 35-45g and age 6-7 weeks were used in experiments.

3.3.3 Ethics Statement:

Experiments were carried out in accordance with Laboratory Animal Research Institute, Division of Earth and Life Sciences, National Institute of Health, USA,

recommendations (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011). Internal Review Board (IRB), ASAB, and NUST approved the procedures.

3.3.4 Study Design:

A 29-day plan was devised to develop Alzheimer's Disease mouse models via oral $AlCl_3$ injection and evaluate the effects of Donepezil hydrochloride and Metformin hydrochloride on oxidative stress in these mice. On the 30^{th} day, the animals were decapitated for expression and histological analyses.

3.3.5 Animals Groups:

All the animals were divided into six groups. Each group consisted of a total of 6 animals of 6-7 weeks of age. The details of all the groups are provided in Table.

Table 3.1: Experimental design of animals with their group division

S/no	Groups	Treatment	Duration
1.	Control group	Distilled water and feed	29 Days
2.	$AlCl_3$ Group	600mg/kg $AlCl_3$	15 Days
3.	Metformin Group	300mg/kg Metformin	14 Days
4.	$AlCl_3$ +Metformin	600mg/kg $AlCl_3$	15 Days
		300mg/kg Metformin	14 Days
5.	Donepezil Group	15mg/kg Donepezil Hydrochloride	14 Days

6.	$AlCl_3$ Group	+	Donepezil	600mg/kg $AlCl_3$		15 Days
				15mg/kg Hydrochloride	Donepezil	14 Days

3.3.6 Study Design:

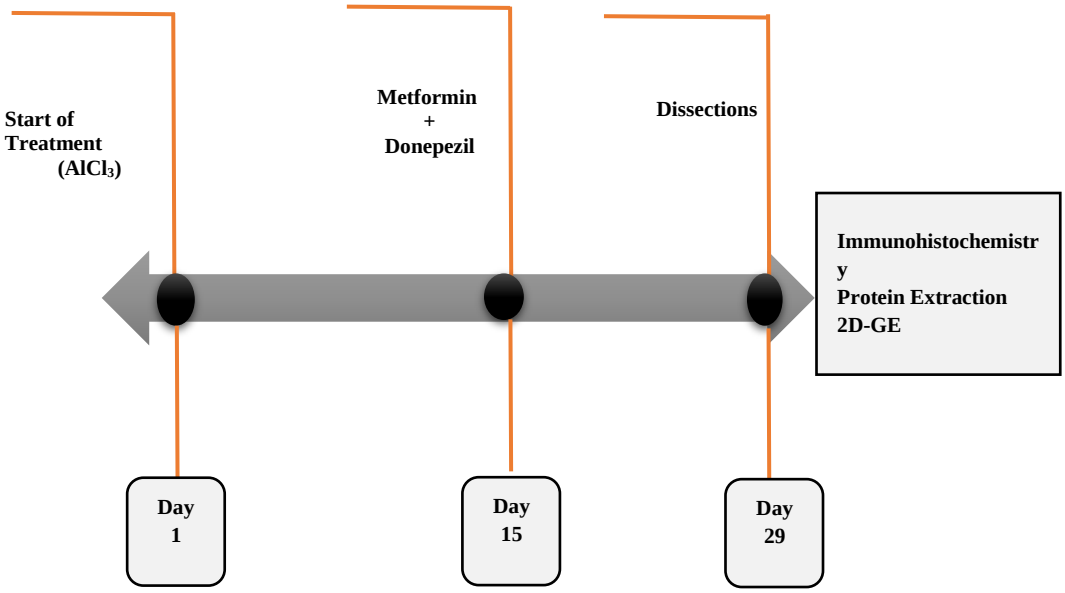

Figure 3.2: Experimental Plan. Balb/c male mice of 6-7 weeks of age received $AlCl_3$ for 15 days. On 16th day mice were switched to oral administration of Donepezil and Metformin Hydrochloride for another 14 days. On day 29 all the mice were sacrificed for protein extraction and their brains were processed for staining and immunohistochemistry.

3.4 Histological Examination of Brain Regional Tissues

3.4.1 Tissue Perfusion/Fixation for Histological Assessment:

Heart perfusion was carried out in accordance with the Gage protocol. Following that, the excised brain tissue was preserved in 4 percent paraformaldehyde for 24 hours at 4 degrees Celsius before being processed for paraffin processing and embedding. Prior to paraffin infiltration, the brain tissue was dehydrated for 24 hours in a series of alcohols (isopropanol), 70% (1 hour), 95% (1 hour), and 100% (1 hour). The brain tissues were then submerged in xylene for 4 hours before being paraffin imbedded in molten paraffin for 4 hours at 60 degrees ^{o}C, hardened in a mould (4 degrees ^{o}C), and cut.

3.4.2 Congo Red Staining:

The Congo red stain (working solution: 49.5 mL Congo Red (Stock) and 0.5 mL 1% NaOH)) was poured on the de-paraffinized brain sections and retained for 20 minutes. The sections were then washed with double distilled water (ddH_2O) and alkaline alcohol for 2 minutes. The sections were then counterstained by haemotoxylin for 30 seconds and further washed with 70% isopropanol for 6 minutes and then with ddH2O. After air-drying (1 h) the slides were mounted by cover slips and later visualized by Optika vision lite2.1 at 4 X, 10 X, 40 X resolution. The images were captured by Optika Vision Lite2.1 image analysis software.

