Targeting Dihydrolipoamide Dehydrogenase (DLD) Interactors for Novel Therapeutic Interventions for Alzheimer's Disease

Jawaria Choudhary
00000327541

Supervisor
Dr. Aneeqa Noor

DEPARTMENT OF BIOMEDICAL SCIENCES
SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING
NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY
ISLAMABAD
DECEMBER 2022

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Author
Jawaria Choudhary
00000327541

A thesis submitted in partial fulfillment of the requirements for the degree of MS Biomedical Sciences

Thesis Supervisor:
Dr. Aneeqa Noor

Thesis Supervisor Signature

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Acknowledgements

My profound gratitude to Almighty **"ALLAH"** for granting me with health and opportunity to accomplish this work and for always blessing me with all that I have ever wished for. Indeed I could have done nothing without Your priceless help and guidance. Whosoever helped me throughout the course of my thesis, whether my parents or any other individual was Your will, so indeed none be worthy of praise but You. My humble gratitude for our beloved **Prophet Muhammad (P.B.U.H)** who has always been an inspiration to me of being a perfect human being.

I am profusely thankful to my beloved parents who raised me when I was not capable of walking and continued to support me throughout in every department of my life. I would also like to express special thanks to my supervisor **Dr. Aneeqa Noor** for her invaluable support and encouragement through my degree and providing me with the guidance and counsel I need to succeed in the MS program. She has been a great mentor in mapping my MS journey, advising on research topic, connecting me with the resources I need, being available and responding to my emails and questions. I would also like to pay special thanks to my co-supervisor **Dr. Saima Zafar** for her tremendous support and cooperation.

I would also like to thank **Dr. Adeeb Shahzad**, and **Dr. Asim Waris** for being on my thesis guidance and evaluation committee. I would like to thank CAD/CAM Lab at Department of Design and Manufacturing Engineering (DME) for providing me with a space to work on my project.

My sincere thanks to my dear friends, **Filza Mustafa and Rameesha Murtaza,** for their immense support throughout my time at NUST. Finally, a token of thanks to my lab fellow, **Fatimah**. Nobody truly understands your frustrations in lab like the person standing right beside you through it all. I would like to pay special thanks to her for being a fantastic lab partner throughout our MS journey, and for her help and continual support.

Dedicated to my exceptional parents, and adored siblings whose tremendous support and cooperation has led me to this wonderful accomplishment

Abstract

Alzheimer's disease is the one of the most common type of dementia, which affects millions of people worldwide. Severe memory loss is a defining feature, with episodic memory being particularly damaged in the initial stages. The majority of occurrences of Alzheimer's disease are random, while risk is increased if specific susceptibility genes are inherited. Alzheimer's disease is linked to a decline in energy metabolism, but it is unclear whether this decline worsens or prevents the condition. The finding that genetically dihydrolipoamide dehydrogenase (DLD) is associated to the late-onset AD serves as further evidence for the significance of energy metabolism in AD. To determine if DLD is an appropriate therapeutic target, *in silico* tools have been used in our study to analyse the interaction of DLD with CAND1, LAMP1, and TPP1 using molecular docking and visualization tools like PyRx, PyMOL, Discovery Studio, and Ligplot++. It was observed that binding sites of 5NHG are surrounded by 68 amino acids, and CAND1 (1191-1200), LAMP1 (216-225), and TPP1 (546-555) were able to interact with the amino acids inside the binding pocket of 5NHG. Protein network analysis showed aberration towards apoptosis. In our study, we also evaluated the binding of ascorbic acid, acetaminophen, and methamphetamine after docking experiment to find therapeutic potential. Ascorbic acid and acetaminophen showed the highest binding energy with 5NHG. The interaction of these drug compounds with 5NHG yielding high binding affinity shows that these compounds can be used to block the binding sites on DLD to prevent the interaction of pathological proteins with DLD that are involved in the up-regulation or down-regulation of DLD in diseased state.

Key Words: *Alzheimer's disease, CAND1, LAMP1, TPP1, Docking, Ascorbic acid, Acetaminophen, Methamphetamine*

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Abbreviations

ABAD	Aβ-binding alcohol dehydrogenase
AD	Alzheimer's disease
AICD	APP intracellular domain
APP	Amyloid precursor protein
Aβ	Amyloid-βeta
BBB	Blood-Brain-Barrier
BCKDH	Branched chain ketoacid dehydrogenase complex
CAND1	Cullin-associated NEDD8-dissociated protein 1
CRL	Cullin ring ubiquitin ligases
CTF	C-terminal fragment
DLD	Dihydrolipoamide dehydrogenase
ETC	Electron transport chain
HD	Huntington's disease
KGDH	α—ketoglutarate dehydrogenase complex
LAMP1	Lysosome-associated membrane glycoprotein 1
MAPT	Microtubule-associated tau protein
NFTs	Neurofibrillary tangles
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase complex
PET	Positron emission tomography
PS1/PS2	Presenilin-1/Presenilin-2
pTau	Phosphorylated tau
ROS	**Reactive oxygen species**
rpAD	Rapidly progressive Alzheimer's disease
sAPP	soluble APP fragment
TPP1	Tripeptidyl-peptidase 1

CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease that worsens over time and cannot be cured and leads to loss of memory, disability in daily life activities, and behavioral instabilities over the progress of the disease (Zvěřová, 2019). It typically starts with subtle symptoms like memory loss and changes in behavior, which gradually worsen over time. After several years, the person may be unable to communicate or understand language, have difficulty regulating bowels, and eventually need help with all aspects of self-care (Sloane et al., 2002). The one of the most prevalent type of dementia is AD, causing 60%-80% of all cases worldwide. Early-onset AD (occurring before age 65) is relatively rare, affecting an estimated 24 million people globally (Erkkinen et al., 2018). Numerous epidemiological studies have found that age is the most significant risk factor for AD and cognitive decline. However, other risk factors such as obesity, hypertension, dyslipidemia, diabetes, cerebrovascular diseases, and metabolic syndrome have also been linked to AD development (Reitz et al., 2011).

There are two main hallmarks of AD: intra-neuronal neurofibrillary tangles (NFTs) of tau and extracellular amyloid-β (Aβ) plaques. The "amyloid cascade hypothesis," proposed by Hardy and Higgins in 1992, is the most widely accepted explanation, however the processes underlying the pathology of AD are still unknown (Hardy & Higgins, 1992). Accumulation of tau and Aβ results in disturbed biological functions, such as transported and packaging systems, oxidative stress, inflammation, and eventually neuronal death (Wirths et al., 2004). It is believed that extracellular Aβ plaque accumulation, resulting from protein misfolding, is one of the most common hallmarks of AD that underlies dementia and neuronal damage and dysfunction (Huang & Mucke 2012).

Mitochondrial dysfunction and oxidative damage have been implicated in the pathogenesis of AD. Many gene expression studies have revealed that AD is associated with impaired mitochondrial bioenergetics, suggesting that mitochondrial dysfunction may play a role in the development of AD (Liang et al., 2008; Rai et al., 2020). According to the "mitochondrial cascade hypothesis," late-onset sporadic AD is mostly caused by mitochondrial dysfunction and that this leads to the accumulation of Aβ and the development of NFTs, which in turn cause synapse degeneration and neuronal death (Swerdlow et al., 2014).

In AD, toxic protein aggregation buildup and decreased mitochondrial axonal transport are related, which causes problems with axonal and synaptic function. A causes mitochondrial

malfunction, which results in reactive oxygen species (ROS) generation in AD. In addition to supplying energy to neurons, mitochondrial oxidative phosphorylation generates a number of ROS, including hydroxyl radicals, hydrogen peroxide (H_2O_2), and superoxide radicals (O_2) (Hoye et al., 2008). ROS-mediated oxidative stress results in mitochondrial malfunction, which in turn causes neuronal damage (Rai et al., 2020). Aβ contributes to ROS production by causing mitochondrial dysfunction in AD. It is clear that Aβ disrupts the electron transport chain (ETC) by lowering key enzyme activity (Casley et al., 2002). NFTs and the microtubule-associated protein Tau (MAPT) may potentially have a role in the aetiology of AD in addition to the deficits brought on by Aβ. For effective transport into neurons, cellular elements such microtubules, lysosomes, mitochondria, and others are needed. Under normal circumstances, Tau stabilises neuronal microtubules (Wang & Liu 2008). Hyperphosphorylated Tau surrounds synaptic connections and prevents mitochondrial transport, resulting in significant amounts of oxidative stress results in impaired mitochondrial metabolism (Rai et al., 2020).

The human dihydrolipoamide dehydrogenase (DLD) gene locus has been connected to a higher risk of developing late-onset AD (Brown et al., 2007). DLD, along with pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (BCKDH), are linked to AD and play a crucial part in energy metabolism. Each of these complexes, which are a component of ketoacid dehydrogenases, contributes in energy metabolism (Carothers et al., 1989). Reduced KGDH activity has been reported in post-mortem brain tissues of people with AD and PD, indicating a connection between neurodegeneration and a decline in KGDH activity (Banerjee et al., 2016). Targeted disruption of DLD in mice can also reduce KGDH and PDH activity, which prevents glycolysis from interacting with the TCA cycle (Luca et al., 2015). Therefore, there is a possibility that DLD activity and the onset of AD are linked.

As the structures of more and more protein targets are made available through crystallography, Nuclear magnetic resonance (NMR), and bioinformatics technologies, there is an increasing need for computational methods and tools that can help to identify and investigate active sites and suggest new therapeutic compounds that can specifically interact and bind to these regions. A new drug's invention takes a tremendous amount of time and money, both of which are unacceptable (Rao & Srinivas, 2011). Numerous studies conducted in recent years have shown the value of *in silico* methods for discovering novel and more effective therapies in drug discovery and development. As a result, developing novel drugs for the management or treatment of patients using a molecular docking technique may be an effective alternative (Umar et al., 2021). Virtual libraries of drug-like molecules are screened to find leads for drug development by anticipating the interaction of small molecules with proteins (Sousa et al.,

2006). In our study, molecular docking was employed to evaluate the role of DLD into the progression of AD. *In silico* techniques have gained a lot of attention recently because of their promise to reduce the time, labour, and expense of drug discovery. Numerous new drugs have been developed as a result of the application of computational approaches (Shaker et al., 2021). In our study, we also evaluated the binding of different drug compounds such as ascorbic acid, acetaminophen, and methamphetamine after docking experiment *in silico.*

1.1 Objectives of the Study

The objectives of the current study are identification of targets, *in silico* analysis for DLD interactors in AD, pathway analysis, and drug screening. The study is conducted to better understand conformational changes that occur after the interactions between DLD and its interactor partners, which will help to understand the AD pathology and to analyse the common interacting proteins between them that are involved in different pathological pathways. This study will provide the bases to identify how this disease can be treated by identifying the potential novel therapeutic interventions and targets for AD.

CHAPTER 2

LITERATURE REVIEW

A number of debilitating, incurable diseases are associated with neurodegeneration. On the basis of morbidity and mortality, neurodegenerative diseases are most prevalent and growing disorders, specifically in elderly population, such as AD, frontotemporal lobar dementia (FTLD), Huntington's disease (HD), Parkinson's disease (PD), progressive supranuclear palsy (PSP), Pick's disease (PiD), and amyotrophic lateral sclerosis (ALS) (Gitler et al., 2017). Although almost all neurodegenerative disorders have overlapping characteristics , individual neurodegenerative diseases have distinguished underlying physiology and heterogeneous clinical presentations. as some of them affect cognitive and memory impairments while others cause difficulty in speech, breathing, and mobility (Canter et al., 2016; Wyss-Coray et al., 2016). The gradual loss of certain susceptible neurons is considered to be the major cause of neurodegenerative disorders, as opposed to certain static neuronal death due to toxic or metabolic disorders. Anatomic distribution of neurodegeneration (for example, spinocerebellar degeneration, frontotemporal degeneration, or extrapyramidal disorders) can distinguish neurodegenerative disorders based on molecular abnormalities and primary clinical hallmarks (for example, parkinsonism, motor neuron disease, dementia) (Dugger et al., 2017). The most known neuropathological disorders are a-synucleinopathies, amyloidosis, and tauopathies. Abnormal concurrence properties of these neurodegenerative disorders are based on protein abnormalities. AD is a neurodegenerative disease associated with gradually progressive functional and cognitive loss, behavioural changes, problems with language, memory loss, and is characterized by the accumulation and formation of amyloid plaques and aggregation of tau in the brain. The most common cognitive symptoms of AD include visuospatial dysfunction, short-term memory, and praxis (Apostolova, 2016).