3.5 Brain Dissection and Isolation of Hippocampus:

Chloroform was used to anaesthetize the mouse. The head was gently extended forward, and surgical scissors were used to make a cut posterior to the ears. A small

incision was made starting at the caudal position and continued into the anterior region of the skull with a hard cut. Using curved narrow forceps, the parietal bone on both sides was rotated and broken off. After that, the forceps were slid beneath the front half of the brain, which was softly removed out of the skull. The brain was immediately placed in a cold Phosphate Buffer Saline (PBS) petri dish with the ventral side of the brain facing the plate.

The olfactory bulb and cerebellum were removed with a knife after the brain was relocated. The cerebral halves were then kept together with little, curved forceps. The forceps were then slowly opened, revealing the cortical halves' entrance. Once a sufficient opening along the middle line was obtained, the forceps were closed at a 30-40^{o} angle. It was rotated counter clockwise and clockwise to separate the left and right hippocampus from the cortex. The dissected hippocampus was then placed in a pre-chilled Eppendorf tube and stored at -80 ^{o}C until use.

3.6 Protein Extraction:

The whole tissue lysates were prepared by suspension in 100 µl of ice-cold lysis buffer (7M urea, 2M thiourea, Titron X 100, 10mM phenyl methyl sulfonyl fluoride (PMSF), 1% d-Dithiothreitol (DTT)), followed by sonication using a UP400S ultrasonic processor (Hielscher Ultrasound Technology). To improve dissolubility, after 1 hour at room temperature, the homogenates were centrifuged for 10 minutes at 4°C. The supernatant was moved and kept at -20 ^{o}C. To increase the yield, 50 µl of lysis buffer was poured to the pellet and repeat the process, two supernatants were then combined and centrifuged for 90 minutes at 14000 rpm. The final supernatant was kept at -80 ^{o}C until it was used.

3.7 Quantification of Protein (Bradford's Assay)

Serial dilutions of bovine serum albumin (1mg/ml) were prepared in duplicate with ddH_2O. The sample was diluted with ddH_2O (1:20) in duplicates. The final volume of each standard/sample was 20 μl and 1 ml of Bradford reagent was added, followed by gentle vertexing. The samples were then incubated for 10 min at room temperature. The absorbance of each sample was measured at 595 nm reagent blank using OPTIMA 300 spectrophotometer.

The standard curve was derived by plotting standard absorbance against concentration. This curve was used for the protein concentration estimation against the observed absorbance.

3.9 2-Dimensional Gel Electrophoresis(2DE):

2DE is one method of examining the proteome. The complex mixture of sample protein is separated in two steps. The first separation method is isoelectric focusing, which includes utilising gel strips with an immobilised pH gradient to separate proteins based on their isoelectric point. Proteins travel to their isoelectric points in an electric field, where they have no net charge. SDS-PAGE is utilised for the next separation, based on their unique masses separate proteins. Proteins must be dyed after separation in order to be visible. Coomassie dazzling blue and silver, both of which have different properties, are two commonly used stains. Image analysis software is then utilised to discover and quantify differences in the resulting gels containing protein spots.

3.10 Silver staining:

Ultra-pure water was used to wash gels for 5 minutes each time. After that, gel was fixed in a solution containing 60% distilled water, 30% ethanol, and 10% acetic acid for two 15-minute washes, followed by two washes with 10% ethanol for five minutes

each and two washes with ultra-pure water for five minutes each. Gel was then sensitised for 1 minute with 50 µl sensitizer and 25 ml water. Gel was rinsed with water again for two minutes. For 30 minutes, gel was coloured with 0.5 mL enhancer and 25 mL water. After that, gel was washed two times with water for 20 seconds each time. For 2-3 minutes, a developer workable solution was applied till bands appeared. The acetic acid solution used was 5%. For 10 minutes, a 5% acetic acid solution was used as a stopping solution.

Chapter 4

RESULTS

4.1 MOLECULAR DOCKING RESULTS:

In this study Patchdock server was used for evaluating the interaction of Metformin and Donepezil with target proteins and the atomic contact energy (ACE) of the drug and target proteins were calculated (Table 4.1). The interactions with highest ACE score were selected and analysed with Biovia Discovery studio and the distance between proteins and the amino acid residues involved in hydrogen bond is noted (Table 4.2). The results are given in the table.

Drug	Protein	Atomic contact energy (ACE)
Donepezil	APOE	-130.89
	PSEN I	-220.37
	Bace1	-195.70
	Ki-67	-183.30
	DCX	-201.82
	AChE	-284.07
Metformin	APOE	-92.70
	PSEN I	-147.60
	Bace1	-72.77
	Ki-67	-114.46
	DCX	-118.77
	AChE	-90.54

Table 4.1: Docking score of interaction between ligands and selected proteins

Ligands	Proteins	Interacting Amino Acids	H-bond (AA)	Distance (H-A, D-A)
	APOE	TRP, TRP		
	PSEN I			
	Ki-67	PRO, GLN, ILE, ILE	PRO	2.34-3.22
			GLN	2.71-3.19
Metformin			ILE	3.03-3.66
			ILE	3.03-3.47
	Bace1	SER, GLN, ARG, ARG, GLU,	SER	2.59-3.51
		GLU	GLN	2.93-3.57
			ARG	3.42-3.86
			ARG	1.68-2.45
	DCX	THR, THR, ASP	THR	1.97-2.78
			THR	3.48-4.06
	AChE	THR, THR, ARG, TYR, GLU,	THR	2.88-3.71
		GLU	THR	2.28-3.08
			ARG	2.84-3.21
			TYR	2.10.2.95
	APOE	GLU, GLN		