2.1 History of Alzheimer's Disease

First time, at the beginning of 20th century (1906), the pathological findings of disease were noticed in a 55 years old woman, named Auguste Deter, by Alois Alzheimer. After 5 years, she died having history of continuous memory loss, language problems, and progressive psychiatric disturbances. Alzheimer noticed two main pathological changes in brain : senile plaques and NFTs (Zvěřová, 2019). Another scientist, Oskar Fischer, explained senile dementia in 12 cases having neuritic plaques in the same year. During that time, Fischer was a member

of Prague School of Neuropathology that was led by Arnold Pick, a psychiatrist who identified the clinical symptoms of Pick's disease (Goedert, 2009).

2.2 Clinical Aspects of Alzheimer's Disease

AD is an irreversible and incurable neurodegenerative disease which leads to the progressive loss of memory, disability in actions of daily living and behavioural instabilities over the disease progress (Zvěřová, 2019). With the primitive symptoms including memory-loss and alterations in behaviour in artful manners, it starts abruptly. The disease increases little by little until, often after ten years or more than ten years, the person is incapable to communicative or understand the language, not able to regulate bowel movement for a long time period and eventually needs help in all phases of self-care (Sloane et al., 2002). Though functional and cognitive disability is the hallmark for AD, latest studies showed that 90% patients of dementia suffer psychiatric and behavioural disturbances over the duration of their disease. In this context the psychiatric instabilities are interpreted enormously and include behavioural instability or cognitive disturbances. They are of different types, over and above distraction, illusions, depression, hallucination, disturbances in sleep and behavioural problems (Reitz et al., 2014).

Clinically, AD slowly progresses into three stages: mild, moderate, and severe, which occurs along the aging processes. AD begins insidiously and is distinguished by onset in patients remaining in the state of clear consciousness, and causes memory loss. One of the most common symptoms of AD is short-term memory loss (Zvěřová, 2019). During AD, impairment in visuospatial tasks, dysfunction in motor neurons, changes in behaviour and personality, and deterioration in verbal communication, are considered as the changes that are slowly added to the initial symptoms. These symptoms of AD are not related to aging and extensive tests are compulsory to identify the AD to eliminate the possibilities of other dementia cases (Houmani et al., 2018).Collateral family history, neuroimaging, neuropsychological evaluation, neurological and physical examination, laboratory tests and medical records play important role in diagnosis of AD (McKhann et al., 2011).

2.3 Epidemiology

The most of dementia cases, 60%-80%, are thought to be caused by AD, affecting estimated 24 million people around the world. However, it can affect people of young age and it is mostly attributed to the elderly people. Between the age of 65 and 85, the prevalence of disease increases 15-folds and increases risks from 0.5% to 6%, and this rate doubles every year. The

AD that occurs before the age of 65 is considered as early-onset AD (Erkkinen et al., 2018). In the whole world, according to an assessment in 2015, dementia affected about 46.8 million individuals. Those new cases were about 30 percent (almost 9.9 million new cases) that was more than the number of cases being presented in the "Report of World Health Organization (WHO)" in 2010. Asia (49%), America (18%) and Europe (25%) were reported having the greatest number of cases (World Alzheimer Report, 2015). This number will keep increasing and will become 74.4 million in 2030 and 131.5 million in 2050 according to an assessment. The greatest number of individuals with dementia are in Africa and East Asia (almost 9.8 million individuals with AD). After this, Europe comes having 7.4 million individuals with dementia (dos Santos et al., 2018).

2.4 Risk Factors

According to many epidemiological studies, age is considered one of the most common risk factor for AD and cognitive decline. AD accelerates with age and age is the potential agent for the cause of dementia shown by the "Diagnostic and Statistical Manual of Mental Disorder (2014)" (dos Santos et al., 2018). The prevalence of AD increases with aging, to an estimated 19% in individuals between the age of 75-84, and to 30%-35% in individuals older than 85 years. The brain volume and weight reduces with age in cognitively normal brain along with the enlargement of ventricles, loss of dendrites and synapses in senile plaques and NFTs accompanied areas (Armstrong, 2019).

There are also other various risk factors that have been found associated with AD, such as obesity, hypertension, diabetes, cerebrovascular diseases dyslipidemia and metabolic syndrome. These factors are involved in pathogenic processes leading to decrease in vascular integrity of blood-brain-barrier (BBB), resulting in BBB break down and causes increases in cytokines, adipokines and neuronal oxidation leading to the formation of senile plaques by the Aβ deposition and NFTs by the abnormal tau phosphorylation (Reitz et al., 2011).

2.5 Hallmarks of AD

AD is characterized pathologically by two hallmarks: intra-neuronal NFTs of tau and extracellular amyloid plaques. The mechanisms that are involved in pathology of AD are still unclear, but the most accepted theory is "amyloid cascade hypothesis" which was proposed by Hardy and Higgins in 1992 (Hardy & Higgins, (1992). According to this hypothesis, the accumulation of pathogenic Aβ isoforms and the cleavage of amyloid precursor protein (APP) are the main factors that play important role in the progressession of AD. Increased

accumulation of Aβ results in increased phosphorylation of tau (ptau) leading to the formation of NFTs causing neuronal death (Amadoro et al., 2011). Different biological functions, such as transporting and packaging systems are disturbed by the accumulation of tau and Aβ resulting in oxidative stress, inflammation which eventually leads to the neuronal death (Wirths et al., 2004). Several significant evidences have been proposed that support amyloid cascade hypothesis, and this hypothesis becoming more clear that the Aβ accumulation starts 10-15 years earlier to the clinical symptoms (Chen, 2016). Understanding the interaction between Aβ, tau and other pathologies of AD is a step towards developing more effective therapies.

2.6 Amyloid-β Protein

In 1906, Alois Alzheimer was the first neuropathologist and psychiatrist who identified the amyloid plaques as a hallmark of AD (Stelzma et al., 1995). Almost after 80 years, Aβ were isolated and distinguished as principal components of amyloid plaques (Glenner & Wong, 1984). Although, the APP cleavage involved in amyloid cascade hypothesis is still not well understood, but we have much better understanding of Aβ functions and its processing. APP gene is located on chromosome 21 and its cleavage is involved in the proteolytic processing of amyloid. This gene codes a large transmembrane protein consisting of 770 amino acids in length. APP has been involved in a number of physiological functions, such as synapse formation, neural plasticity, and iron transport, although its endogenic function is not completely understood. The transmembrane function of APP is involved in adhesion of cell molecule and cell signalling receptor, and also plays role in neurodevelopment (Turner et al., 2003).

Two enzymes are involved in the cleavage of APP i.e. α-secretase and β-secretase. Cleavage of APP by α-secretase results in soluble APP fragment (sAPPα) and C-terminal fragment (CTF) C83 (Chen, 2016). sAPPα has been shown to be involved in memory and learning. In AD patients, worse memory and learning performance is correlated with the reduced levels of sAPPα (Almkvist et al., 1997). In contrast, increased production of sAPPα has been reported to improve memory and learning, and is involved in cell proliferation, axonal transport and enhance neuronal plasticity (Baratchi et al., 2012). Cleavage of APP by the other proteolytic enzyme β-secretase results in soluble APP fragment. (sAPPβ) and CTF C99. (sAPPβ) plays role in neuronal differentiation and maturation (Freude et al., 2011).

An additional enzyme γ-secretase is involved in the further cleavage of CTF fragments, either they are produced by α-secretase or β-secretase, resulting in an APP intracellular domain (AICD) and small peptide fragment P3, from C83 or C99 CTF. The AICD affects a large

number of cellular functions and interact with over 20 known proteins (Müller et al., 2008). AICD has been identified in JNK and p53 mediated apoptosis and is reported to cause cell death in transfected cells in vitro (Ozaki et al., 2006). In the perspective of AD, small peptide P3 produced along AICD is not completely understood, some studies reports P3 reactivity is significantly higher in AD brains and is involved in the oligomeric formation of Aβ species (Streltsov et al., 2011). As α-secretase is involved in the APP cleavage which contribute in some form of AD, the scientists mostly focus on Aβ production by γ-secretase. The Aβ peptides formed by the cleavage of C99 by γ-secretase vary in amino acid length, as 38, 40 or 42 amino acids. There are some evidences that the higher intensity of Aβ42 plays a major role in the onset and progression of AD, cognitive impairment, and neuronal atrophy (Younkin, 1998; Cole & Vassar, 2007).

Overexpressing of catalytic subunits of γ-secretase, presenilin-1 and presenilin-2 (PS1, PS2), also increase the production of Aβ42 resulting in production of pathogenic Aβ. Aβ contributes to disease in different forms, initially Aβ monomers are soluble peptides cleaved by γ-secretase (Chen, 2016). When Aβ monomers start to accumulate in higher levels, they undergo conformational shift into β-sheets resulting in aggregation of Aβ oligomers. Aβ is considered a strong indicator for the formation of plaques, cognitive impairment, and synaptic loss in AD patients. Aβ accumulates intracellularly resulting in apoptosis, disturb axonal transport and block synaptic transmission (Pigino et al., 2009). AD patients with higher levels of Aβ cause the disease with higher severity by shifting from soluble to insoluble Aβ and shift from Aβ38 to Aβ42 (Wang et al., 1999).

2.7 Tau Protein

In the mid of 1970s, MAPT was first identified as a microtubule assembly factor. The main physiological function of tau is the stabilization and assembly of microtubules, which results in the proper functioning of neurons (Weingarten et al., 1975). Tau protein is commonly found in axons and cytosol of neurons (Sajjad et al., 2018) . This protein has a greater tendency to form self-aggregative beta-sheets which results in the formation of NFTs (Sabbagh & Dickey, 2016). Most neurodegenerative diseases are caused by the abnormal accumulation and aggregation of proteins in the surrounding areas of neurons. Moreover, the abnormal accumulation of intraneuronal tau protein is considered the most crucial pathological feature in a broad spectrum of neurodegenerative diseases. The aggregation of tau protein is not only found in AD but also in other neurodegenerative diseases, such as frontotemporal dementia

with parkinsonism-17 (FTDP-17), PSP, FTLD, corticobasal degeneration (CBD), and PiD. These neurodegenerative are collectively known as tauopathies (Wolfe, 2012).

Tau protein is mainly divided into four functional domains: N-terminal domain which is also known as projection domain, proline-rich regions (PRR), microtubule binding region (MTBR) and C-terminal domain (Barbier et al., 2019). The human MAPT gene is located on the chromosome 17q21, consisting of 16 exons. Out of 16 exons, one exon acts as promoter, eight exons are constitutive exons Exon 1 (E1), E4, E5, E7, E9, E11, E12, and E13, and, three exons are alternatively spliced exons E2, E3, and E10. Six isoforms of tau are formed by the alternative splicing of E2, E3, and E10, namely 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, and 2N4R. Alternative splicing of exons 2 and 3 results in 0N, 1N and 2N inserts at N—terminal and splicing of exons results in either 3R or 4R inserts in MTBR domain (Muralidar et al., 2020). Exons 4A, 6, and 8 are absent in central nervous system and are only found in the mRNA of peripheral tissues, while E14 is a part of 3' untranslated mRNA region of tau (Andreadis, 2005).

Tau has acidic amino-terminal region (N-terminal) and positively charged proline-rich region on one half of the protein, while on the other half tau has carboxy-terminal (C-terminal). In the middle region of tau, negatively charged microtubules binding repeats are present which are bound tightly to the positively charged proline-rich region (Lee et al., 1989). Any pathological change, abnormality or dysfunction in the function of tau results in the destabilization of microtubules resulting in neuronal cell death which ultimate leads to tauopathies (Muralidar, et al., 2020). Tau protein translation is unusual as tau mRNA is transported to proximal axon where translation process occurs, and enhances the polarity of neurons. When tau gets phosphorylated at Ser214 and Ser262, it results in tau destabilization and detachment from microtubules in AD. Even though tau phosphorylation is important for the cell polarity inception, but hyperphosphorylation and overactivity of microtubules leads to cell death (Mandelkow, 1998) . Hence, loss of tau's role in the stabilization of microtubule disrupts axonal transport, and dysfunction in the function and structure of cytoskeleton leads to neurodegeneration resulting in pathological disorders (Roy et al., 2005).

2.8 Metabolic Impairment

Brain occupy on average 2% of a person's body weight, a resting awake person uses about 25% of body's glucose, and consumes about 20% of body's oxygen. Even mild changes in energy metabolism in human brain are closely associated with a disturbance in nervous function as the

brain is one of the most energy-consuming organs. Among the earliest and most consistent early signs of AD is impaired energy metabolism (Wang et al., 2020).

It is believed that extracellular Aβ plaques accumulation resulting from protein misfolding is one of the most common hallmarks of AD that causes dementia and neuronal impairment (Huang & Mucke, 2012). Neuroimaging studies of AD brains have found impairments in glucose metabolism and reduced mitochondrial enzyme activity at the latter stages of the AD (Mosconi, 2012). It is unclear how these observations are related to one another in terms of cause and effect, as poor energy metabolism may cause protein misfolding that results in the accumulation of Aβ plaques, but the other way around, Aβ production and accumulation can also damage energy metabolism (Calkins & Reddy, 2011). There has been debate over whether the decrease in energy metabolism causes AD or acts as a protective mechanism against its symptoms. A major problem with assigning causality to the disease is the fact that studies on post-mortem brains were conducted at a late stage of disease (Ahmad & Ebert, 2021).

2.8.1 Impaired Metabolism Regulates Mitochondrial Dysfunction

Several gene expression studies have consistently shown mitochondrial-related metabolic defects in AD, providing strong evidence for impaired mitochondrial bioenergetics (Liang et al., 2008**).** Energy deficiency is caused by mitochondrial dysfunction in the early stages of AD, which causes the disease to progress. Researchers have demonstrated that mitochondrial dysfunction and oxidative damage may play a role in AD pathogenesis (Rai et al., 2020). According to the mitochondrial cascade hypothesis, the primary cause of late-onset sporadic AD is mitochondrial dysfunction, which leads to the build-up of Aβ and the development of NFTs, which in turn cause synapses to degenerate and neuronal death (Swerdlow et al., 2014). Neurons require a constant supply of energy to maintain energy homeostasis so they can function properly. As a result of insufficient glycolysis capacity, neurons rely primarily on aerobic oxidative phosphorylation for their energy needs (Yellen, 2018).

Aside from providing neurons with energy, mitochondrial oxidative phosphorylation produces several ROS, such as, hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2), and superoxide radicals (O_2^-). Superoxide dismutase, catalase, superoxide reductase, thioredoxin/thioredoxin reductase, glutathione peroxidase, glutathione peroxidase, and peroxiredoxin are examples of endogenous antioxidant defence systems that neutralise ROS (Hoye et al., 2008). During the antioxidant defence system, there is an equilibrium between ROS and antioxidants (Tan et al., 2018). It becomes impossible to neutralize ROS in excessive amounts when the equilibrium is disrupted by excessive ROS production. As a consequence of ROS-mediated oxidative stress,

mitochondrial dysfunction occurs, which in turn results in neuronal damage (Rai et al., 2020). As a result of a high amount of ROS being produced, the lipids, proteins, and DNA of mitochondria are significantly damaged (Bhat et al., 2015). During this period of heavy oxidative stress, mitochondria are susceptible.

It has been shown that mitochondria are susceptible to oxidative stress due to the lack of histones (Tan et al., 2018). In addition, mitochondria can only repair mitochondrial DNA with a very limited fidelity and capacity. These above-mentioned changes in energy metabolism are revealed by positron emission tomography (PET) imaging of the brain. Biochemical assays show diminished mitochondrial enzymatic activity and PET imaging indicates reduced glucose uptake by neurons (Mosconi, 2013). It is therefore believed that mitochondrial abnormalities cause amyloid plaques and hyperphosphorylated tau (pTau) to form atypically processed amyloid precursor protein (APP).

2.8.2 Mitophagy in AD

A mitochondrial cell is dynamic in that its size, shape, number, and location constantly change. Mitochondria are capable of dividing, joining together, and moving throughout the cytoplasm, resulting in their various shapes (Misrani et al., 2021). Fission (division) and fusion, which are both crucial components of mitochondrial biology and quality control (Fu et al., 2019), are two distinct, tightly regulated adverse events that make up the majority of these processes, which are together known as mitochondrial dynamics (Chu, 2019).

For mitochondrial morphology, as well as for cell viability and synaptic activity, equilibrium between fission and fusion is crucial. Various mitochondrial dysfunctions can lead to neural defects, including mitochondrial motility, fission, fusion, and turnover. Mitochondrial mobility and positioning are closely related to the fission/fusion process. Alterations in mitochondrial morphology and mobility as a result of abnormal mitochondrial fission or fusion are also responsible for influencing mitochondrial distribution and mobility (Chen & Chan, 2009). It has been shown that various neurodegenerative disorders such as AD, HD, and PD can be characterized by fission and/or fusion process disruption (Stanga et al., 2020).

2.8.3 Abnormal Mitochondrial Transport in AD

Mitochondrial transport mechanism ensures that mitochondria are distributed properly throughout the cell. There are proteins located inside mitochondrial membranes that transport molecules into and out of the organelles and other factors such as ions (Ruprecht et al., 2019). The actin cytoskeleton is primarily responsible for mitochondrial transport in budding yeast,

and both microtubules and actinare responsible in mammalian cells (Misrani et al., 2021). Mitochondria can be inherited and recruited properly through these transport mechanisms. ATP is transported to synapses by mitochondrial transport, axonal growth is promoted by mitochondrial transport, and Ca^{2}+ buffering, mitochondrial repair, and degradation is enabled by mitochondrial transport (Lin & Sheng, 2015).

A variety of cellular signals, including Ca^{2}+, ATP, ROS, oxygen levels, and nutrients control the movement and position of mitochondria in neurons. Milton/Miro proteins have been discovered as mammalian adaptors for kinesin-mediated transport of mitochondria; however, additional adaptorand motor proteins also function in axonal trafficking of mitochondria; this ensures appropriate distribution of mitochondria in the cell (Melkov & Abdu, 2018). There are several human neurodegenerative conditions associated with defective mitochondrial axonal transport, including Charcot-Marie-Tooth disease, spastic paraplegia, AD, hypertension, and PD (Flannery & Trushina, 2019). There is a correlation between impaired mitochondrial axonal transport and toxic protein aggregate accumulation in AD, which results in disturbed axonal and synaptic function. A disturbance produced in mitochondrial motility is closely linked to an unbalanced fission/fusion process, higher levels of both Aβ and pTau, and ROS, although the specific molecular pathways underpinning impaired mitochondrial transport in AD are still unclear (Misrani et al., 2021).

2.8.4 Amyloid-β Toxicity–Associated Mitochondrial Dysfunction

Aβ causes mitochondrial dysfunction, which results in ROS production in AD.It is clear that Aβ disturbs the electron transport chain (ETC) by decreasing the activities of important enzymes (Casley et al., 2002), which ultimately causes mitochondrial functional dynamics to be disturbed (Johri & Beal, 2012). There are three main pathological changes causing these changes: oxidative stress, mitochondrial axonal transportation dysfunction, and mutations of mDNA (Baloh, 2008). Additionally, intracellular Aβ disrupts the oxidative phosphorylation and the generation of ROS in the mitochondria (Hirai et al., 2001), lowering the potential of the mitochondrial membrane potential , complex IV function, and ATP synthesis (Nunomura et al., 2001). Multiple AD transgenic mice models have these mitochondrial abnormalities, including mice expressing mutants of APP (mAPP) and presenilin-1 (PSEN1).

A large amount of H_2O_2 and nitric oxide (NO) is produced by these mutants in addition to an increase in the oxidation of proteins and lipids. These deficiencies are linked to age-associated Aβ deposition, which induces oxidative stress (Rai et al., 2020). Aβ also binds to Aβ-binding alcohol dehydrogenase (ABAD), which is part of the mitochondrial short-chain dehydrogenase

reductase family. By inhibiting ABAD, neurons are protected from apoptosis and ROS production (Yao et al., 2011).

2.8.5 Hyperphosphorylated Tau–Associated Mitochondrial Dysfunction

Apart from Aβ-induced impairments, MAPT and NFT could also contribute to AD pathogenesis. Cellular components such as microtubules, lysosomes, mitochondria, and others are required for efficient transport inside neurons. Neuronal microtubules are stabilized by Tau under physiological conditions (Wang & Liu, 2008). It has been shown that tau proteins undergo phosphorylation along with O-glycosylation (Martin et al., 2011). The depolymerization and polymerization of microtubules is disrupted by hyperphosphorylation of Tau, resulting in microtubule detachment. Additionally, phosphorylated Tau (pTau) aggregates and fibrils accumulate in degenerating neurons, causing cognitive impairment in AD (Kerr et al., 2017).

Pathology caused by NFT and mitochondrial dysfunction also excretes pTau in a similar way to Aβ pathology. The accumulation of pTau in cells is caused by mitochondrial dysfunction causing ROS to form which causes lipid peroxidation of the membranes (Tönnies & Trushina, 2017). A large amount of oxidative stress is created when hyperphosphorylated Tau surrounds synaptic junctions and inhibits mitochondrial transport. Tau N-terminals are found in AD mitochondria, and decreased expression of COX and cyclooxygenase-IV (COX IV) results in impaired mitochondrial metabolism (Rai et al., 2020).

2.8.6 Dihydrolipoamide Dehydrogenase

The DLD gene locus has been linked to an increased risk of late-onset AD (Brown et al., 2007). The DLD enzyme is a component of three complexes, α—ketoglutarate dehydrogenase complex (KGDH), pyruvate dehydrogenase complex (PDH), and branched chain ketoacid dehydrogenase complex (BCKDH). Clinical studies have shown that AD patients have decreased levels of cerebral glucose uptake, glucose-dependent energy production, and associated enzyme activity, including pyruvate dehydrogenase (PDH) and α-ketoglutare dehydrogenase (KGDH). The role of glucose energy metabolism in disease progression is shown by the decreased energy metabolism (Bubber et al., 2005). These complexes are part of ketoacid dehydrogenases and each of the complex contributes to the metabolism of energy (Carothers et al., 1989). It has been observed that there is an association between a decrease in KGDH activity and neurodegeneration, as reduced KGDH activity has been documented in

post-mortem brain tissues of individuals with AD and PD (Banerjee et al., 2016). KGDH activity is suppressed to mimic the reduction in glucose utilization in the cortex by creating mice homozygous for a knockout mutation of one of its subunits (E2). In mice, targeted disruption of DLD can also decrease KGDH and PDH activity, which inhibits the interaction of glycolysis with the TCA cycle (Luca et al., 2015). Thus, a clear possibility that DLD activity and the development of AD are directly related.

2.9 *In silico* Methods and Drug Discovery

In recent years, numerous studies have revealed that *in silico* techniques are useful in finding newer and more effective therapeutics in drug discovery and development. As a result, a molecular docking approach could be a viable alternative for the advancement of novel drugs for the management of/treatment of patients (Umar et al., 2021). A molecular docking (MD) algorithm is a computational process for predicting noncovalent interactions between macromolecules, or more commonly between receptors (macromolecules) and ligands (small molecules), starting with their unbound structures, MD simulation structures, homology models, etc. It aims to predict the conformations bound to a protein and the binding affinity (Trott & Olson, 2010).

In order to develop drugs, the binding of small molecules to proteins is predicted by virtual libraries of drug-like molecules (Sousa et al., 2006). In our study, molecular docking approach was used to evaluate the role of DLD into the progression of AD. We looked into the binding energies (kcal/mol) and molecular interactions of three interacting proteins, Cullin-associated NEDD8-dissociated protein 1 (CAND1), Lysosome-associated membrane glycoprotein 1 (LAMP1), and Tripeptidyl-peptidase 1 (TPP1) with DLD. In past, it has been successful to develop new drugs using conventional drug discovery strategies (Tang et al., 2006), but it takes more than 12 years for a lead to be identified to be tested with clinical trials costing approximately $1.8 billion USD on average (Paul et al., 2010).

Due to their potential to reduce time, labor, and costs associated with drug discovery, *in silico* approaches have recently attracted significant interest. The use of computational methods has led to the development of many new drugs (Shaker et al., 2021). In our study, we also evaluated the binding of different drug compounds such as ascorbic acid, acetaminophen, and methamphetamine after docking experiment *in silico.*

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Following *in silico* based Software and online tools have been used in this study.