Donepezil	PSEN I	PHE, LEU, ARG, TYR	ARG	2.55-3.54
	Ki-67	PRO,LYS,LYS,PHE,PHE,ILE,ILE,ILE,ASP	ILE	3.40-3.85
	Bace1	ALA,VAL,TRP,ARG,TYR,VAL,GLY,GLN,GLY	GLY	2.07-2.92
			GLN	3.18-3.70
			GLY	2.82-3.70
	DCX	LYS, LYS, LYS, LYS, ALA, GLU, VAL, VAL, LYS, LYS, ALA	LYS	1.60-2.34
			LYS	1.58-2.48
			ALA	2.58-3.25
	AChE	TRP, TRP, VAL, PHE, TYR, TYR, PHE, TYR, TYR		

Table 4.2. Interacting residues and Hydrogen bonds between ligands and selected proteins

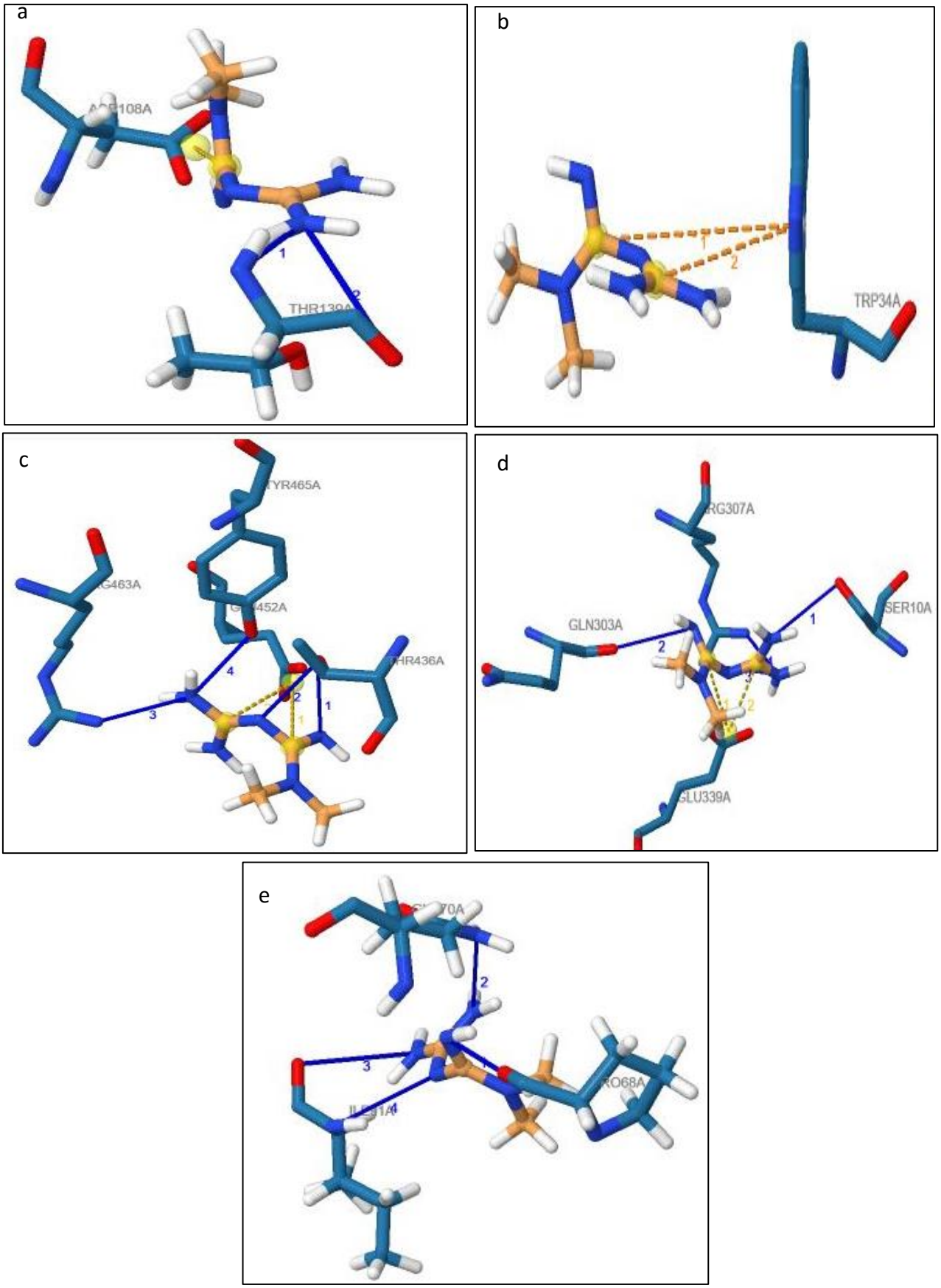

Figure 4.1: Docking and Visualization of interaction of Metformin with (a) ApoE (b) Bace1 (c) Ki-67 (d) DCX and (e) AChE.

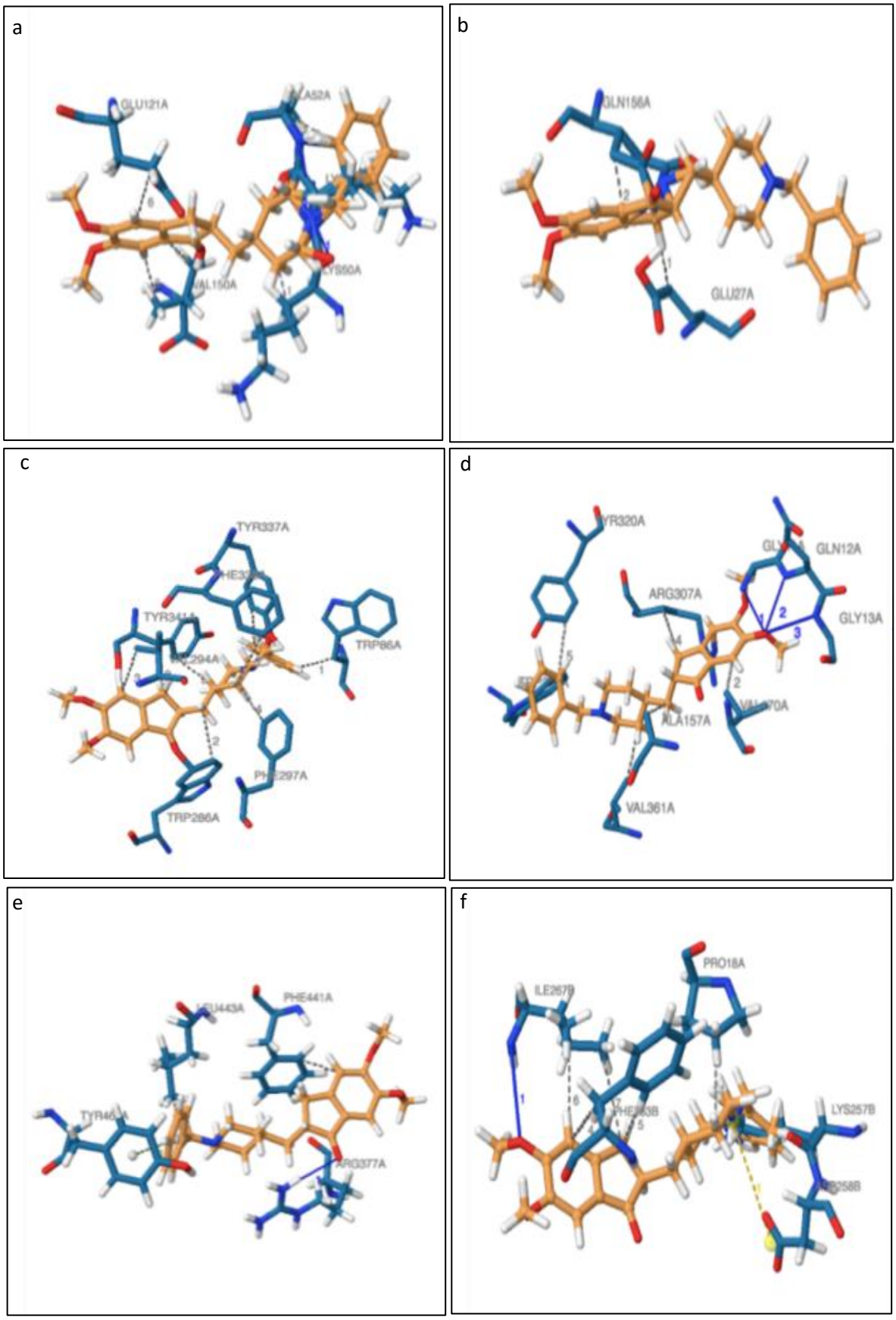

Figure 4.2: Docking and Visualization of interaction of donepezil with (a) APOE (b)Bace1 (c) Ki-67 (d) DCX (e) PSEN I (f) AchE.

4.2 PTMs Predictions:

Potential glycosylated sites in all the target proteins were predicted using NetOGlyc 4.0 and NetNGlyc 1.0 for O glycosylation and N-glycosylation respectively. The threshold value was set at 0.5 and the scores higher than 0.5 were considered to be glycosylated. The results of predicted glycosylated sites are given in the supplementary table S1and S2.

4.3 Histological Studies:

4.3.1 Congo Red Staining

The hippocampus of each research group was histopathologically examined to ascertain if there were any morphological changes in the affected area (hippocampus). As a result, the Congo Red stain revealed the prevalence of Amyloid beta plaques in AlCl3 treated groups versus controls. The presence of Amyloid beta plaques was lower in the Metformin-treated post-AlCl3 exposure group compared to the donepezil-treated post-AlCl3 exposure group. The metformin-treated and control groups are very comparable.