3.1.1 Software

Table 1. List of Software. The software used in this study are enlisted with their applications and developer companies.

Software	Applications	Developer	References
ChemDraw Pro 12.0	Drawing tool to draw 2D structures of Ligands	PerkinElmer	
Discovery Studio	Cleaning of proteins, visualisation and 2D analysis	BIOVIA	Jejurikar et al., 2021
PyRx	Molecular docking	Source Forge	Pawar et al., 2021
PyMol	3-D Molecular visualisation of protein-ligand complex	Schrödinger, Inc.	Wang et al., 2021
LigPlot+ v.2.2	2-D visualisation of protein-ligand complexes	European Bioinformatics Institute	Wallace et al., 1995

3.1.2 Online Tools

Table 2. List of Online Tools. The online tools used in this study are enlisted with their applications.

Online Source	Applications	References
Universal Protein Resource (UniProt)	Database of protein sequence and functional information	The UniProt Consortium, 2021
String	Database and online resource for protein-protein interactions that are known and predicted	Szklarczyk et al., 2021
RCSB Protein Data Bank (*PDB)*	Database for 3-D structures of proteins	Zardecki et al., 2020
Biological General Repository for Interaction Datasets (BioGRID)	Database of protein-protein interactions	Oughtred et al., 2021
PubChem	2-D Structures of drug compounds	Kim et al., 2016
Comparative Toxicogenomics Database (CTD)	Database for chemical compounds	Davis et al., 2021
DrugBank	Database containing drugs and drugs targets	Wishart et al., 2018

3.2 Methodology

3.2.1 Ethics Statement

The Department of Neurology at University Medical Center, Göttingen provided all sAD, rpAD, and control brain samples after informed consent was obtained from patients or their guardians. Göttingen's local ethics committee approved the study (No. 24/8/12).

3.2.2 Brain Samples Collection

Samples of the frontal cortex were obtained from healthy control group, AD patients, and rapidly progressive AD (rpAD) patients. Molecular analyses were performed on snap-frozen (1 cm thick) tissue sections from one hemisphere. The diagnosis of sAD was made using the criteria for the Consortium to Establish a Registry for AD (CERAD). A Tris-Triton lysis buffer was used to homogenize brain tissue (10% w/v). An assay using Bradford's methods was used to quantify the protein concentration in lysis buffers containing Tris-Triton and Urea-Thiourea (Bradford et al., 1976).

3.2.3 Immunoprecipitation

The IP of DLD was performed with Dynabeads (1.5 mg for each 0.5 mg of protein in the sample). Dynabeads were washed with 0.3% CHAPS and then incubated with 4 µl of DLD antibody, ab133551 (abcam, US), for 30 min at 4°C. The interactors were eluted in Laemmli buffer and were stored at -20°C until analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI MS/MS).

3.2.5 Selection of Protein and Ligands

A total of 271 proteins were identified that interacted with DLD protein. In control groups, two proteins were identified, seven proteins in AD, nine proteins in control and AD, and seventeen proteins were identified in 2 out of 3 control healthy group, AD and rapidly progressive AD (rpAD) groups interacting with DLD shown in Figure 1. In our study, we used CAND1, LAMP1, and TPP1 for *in silico* interaction with DLD on the basis of their functional domains and how they are involved in the aggregation of AD.

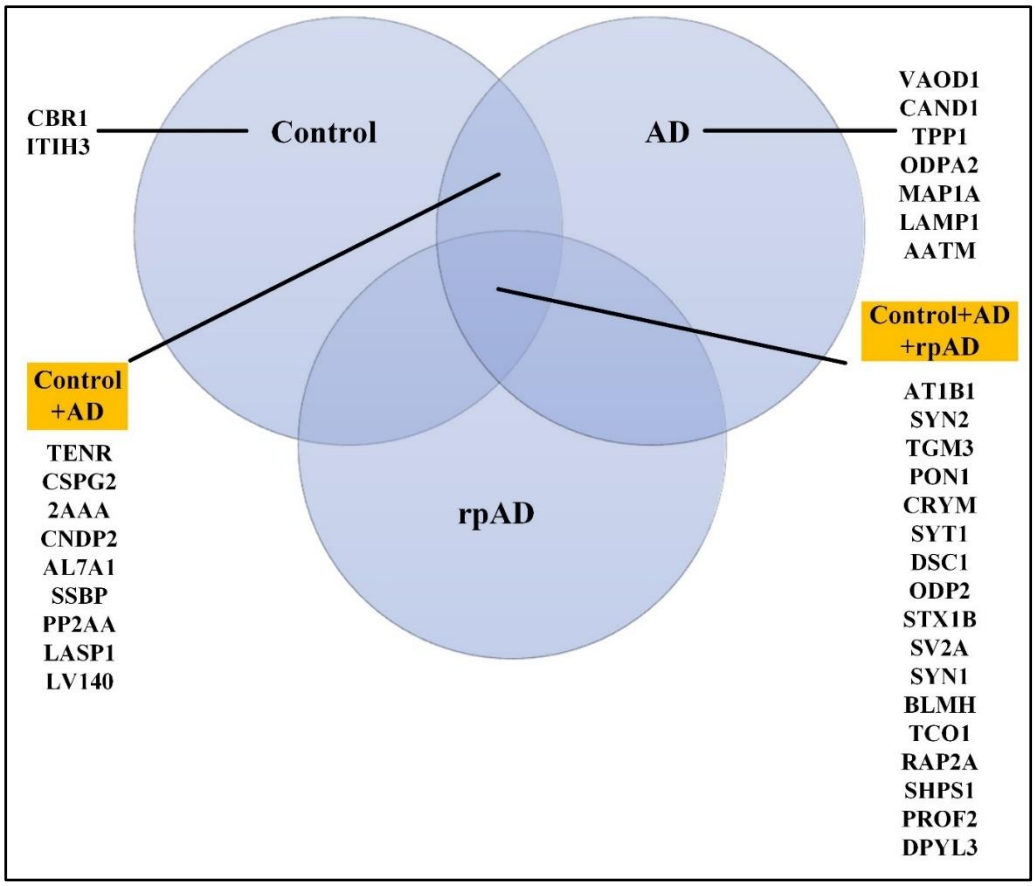
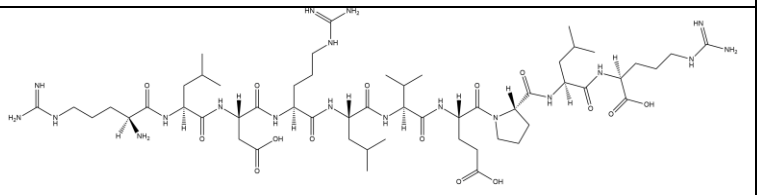
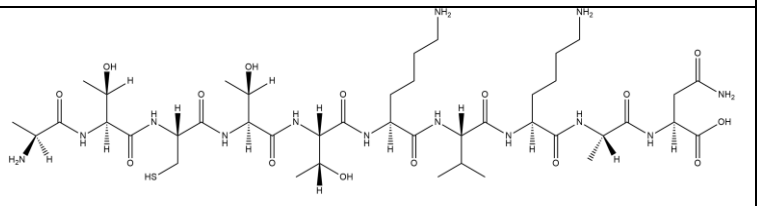


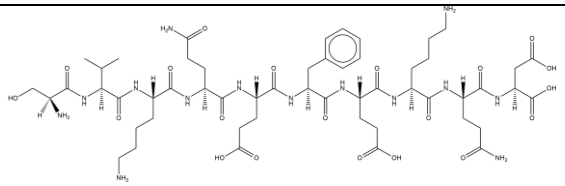
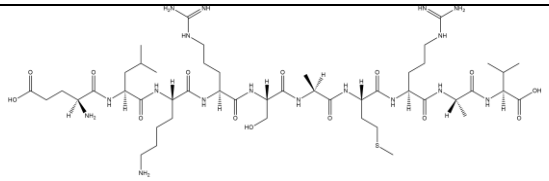
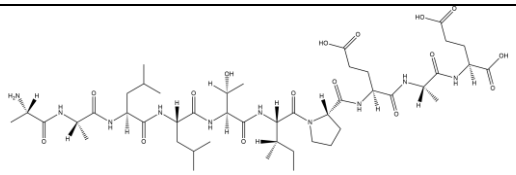
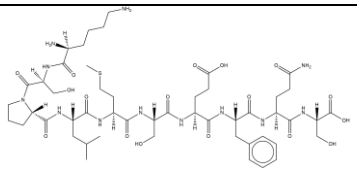
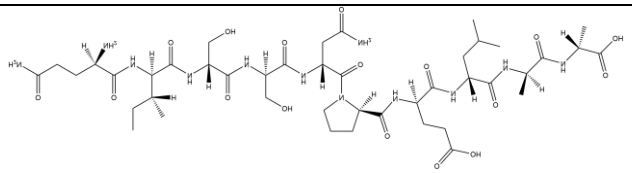
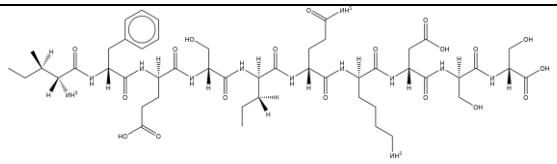
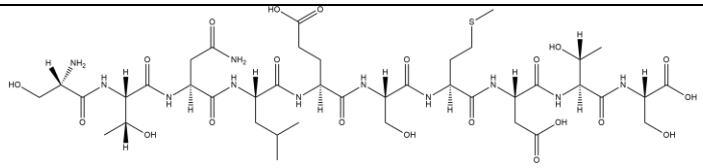
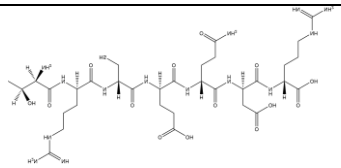
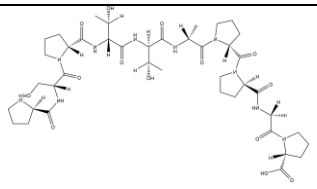
Figure 1. Venn diagram of Common DLD Interactors. Two proteins in healthy control group, 7 proteins in AD, nine proteins in control and AD and 17 proteins were identified in 2 out of 3 control healthy group, AD and rpAD groups interacting with DLD.

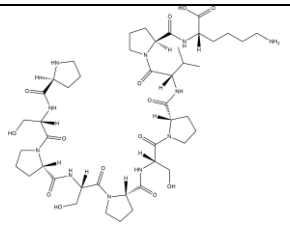
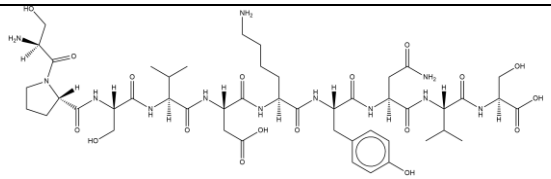
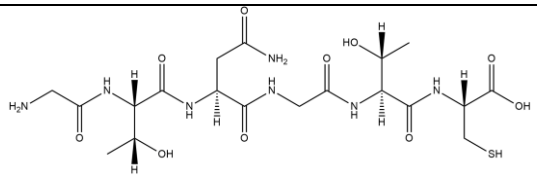
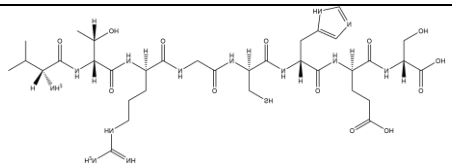
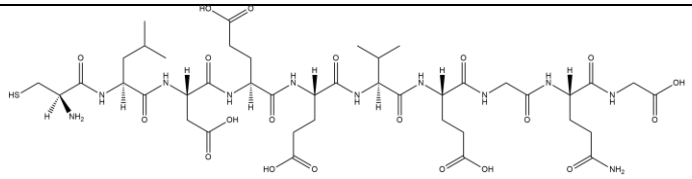
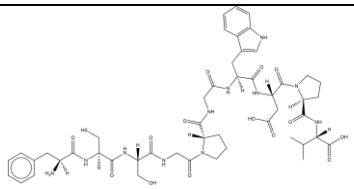
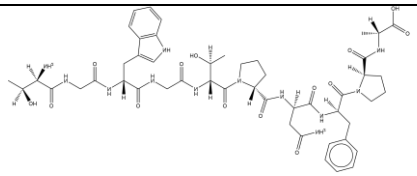
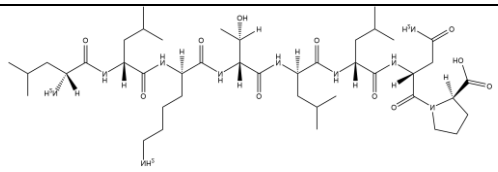
3.2.6 Preparation of Ligands

ChemDraw Pro 12.0 software was used to draw 2-D structures of ligands and were saved in .sdf format.

Table 3. Structures of ligands. 2-D structures of CAND1, LAMP1, and TPP1 selected for current study.

Ligands	Sequence	2D Structure
Cullin-associated NEDD8-dissociated protein 1 (CAND1)	RLDRLVEPLR (1141-1150)	
	ATCTTKVKAN (1151-1160)	

	SVKQEFEKQD (1161-1170)	
	ELKRSAMRAV (1171-1180)	
	AALLTIPEAE (1181-1190)	
	KSPLMSEFQS (1191-1200)	
	QISSNPELAA (1201-1210)	
	IFESIQKDSS (1211-1220)	
	STNLESMDTS (1221-1230)	
Lysosome-associated membrane glycoprotein 1 (LAMP1)	TRCEQDR (189-195)	
	PSPTTAPPAP (196-205)	

	PSPSPSPVPK (206-215)	
	SPSVDKYNVS (216-225)	
	GTNGTC (226-231)	
Tripeptidyl-peptidase 1 (TPP1)	VTRGCHES (518-525)	
	CLDEEVEGQG (526-535)	
	FCSGPGWDPV (536-545)	
	TGWGTPNFPA (546-555)	
	LLKTLLNP (556-563)	

3.2.7 Preparation of Protein Molecule

The 3-D crystal structure of the human DLD protein (5NHG) was retrieved from the RCSB PDB in .pdb format. The protein molecules were further purified by using Discovery Studio software. The Hetatms and water molecules were removed and active sites selected from the

protein report given by the software, as shown in Figure 2. The protein structure (5NHG) was then stored in .pdb format for further analysis.

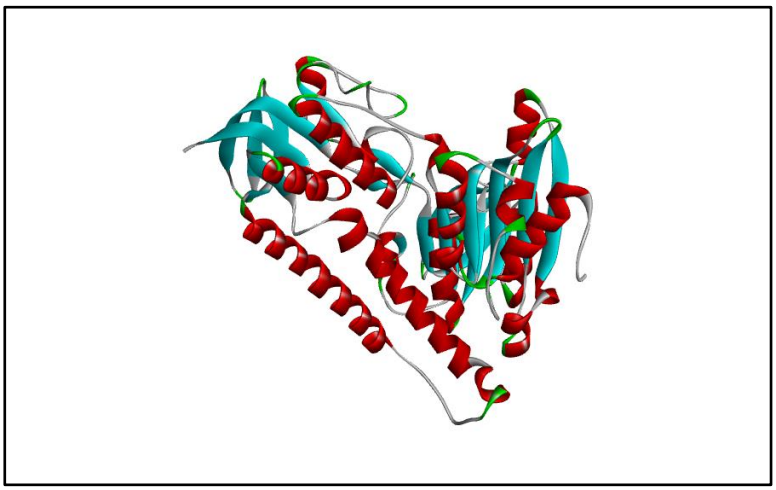


Figure 2. 3-D Structure of DLD. Retrieved from PDB under the ID; 5NHG and prepared crystal structure of DLD using Discovery Studio by removing water molecules and Hetatms.

3.2.8 Energy Minimization

In PyRx (version 0.8), Open Babel was used to optimise the ligands in simple data format (SDF), using the "Merk Molecular Force Field 94 (MMFF94)" to energetically transform them into the most stable forms. Ligands were converted to PDBQT format for further docking analysis.

3.2.9 Molecular Docking

Trott and Olson's docking procedure was used to perform the molecular docking (Trott & Olson, 2010). In PyRx, protein molecules was converted into macromolecules in PDBQT format. Vina Wizard was used for the molecular analysis to determine how the selected ligands interact with our target protein. Ten configurations for each protein-ligand complex were produced following the completion of the molecular docking analysis. the best docking value was considered to be the one with the smallest bind and "Root Mean Square Deviation (RMSD)".

3.2.10 Protein-Ligand Interaction

The docked molecules were uploaded in PyMol in .pdb format to create a ligand-receptor complex, which was then used to analyse the interaction between the ligand and receptor. The Discovery Studio was then used to visualise 2-D structure of protein-ligand interactions, after

which Ligplot++ was used to visualise the 2-D structure of protein-ligand complex containing hydrogen bonds and hydrophobic interactions.

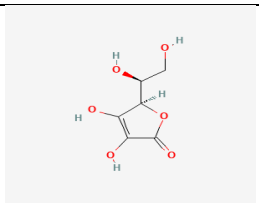
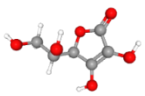
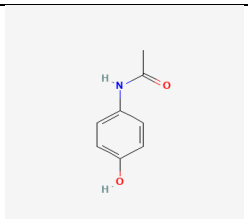
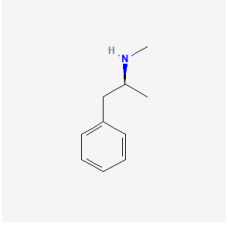
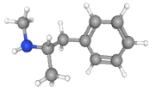
3.2.11 Network Analysis for the Identification of Common Interacting Proteins

A protein-protein interaction network helped identify the interaction of each protein with another protein with a different molecular function in a diseased state. String and BioGRID databases were used to evaluate the proteins that interact with other.

3.2.12 Drug Screening

The drug likeliness ligands were identified using CTD database and Drug Bank database. Three compounds were identified: ascorbic acid, methamphetamine, and acetaminophen. PubChem database was used to download the 2-D structure of drug likeliness ligands and were docked with protein molecule in PyRx to determine the interactions between them. PyMol was used to make a protein-ligand complex, and for further visualisation and analysis Discovery Studio and LigPolot++ were used.

Table 4. Structures of Drug Compounds. 2-D and 3-D structures of potential therapeutic compounds retrieved from PubChem

Drug Compounds	2-D Structure	3-D Structure
Ascorbic Acid		
Acetaminophen		
Methamphetamine		

CHAPTER 4

RESULTS

DLD was downloaded from PDB database under the PDB ID: 5NHG and was selected as a target protein. Proteins that interacted with DLD differentially in AD state were selected as candidate ligands, i.e., CAND1, LAMP1, and TPP1.

4.1 Molecular Docking Analysis

Molecular docking is a process in which ligands attach to the targeted proteins resulting in a new complex, shown in Figure 3. All of the three ligands were docked against our targeted protein 5NHG in PyRx Vina Wizard according to Trott & Olson (2009) and formed new complexes.

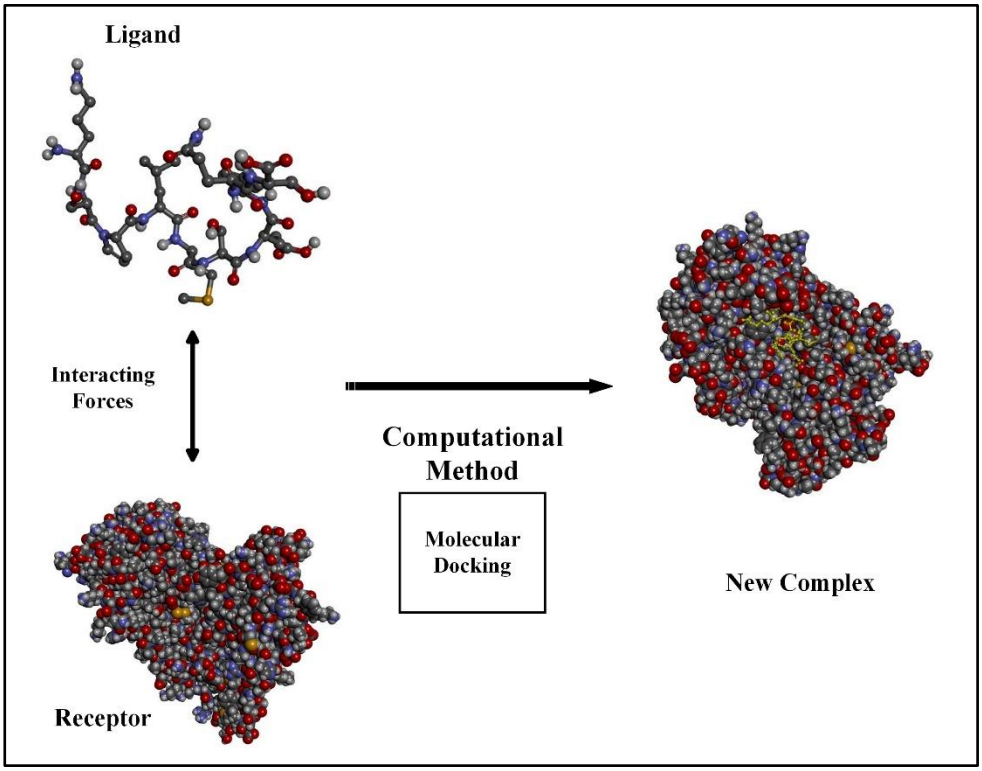


Figure 3. Ligand and receptor complex. Ligand and receptor molecules bind together into a new large molecular complex through the process of molecular docking.

The highest binding energy (kcal/mol) that result after docking was considered as the most suitable docking model of the ligands as presented in Table 5.

Table 5. Binding energy values of protein molecule and ligands. Binding energy values of CAND1, LAMP1, and TPP1 with 5NHG evaluated by PyRx docking

Ligands	**Binding Domains Sequence**	**Targeted Protein**	**Binding Energy (kcal/mol)**
Cullin-associated NEDD8-dissociated protein 1 (CAND1)	RLDRLVEPLR(1141-1150)	5NHG	-7.2
	ATCTTKVKAN (1151-1160)		-6.3
	SVKQEFEKQD (1161-1170)		-6.8
	ELKRSAMRAV (1171-1180)		-6.7
	AALLTIPEAE (1181-1190)		-6.8
	KSPLMSEFQS (1191-1200)		-7.6
	QISSNPELAA (1201-1210)		-5.1
	IFESIQKDSS (1211-1220)		-7.5
	STNLESMDTS (1221-1230)		-6.1
Lysosome-associated membrane glycoprotein 1 (LAMP1)	TRCEQDR (189-195)	5NHG	-7.1
	PSPTTAPPAP (196-205)		-7.8
	PSPSPSPVPK (206-215)		-5.9
	SPSVDKYNVS (216-225)		-8.6
	GTNGTC (226-231)		-7.1
Tripeptidyl-peptidase 1 (TPP1)	VTRGCHES (518-525)	5NHG	-8.7

	CLDEEVEGQG (526-535)		-7.9
	FCSGPGWDPV (536-545)		-10.2
	TGWGTPNFPA (546-555)		-10.8
	LLKTLLNP (556-563)		-7.1

CAND1 domain sequences are docked with 5NHG, CAND1 (1191-1200) produces the highest binding energy of -7.6 kcal/mol when docked with 5NHG. Similarly, when LAMP1 sequences are docked, LAMP1 (216-225) yields the highest binding energy of -8.6 kcal/mol with 5NHG. Also, TPP1 (546-555) shows the highest binding energy of -10.8 kcal/mol when docked with 5NHG respectively. The interaction of sequences of ligand proteins with 5NHG yielding high binding affinity shows how AD can be caused by the apoptotic regulation of these interacting proteins.