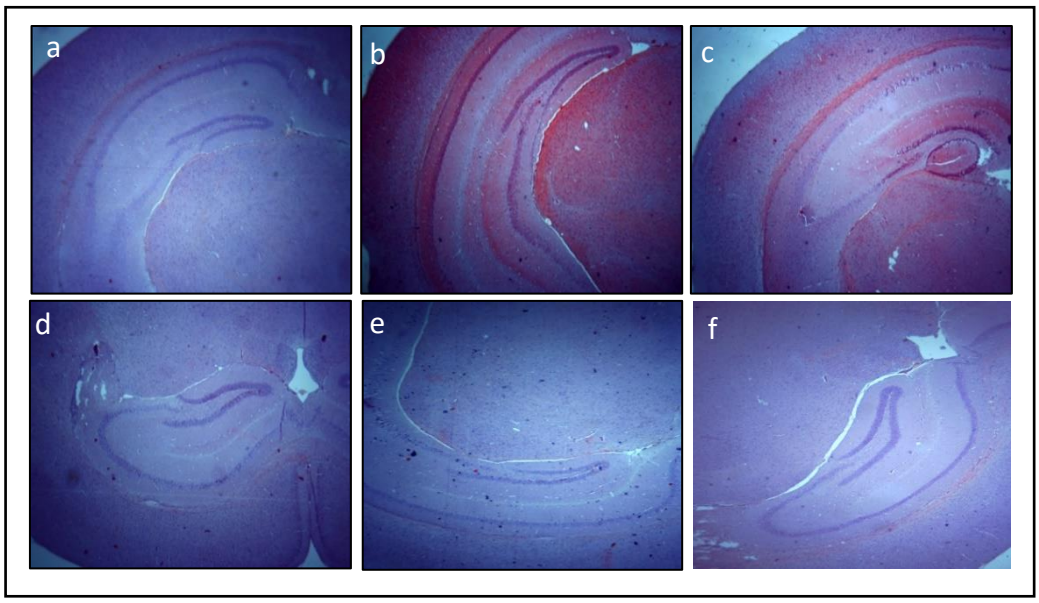

Figure 4.3: Congo Red staining of section of Hippocampus: (a) control group (b) Alcl3 treated group (c) Donepezil treated group (d) Metformin treated group (e) Alcl3 + Donepezil treated group (f) Alcl3 + Metformin treated group. Original magnifications 4X.

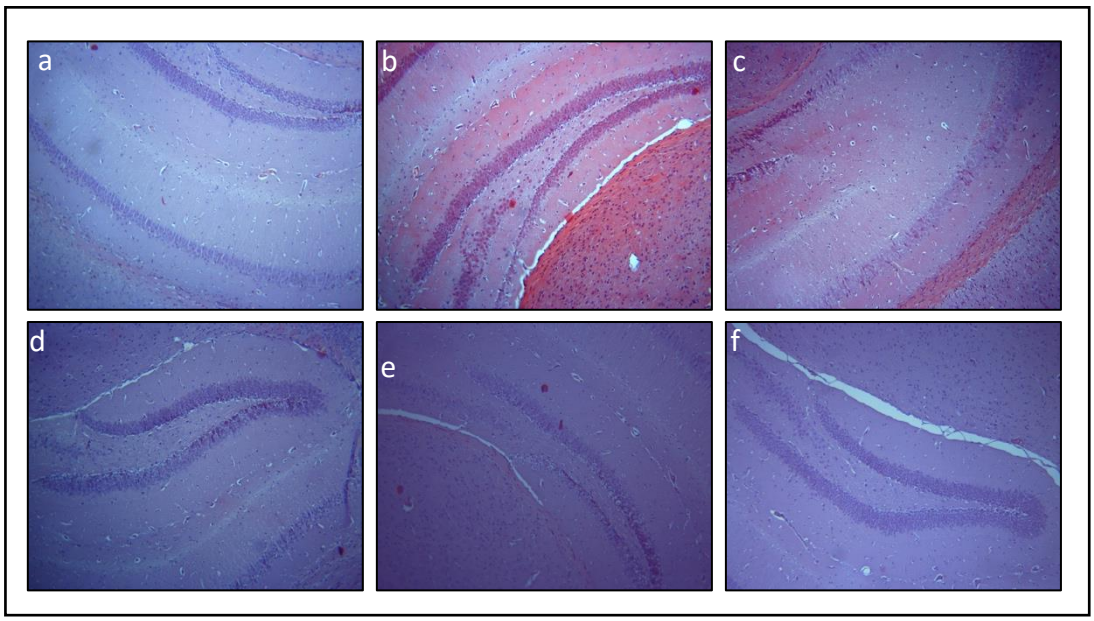

Figure 4.4: Congo Red staining of section of Hippocampus: (a) control group (b) Alcl3 treated group (c) Donepezil treated group (d) Metformin treated group (e) Alcl3 + Donepezil treated group (f) Alcl3 + Metformin treated group. Original magnifications 10X.