4.2 Binding Energy Evaluation

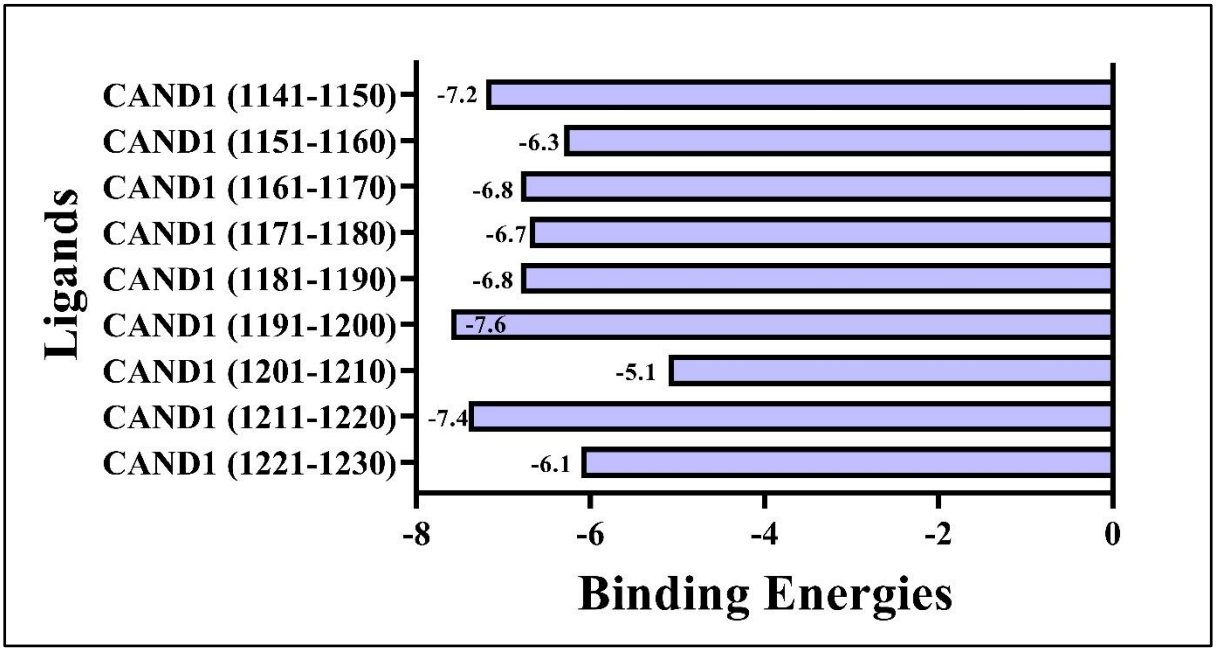


Figure 4. Binding energy values of CAND1 with 5NHG. Binding energy as a result of docking results of CAND1 with 5NHG evaluated by PyRx. CAND1 (1191-1200) produces the highest binding energy of -7.6 kcal/mol when docked with 5NHG.

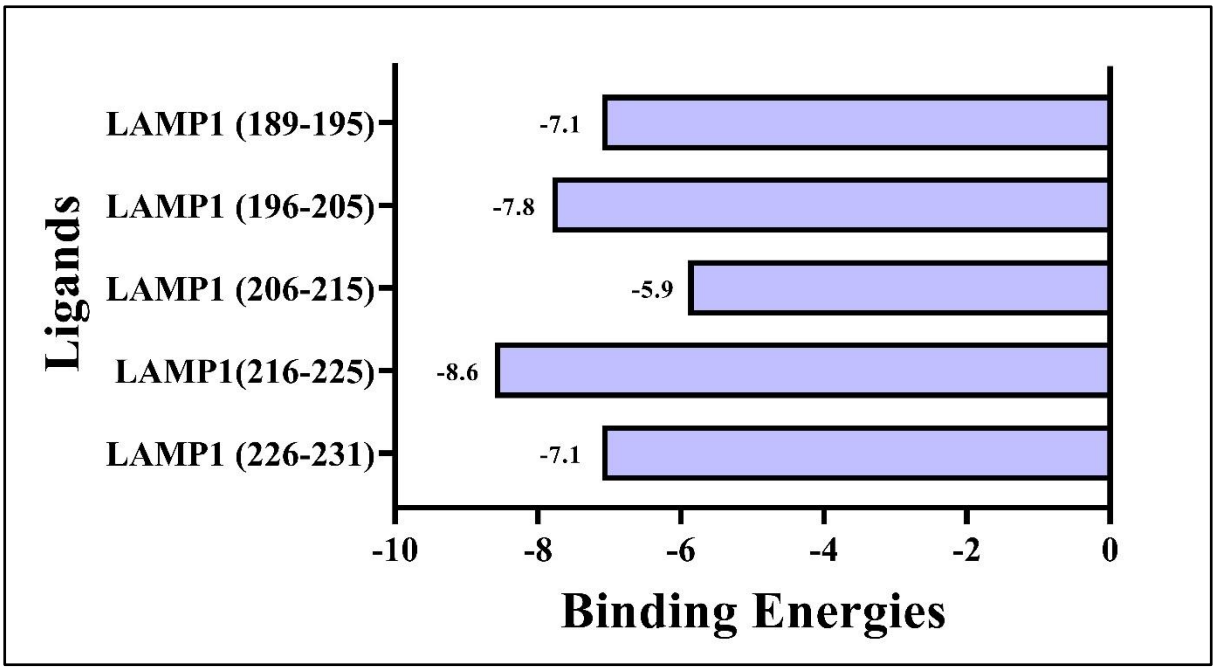


Figure 5. Binding energy values of LAMP1 with 5NHG. Binding energy as a result of docking of LAMP1 with 5NHG evaluated by PyRx. LAMP1 (216-225) yields the highest binding energy of -8.6 kcal/mol with 5NHG.

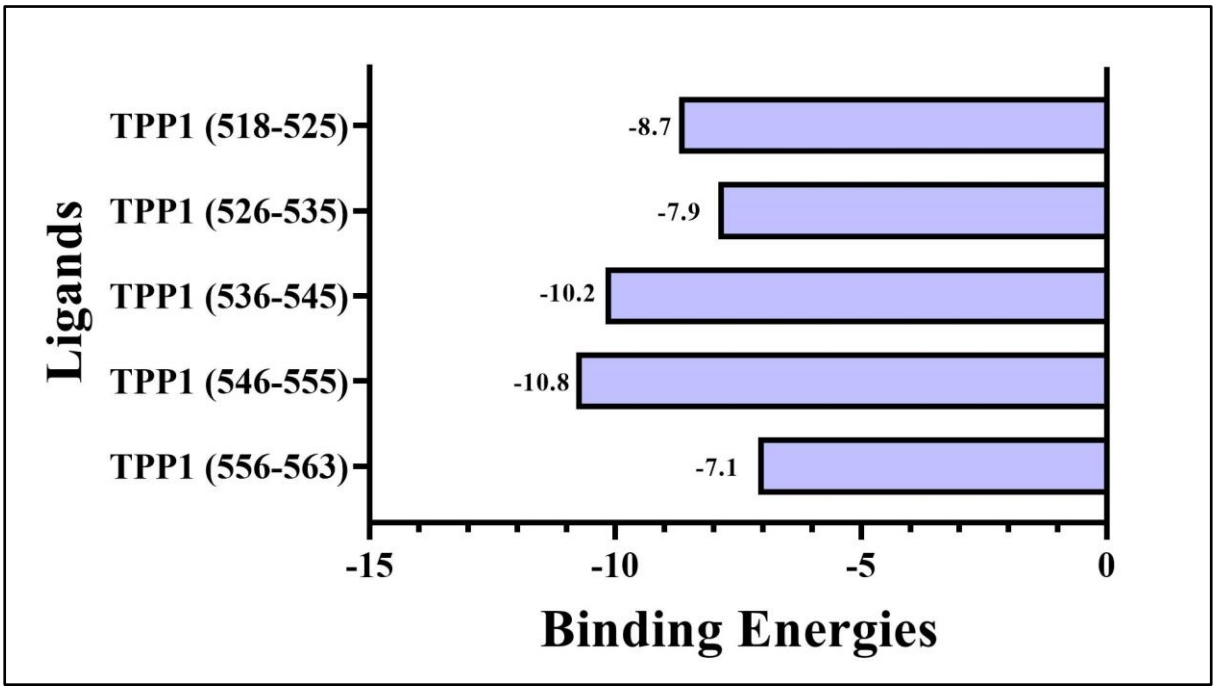


Figure 6. Binding energy values of TPP1 with 5NHG. Binding energy as a result of docking of TPP1 with 5NHG evaluated by PyRx. TPP1 (546-555) yields the highest binding energy of -10.8 kcal/mol when docked with 5NHG respectively.

4.3 Molecular Interaction Analysis

The binding sites of 5NHG are surrounded by 68 amino acids including, GLY 13,SER 14, GLY 15, GLY 17, PRO 16, GLY 18, GLU 36, LYS 37, ASN 38, GLU 39, GLY 43, THR 44, CYS 45, VAL 48, GLY 49, CYS 50, LYS 54, GLY 117, TYR 118, LYS 120, THR 130, LYS 131, ALA 132, ALA 147, THR 148, GLY 149, SER 150, GLU 151, VAL 152, THR 153, GLU 162, SER 167, THR169, GLY 187, VAL 188, GLU 192, GLY 215, VAL 216, GLY 217, CYS 277, ILE 278, ARG 280, ARG 281, PRO 282, PHE 283, ARG 299, ARG 301, GLY 319, ASP 320, VAL 322, ALA 323, GLY 324, PRO 325, MET 326, LEU 327, ALA 328, HIS 329, ALA 331, ASN 352, VAL 354, SER 356, VAL 357, TYR 359, TRP 366, VAL 367, GLY 368, LYS 369, and GLN 373. The PyMol and Discovery Studio were used to observe the ligands interactions into the binding site of protein 5NHG.

4.3.1 CAND1 (1191-1200) Interaction Analysis

It was observed that CAND1 (1191-1200) interacted with the 32 amino acids inside the binding pocket of 5NHG including, PRO 16,GLY 13,SER 14, GLY 15, GLY 17, GLY 18, LYS 37, GLU 36, ASN 38, GLU 39, GLY 43, THR 44, VAL 48, GLY 117, TYR 118, LYS 120, THR 130, LYS 131, ALA 132, ALA 147, THR 148, GLY 149, SER 150, GLU 151, VAL 152, GLU 162, SER 167, THR169, ARG 280, PHE 283, GLY 319, and ALA 331, shown in Figure 7.

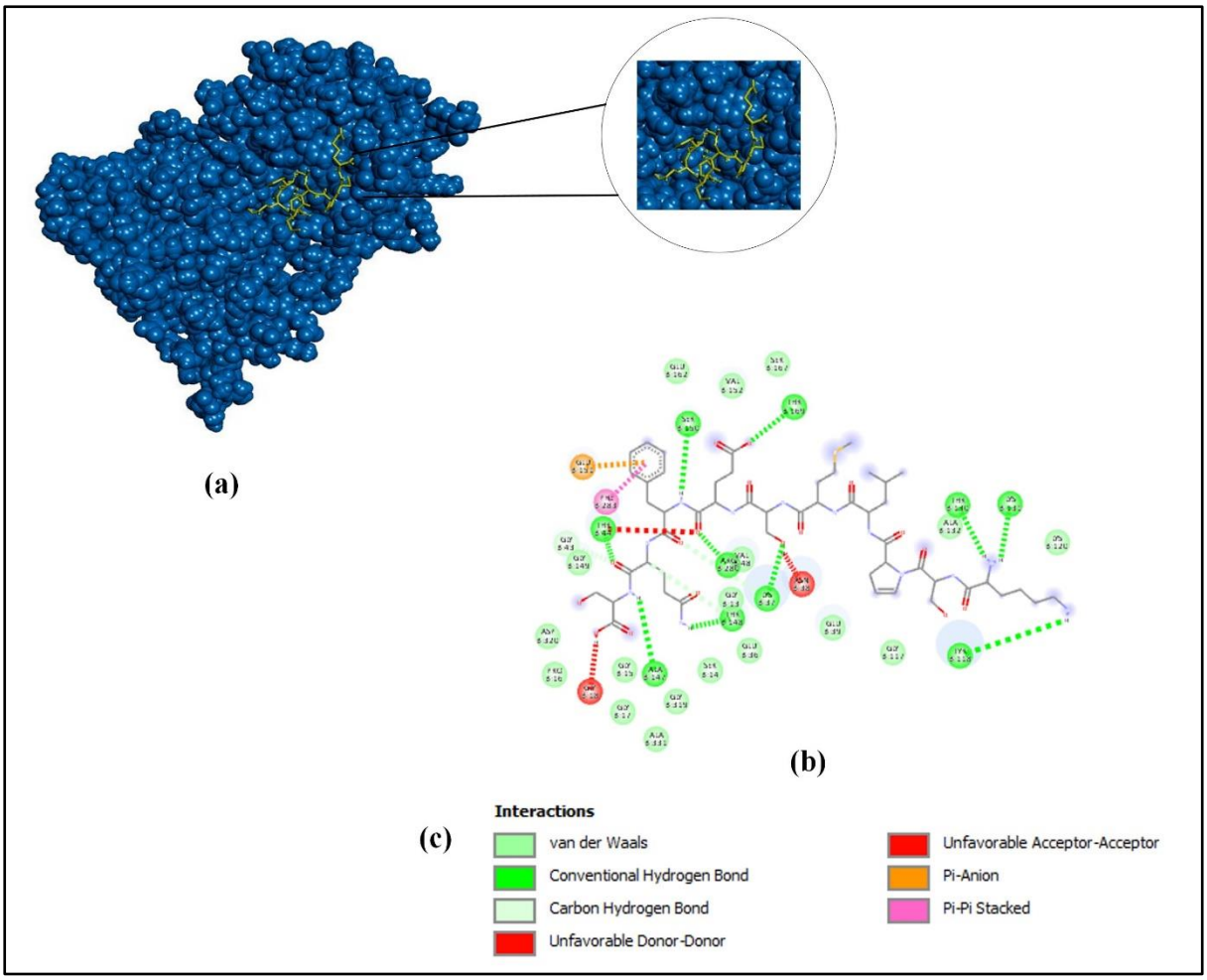


Figure 7. Molecular interaction analysis of CAND1 with 5NHG. (a) Surface illustration of DLD (PDB: 5NHG) show the binding configuration of docked CAND1 (1191-1200) (Yellow) **(b)** 2-D visualisation of Protein-Ligand interactions **(c)** Interactions produced in result of docking.

Three unfavourable donor-donor and acceptor-acceptor interactions were observed with GLY 18, ASN 38, and THR 44. A Pi-Anion with GLU 151 and a Pi-Pi Stacked with PHE 283 interactions were also observed. Two Carbon Hydrogen Bonds were established with ARG 280 and THR 148. Ten Conventional Hydrogen Bonds were observed with LYS 37, THR 44, TYR 118, THR 130, LYS 131, ALA 147, THR 148, SER 150, THR 169, ARG 280. The remaining interactions were van der Waals with PRO 16,GLY 13,SER 14, GLY 15, GLY 17, GLU 36, GLU 39, GLY 43, VAL 48, GLY 117, LYS 120, ALA 132, GLY 149, VAL 152, GLU 162, SER 167, GLY 319, ALA 331.

4.3.2 LAMP1 (216-225) Interaction Analysis

It was observed that LAMP1 (216-225) interacted with 33 amino acids inside the binding pocket of 5NHG including, CYS 45, GLY 49, CYS 50, LYS 54, SER 150, GLY 187, VAL

188, ILE 189, GLU 192, VAL 216, GLY 217, CYS 277, ILE 278, GLY 279, ARG 280, ARG 299, ASP 320, GLY 324, PRO 325, MET 326, ALA 328, HIS 329, TYR 351, ASN 352, VAL 354, SER 356, VAL 357, TYR 359, TRP 366, VAL 367, GLY 368, LYS 369, and GLN 373, shown in Figure 8.

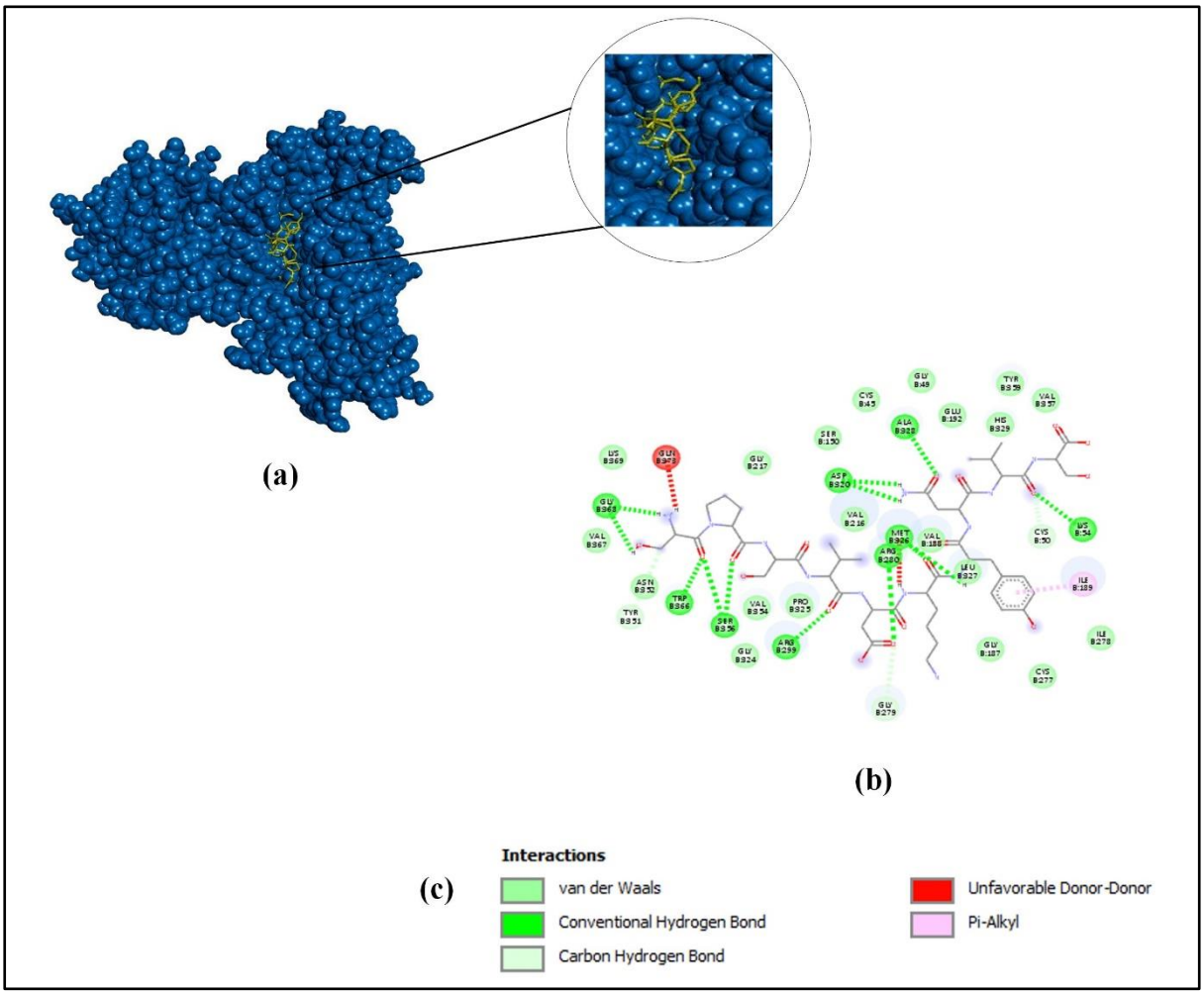


Figure 8. Molecular interaction analysis of LAMP1 with 5NHG. (a) Surface illustration of DLD (PDB: 5NHG) shows the binding configuration of docked LAMP1 (216-225) (Yellow) **(b)** 2-D visualisation of Protein-Ligand interactions **(c)** Interactions produced in result of docking.

Two Unfavourable Donor-Donor interactions with MET 326 and GLN 373 were observed. One Pi-Alkyl with ILE 189 and three Carbon Hydrogen Bonds were formed with CYS 50, GLY 279, and TYR 351. Twelve Convention Hydrogen Bonds were yielded with LYS 54, ARG 280, ARG 299, ASP 320, MET 326, ALA 328, SER 356, TRP 366, and GLY 368. The remaining interactions were van der Waals with CYS 45, GLY 49, SER 150, GLY 187, VAL 188, GLU 192, VAL 216, GLY 217, CYS 277, ILE 278, GLY 324, PRO 325,,HIS 329, ASN 352, VAL 354, VAL 357, TYR 359, VAL 367, LYS 369.

4.3.3 TPP1 (546-555) Interaction Analysis

It was observed that TPP1 (546-555) interacted with 31 amino acids inside the binding pocket of 5NHG including, THR 44, CYS 45, GLY 49, CYS 50, SER 150, THR 153, GLY 187, VAL 188, ILE 189, GLY 215, VAL 216, GLY 217, CYS 277, ILE 278, GLY 279, ARG 280, ARG 281, PRO 282, ARG 299, ARG 301, ASP 320, VAL 322, ALA 323, GLY 324, PRO 325, MET 326, LEU 327, ALA 328, SER 356, TRP 366, and GLN 373, shown in Figure 9.

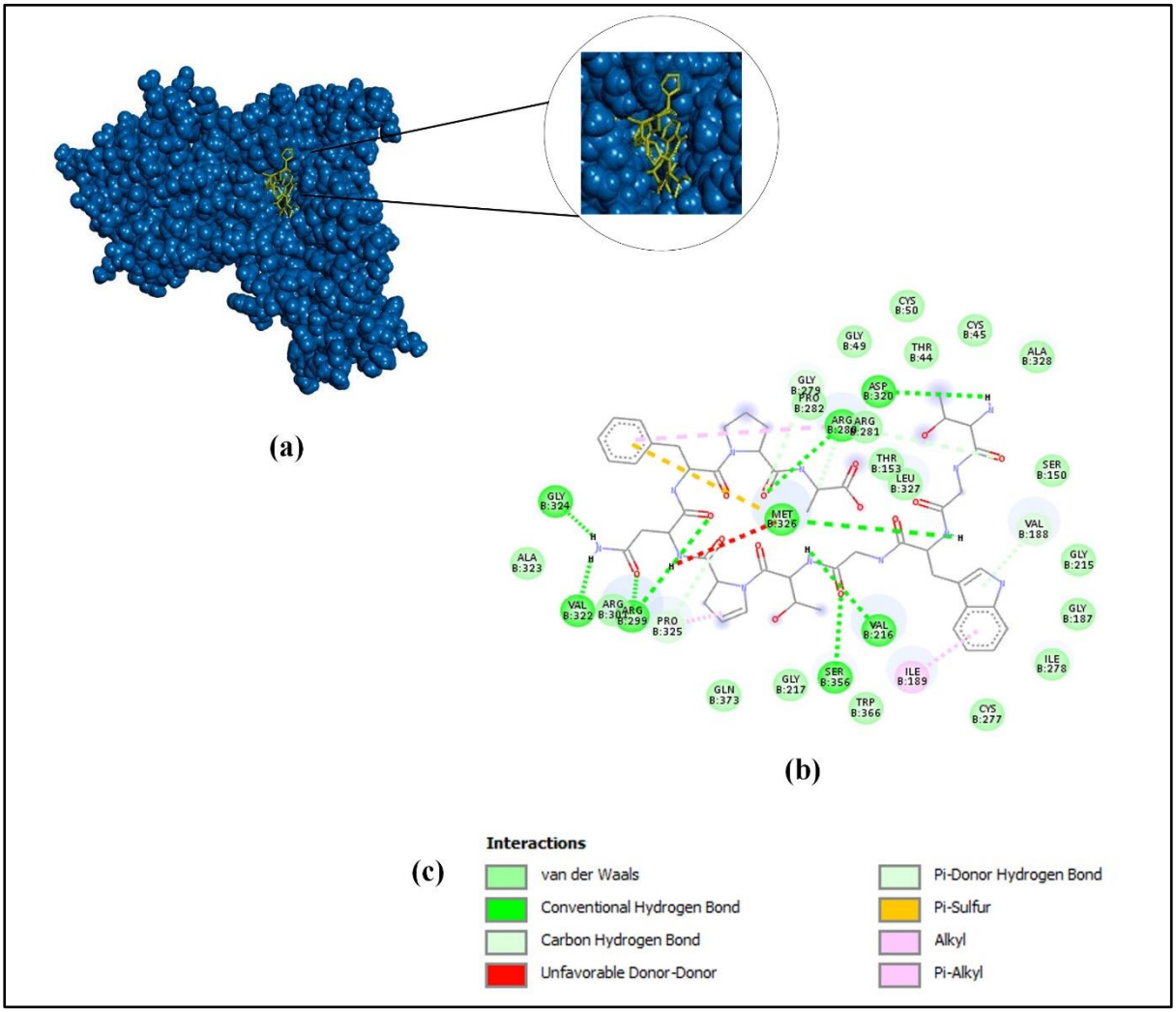


Figure 9. Molecular interaction analysis of TPP1 with 5NHG. (a) Surface illustration of DLD (PDB: 5NHG) show the binding configuration of docked TPP1 (546-555) (Yellow) **(b)** 2-D visualisation of Protein-Ligand interactions **(c)** Interactions produced in result of docking.