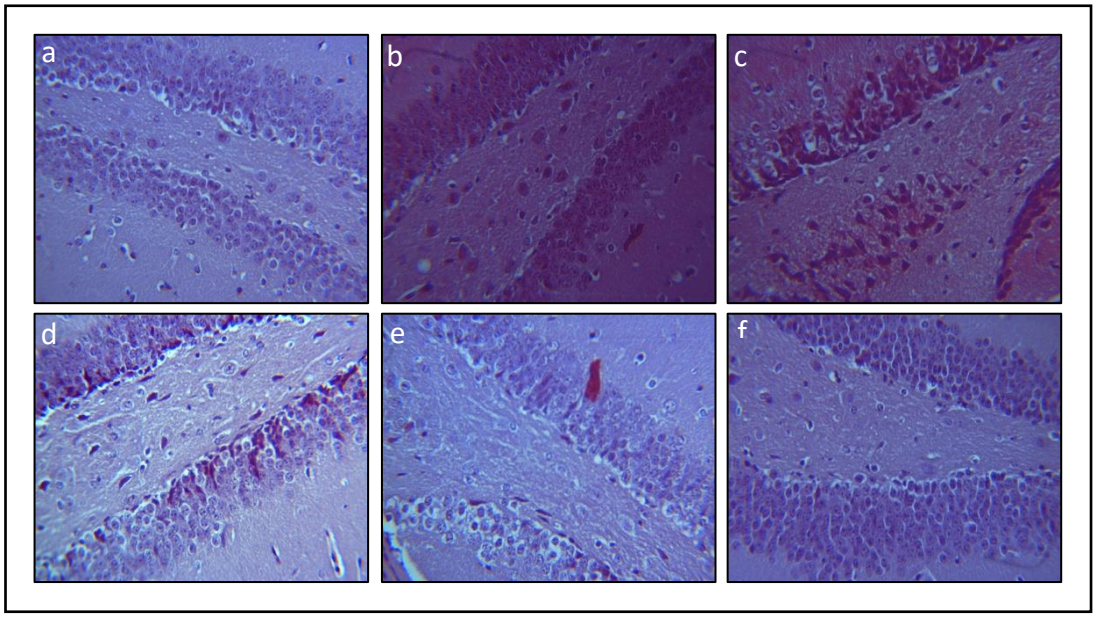

Figure 4.5: Congo red-stained sections of Hippocampus for visualization of amyloid plaques: The micrographs represent the following groups: (a) control group (b) Alcl3 treated group (c) Donepezil treated group (d) Metformin treated group (e) Alcl3 + Donepezil treated group (f) Alcl3 + Metformin treated group. Original magnifications 40X.

4.4 Protein Expression Analysis:

4.4.1 Protein Quantification

The protein concentration of every sample was calculated by plotting the absorbance value of the coloured reaction product on the standard curve. The intensity of the coloured result is proportional to the protein concentration of the sample.

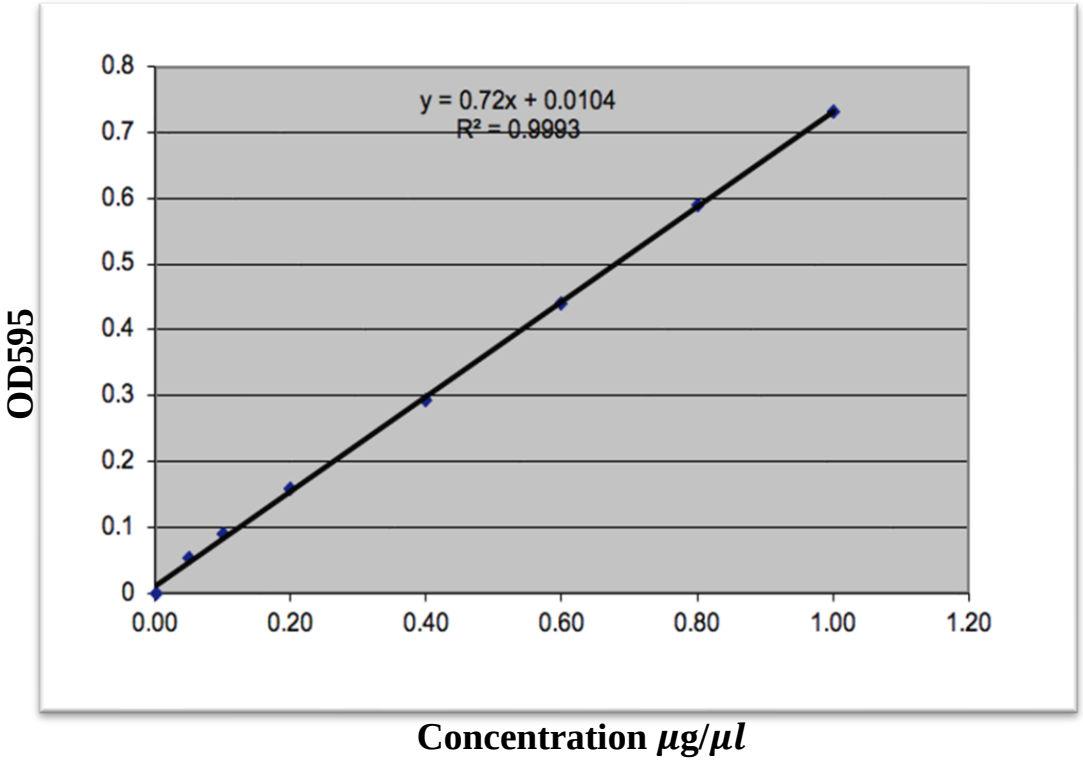

Figure 4.6: Bradford standard curve plotted for eight standard values. Concentration was plotted on the x-axis (independent variable). Absorbance measured at 595 nm was plotted on the y-axis (dependant variable). This graph represents linear regression for the eight points. The obtained linear regression value was 0.9993(R^2=0.9993).

4.5 Changes in Protein Expression linked with Alzheimer's Disease:

In search of molecular signatures linked in the progression of Alzheimer's disease, at first examined the expression levels of proteins representing different regions of the AD brain and discovered significant changes in the expression levels of certain proteins involved in various cellular processes. The 2DE gel images show hippocampal proteins

whose levels were altered in comparison to the control group, which served as a baseline for comparison with the other AlCl3-treated and two drug-treated groups. A total of six significant spots were detected on the 2D-GE gels, and the proteins which were identified in all the four groups were revealed that these proteins are differentially expressed in $AlCl_3$ treated group when compared with control, $AlCl_3$ + Donepezil and $AlCl_3$ + metformin treated group. AD hippocampus showed downregulation of proteins at first three spots identified as compared to the control and other drug treated groups ($AlCl_3$ + Donepezil and $ALCl_3$+Metformin). Furthermore, Metformin upregulated the levels of proteins at spot 1 and 2 as compared to the standard drug donepezil while at spot 3 the effect of donepezil and metformin on proteins upregulation was approximately equal.

Moreover, the AD hippocampus showed upregulation of proteins at spot 4, 5 and 6 in comparison with the control group. As the proteins at these spots are downregulated in the control group the drug treated groups ($AlCl_3$+Donepezil and $ALCl_3$+Metformin) show a positive impact on downregulation of proteins at these spots.

The expression graphs based on the normalised volume of protein spot from Delta 2D also reveal the differential expression of these proteins.

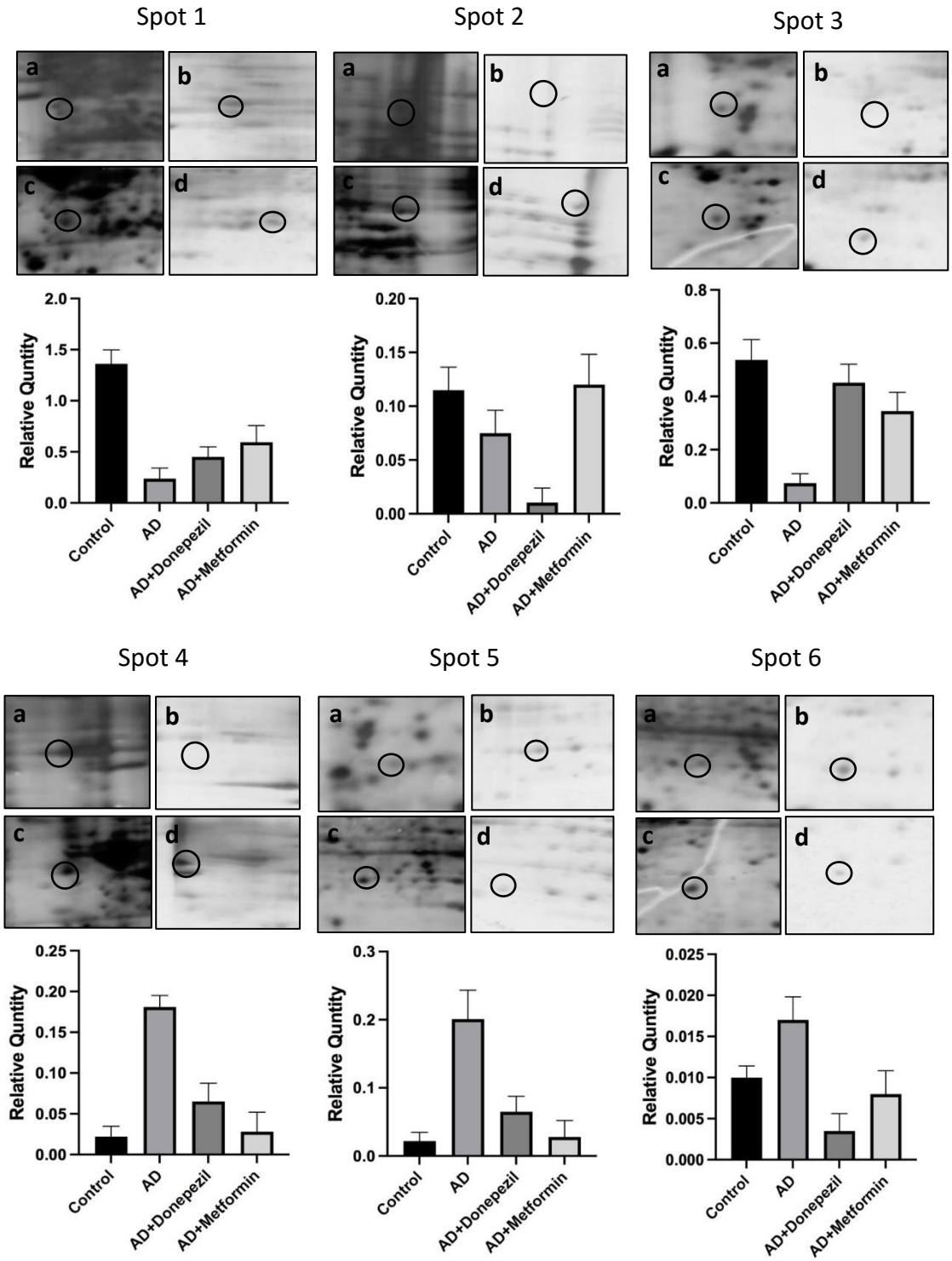

Figure 4.7: Representative 2-DE spots of differentially expressed proteins in AD hippocampus. (a) Control (b) AD (c) Donepezil (d) Metformin treated. Total protein extracted with Titron X 100 and urea from hippocampus were separated by small gel 2-DE (IPG strips pH 3-10 NL, 7 cm) pH 3-10 NL, 7 cm) and silver stained. Delta 2D image analysis software 4.0 (Decodon) was used to detect spots. The statistical analysis was carried out using one-way ANOVA, and spots were chosen based on normalised volumes. ($p = \leq 0.05$).

Chapter 5

DISCUSSION

The current study was carried out to evaluate and compare the therapeutic effect of Metformin. a widely used insulin sensitizing anti-diabetic drug with donepezil a classical drug for AD that is involved in cholinesterase inhibition and upregulating acetylcholine thus enhancing cholinergic transmission. We also docked metformin with some of the important proteins involved in AD and compared their interaction with that of donepezil.

A significant amount of research has shown that metformin a common medication for type 2 diabetes is involved in enhancing cognition by exerting neuroprotective and antioxidant effects. Recent studies have demonstrated the positive effects of metformin on cognition in patients with diabetes establishing that metformin may be useful in treating Dementia and AD (Alagiakrishnan k, 2013; Patrone C, 2014). Metformin was found to be involved in restoring the abnormal blood brain barrier transport of amyloid beta (Aβ) and improving cognition in diabetic mice. Moreover, in human it was found that metformin showed positive impacts on prevention of dementia in older diabetic patients (Koening AM, 2017; Orkaby AR, 2017).

The deposition of Aβ in the brain is one of the major pathological hallmarks of AD, which leads to a chain of reactions and causes apoptosis in neurons (Imfeld et al., 2012). It is also involved in the production of oxidative stress and inflammation in the brain mostly by increasing the formation of superoxide anions and also reducing the SOD activity. The histopathological assessment in the present study showed that metformin treated post $AlCl_3$ exposure group showed a decrease in Amyloid beta plaques as

compared to donepezil treated group. Metformin reversed the morphological alterations in the hippocampus of $AlCl_3$ induced AD mouse models. These findings were consistent with previous studies. A recent study conducted by Oliveira and group on diabetic mice showed that the metformin enhanced spatial memory by reducing the deposition of p-Tau and Aβ plaques and inhibited neuronal apoptosis.(Oliveira et al., 2021). Similarly, another study investigated the effects of metformin on Aβ associated pathologies in APP/PS1 mice and showed that metformin reduced the Aβ burden by promoting its phagocytosis and increasing the autophagy in microglia (Y. Chen et al., 2021).

Oxidative stress is another important hallmark and one of the early events in AD (Christen, 2000). The oxidative stress and the accumulation of Amyloid plaques are closely related as the overexpression of mutated amyloid precursor protein in transgenic mice have shown to increase the production of reactive oxygen species thus enhancing oxidative stress in brain , Moreover the increased oxidative stress in turn leads to more Aβ deposition due to impaired antioxidant defence system (Y. Zhao & Zhao, 2013).Metformin have been showed to reduce oxidative stress in hippocampus and prevent cognitive damage (Alzoubi et al., 2014). Metformin exerts its antioxidant function by activating the neuronal AMPK which is involved in sensing and resolving ROS in mitochondria (Rabinovitch et al., 2017).

A proteomic approach was utilised to detect the differential expression of proteins in the AD and control groups in order to better understand the molecular processes of $AlCl_3$-induced AD. The Proteomic protocol showed expression alterations in $AlCl_3$ treated AD groups compared to controls. Following gel imaging and band identification, about 7 protein bands were detected using quantitative intensity analysis.

Protein expression levels differed significantly between the $AlCl_3$-treated and control groups. The 2D-GE results revealed the downregulation of certain proteins in hippocampus while some of the proteins in the same region were found to be upregulated. These differentially expressed proteins maybe involved in different cellular processes, neuropathology, inflammation, oxidative stress and other clinical impairment. Metformin showed significant impact on reversing the abnormal expression of proteins in comparison with the standard drug donepezil.

In the current study also attempted to find the comparative interaction of Metformin and donepezil against AD proteins (ApoE, PSEN I, BACE1, Ki-67, DCX and AChE) using Patchdock server. Metformin showed strong binding affinity with DCX, PSEN I and Ki-67 showing stable conformation in comparison with donepezil. The PSEN1 is an important gene involved in AD pathology. The amyloid hypothesis suggests that PSEN1 contributes to AD pathology by increasing Aβ production. Metformin also showed comparable interaction with BACE1, which is another critical protein associated with AD. Studies have shown that metformin decreases the expression of BACE1 and as a result reduces the production of BACE1 cleaved products and Aβ (Hettich et al., 2014)

Conclusion

In currant study we evaluate the comparative effects of a widely prescribed antidiabetic drug Metformin in comparison with Donepezil a classical drug for AD. A large number of research studies have highlighted the molecular association between Diabetes Mellitus Type 2(T2DM) and AD which has led to investigate the effects of several anti-diabetic drugs against AD. Interestingly our study showed that the Metformin reduced Amyloid beta plaques formation and normalized the abnormal changes in protein expression patterns, thus improving cognitive function. Further elucidation is required to provide a better understanding of molecular pathways involved before the therapeutic application of metformin can be expanded to treat AD.

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APPENDIX

Poster Presentation (3rd Position Holder)

Sakina, Dr. Saadia Zahid. (2021), Comparative Molecular Docking Analysis of Donepezil and Anti-Diabetic Drug Metformin Against Alzheimer's Disease Proteins. 9th International Conference on Biological and Computational Sciences (C-BICS 2021)

Abstract

Alzheimer's disease (AD) is a neurological polygenic condition that progresses over time which effect about 45 million people worldwide. Which is mostly characterized by the deposition of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs) in the brain. Drugs approved by the FDA for AD temporarily slower the progression of AD, however there are no therapeutic drugs that permanently inhibit AD progression or reverse the neural degradation caused by AD. One of the drugs called Donepezil inhibit cholinesterase and in turn up-regulates acetylcholine thus enhancing cholinergic transmission. Currently, there is a fast increase in studies pointing towards a connection between Diabetes Mellitus Type 2 (T2DM) and AD. This relation between AD and T2DM has led to the use of many anti-diabetic drugs against AD. In this study we performed molecular docking to compare the binding of Donepezil and Metformin (GlucophageR, 1, 2-dimethylbiguanide hydrochloride) which is most often used oral medicine for T2DM and an insulin sensitizing drug with some of the important proteins involved in AD such as Ki-67, ApoE, DCX, Presenilin, and Bace1. The docking results indicated a higher binding affinity of Donepezil the standard drug with the target proteins of AD, however the metformin also showed comparable binding affinity. The target proteins Presenilin, DCX and Ki-67 showed the most stable conformations.

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Comparative Study of Effects of Metformin and Donepezil in $AlCl_3$ Induced Oxidative Stress in the Brain of AD Mouse Models

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