Three Alkyl and Pi-Alkyl interactions with ILE 189, ARG 280 and PRO 325, one Unfavourable Donor-Donor with MET 326, and one Pi-Sulfur interaction with MET 326 were observed. Three Carbon Hydrogen and Pi-Donor Hydrogen Bonds were formed with Val 188, GLY 279, and PRO 325. Nine Convention Hydrogen Bonds were yielded with VAL 216, ARG 280, ARG 299, ASP 320, VAL 322, GLY 324, MET 326, and SER 356. The remaining interactions were van der Waals with THR 44, CYS 45, GLY 49, CYS 50, SER 150, THR 153, GLY 187, GLY

215, GLY 217, CYS 277, ILE 278, ARG 281, PRO 282, ARG 301, ALA 323, LEU 327, ALA 328, TRP 366, and GLN 373.

4.4 2-D Protein-Ligand Complex Interaction Analysis

The protein-ligand complex interaction was evaluated using the Ligplot software. PDB file complexes made and retrieved from PyMOL, were analyzed by using Ligplot. Protein-ligand complexes in 2-D have been shown in Figure 10. Ligplot++ Software was used to visualise the 2-D structure of protein-ligand complex containing hydrogen bonds, and hydrophobic interactions.

5NHG-CNAD1 (1191-1200) complex was able to produce eleven hydrogen bonds at a distance of 3.27Å with Gly15, 2.99Å with Lys37, 3.3Å with Asn38, 2.25Å with Thr44, 3.24Å with Thr130, 2.99Å with Lys 131, 3.15Å with Thr148, 3.03Å with Ser150, 3.39Å with Ser167, 2.25Å with Thr169, and 2.78Å with Arg280 respectively. Thirteen hydrophobic interactions were also observed such as, Gly13, Glu36, Glu39, Gly43, Gly117, Tyr118, Ala132, Ala147, Val148, Gly149, Glu151, Val152, and Phe283.

5NHG-LAMP1 (216-225) complex was able to form twelve hydrogen bonds at a distance of 2.80Å with Lys54, 2.95Å with Cys277, 3.13Å with Arg280, 3.10Å with Arg299, 2.90Å with Met326, 3,10Å with Val357, 2.14Å with His329, 3.11Å with Asp333, 2.80Å with Gly368, 3.18 Å with Asn352, 3.32Å with Val354, and 3.13Å with Ser356. Eighteen hydrophobic interactions were also observed such as, Cys50, Gly187, Val188, Ile189, Glu192, Val216, Gly217, Ile278, Gly279, Asp320, Gly324, Pro325, Leu327, Ala328, Tyr351, Tyr359, Trp366, and Val367.

5NHG-TPP1 (546-555) complex was able to produce four hydrogen bonds at a distance of 3.26Å with Val216, 3.18Å with Asp320, 2.85Å with Gly324, and 2.94Å with Ser356. Nineteen hydrophobic interactions were also observed such as, Thr44, Cys45, Cys50, Thr153, Gly187, Val188, Ile189, Gly215, Gly217, Cys277, Gly279, Arg280, Arg281, Arg299, Arg301, Pro325, Met326, Leu327, and Trp366.

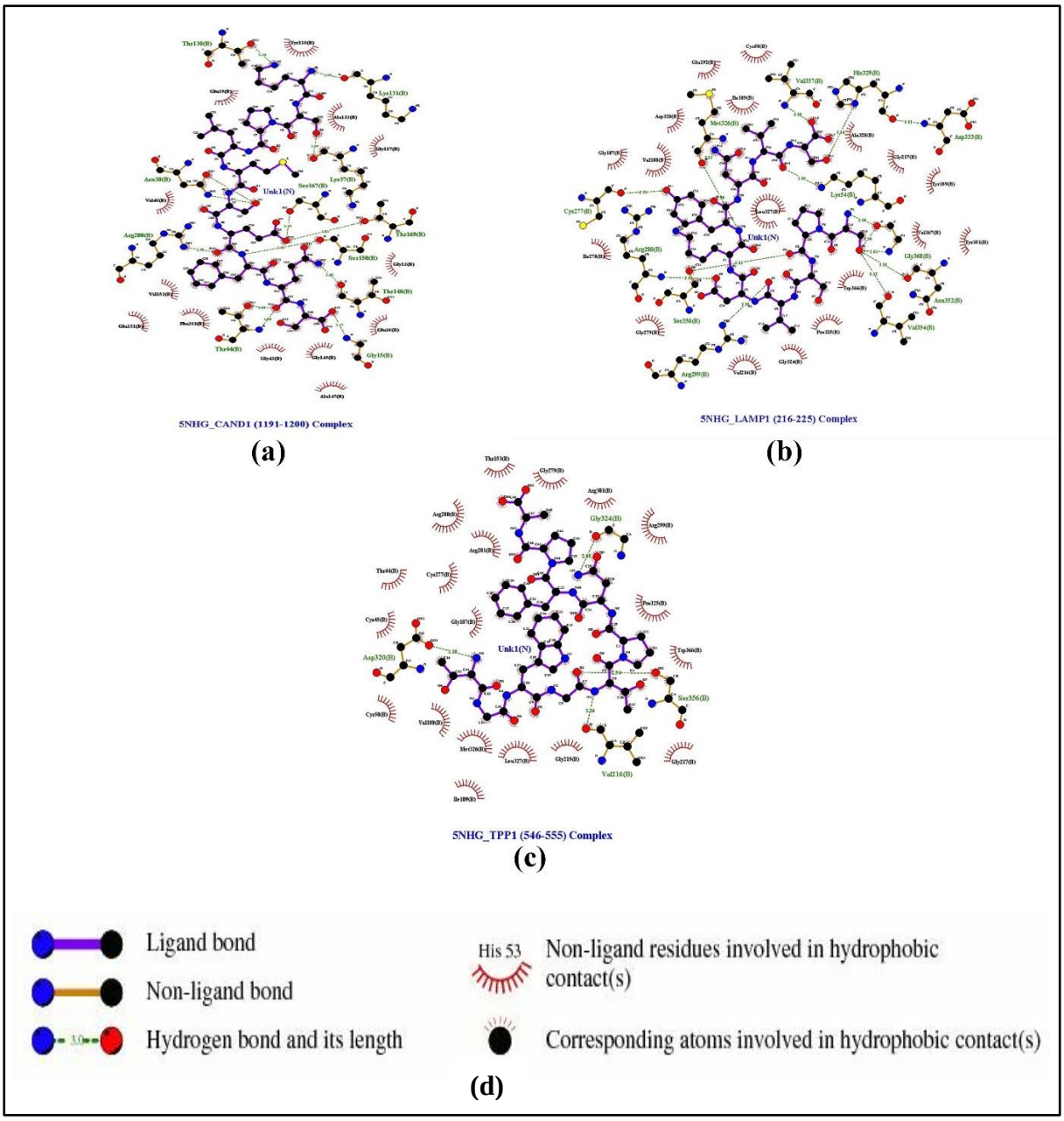


Figure 10. Protein-ligand complex interaction analysis. 2-D illustration of the interactions between **(a)** 5NHG-CAND1 (1191-1200) complex, **(b)** 5NHG-LAMP1 (216-225) complex, **(c)** 5NHG-TPP1 (546-555) complex. **(d)** The meaning of the items on the diagram**.** Spoked red arcs indicate hydrophobic interactions, green dashed lines indicate hydrogen bonds with distance in angstrom (Å). Purple line indicates ligand bond and yellow line indicates non-ligand bond.

4.5 Protein-Protein Network Analysis

The protein-protein interaction analysis revealed the interaction of proteins contributing to a pathological pathway. BioGRID and String databases were used for the protein network analysis. 451 interacting proteins of DLD, 863 interacting proteins of CAND1, 624 interacting proteins of LAMP1, and 161 interacting proteins of TPP1 were found. It was found that 140 interacting proteins of DLD, 164 of CAND1, 190 of LAMP1, and 46 interacting proteins of

TPP1 are common with each other and are involved in different molecular or biological pathways. Some of the common proteins that are involved in pathological pathways in AD are PDHB, FASN, AIFM1, AIMP2, HSD17B10, ZC3HAV1, ACBD3, EHD1, PRKAR2A, PARK2, DNAJA1, MYC, BAP1, TUBA1C, HNRNPH1, SLCLA5, BCKDHB, and EGFR, shown in Figure 11. These common interacting proteins have been found in different molecular pathways that are involved in inhibition of cell proliferation, apoptotic cell-death pathway, mitochondrial dysfunction, neurogenesis, and neural metastasis, as shown in Figure 12.

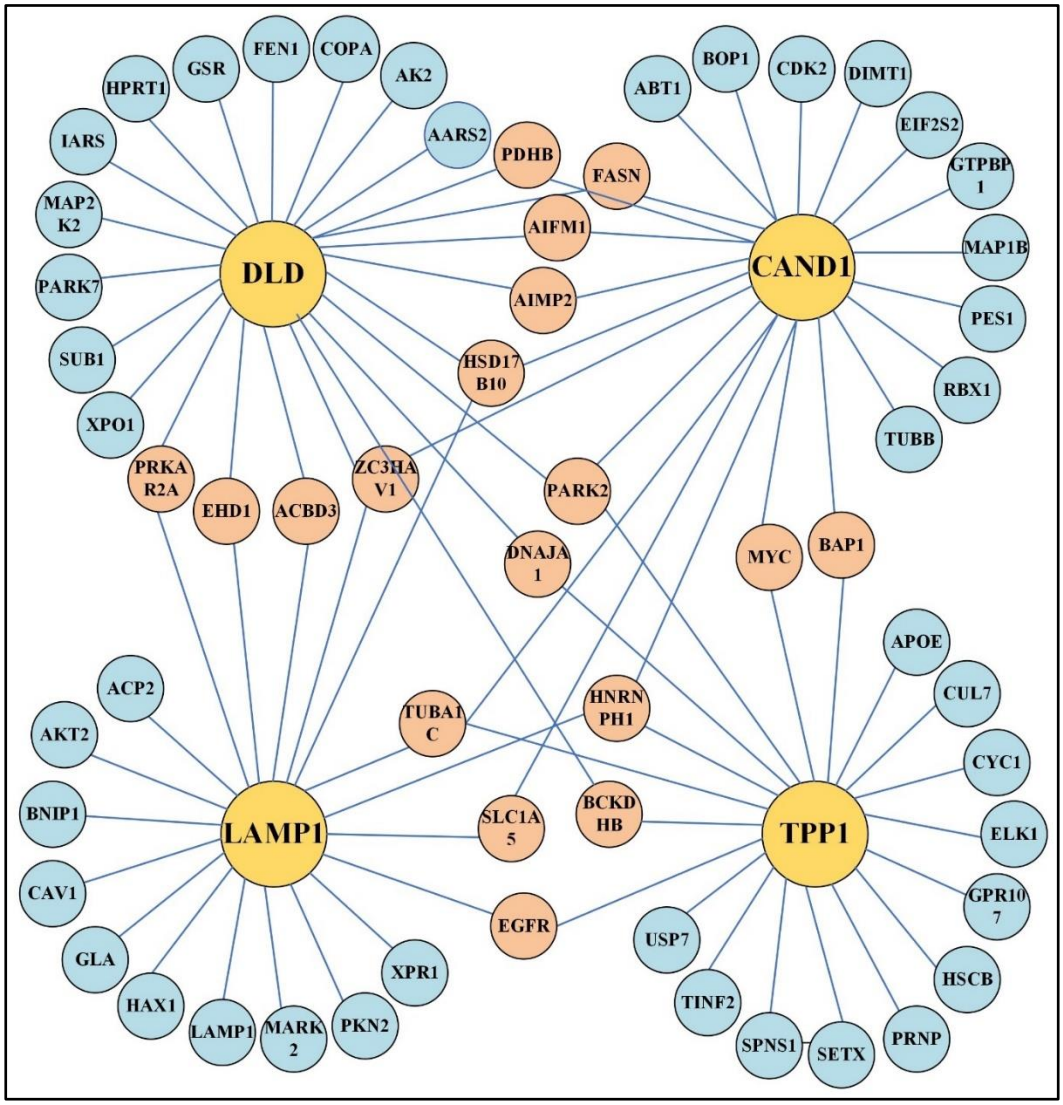


Figure 11. Protein-Protein interactions analysis. Protein-Protein Network containing four major proteins (yellow) shows the common interacting proteins (Pink) present between them that are involved in pathological pathways in AD and uncommon proteins (Blue)

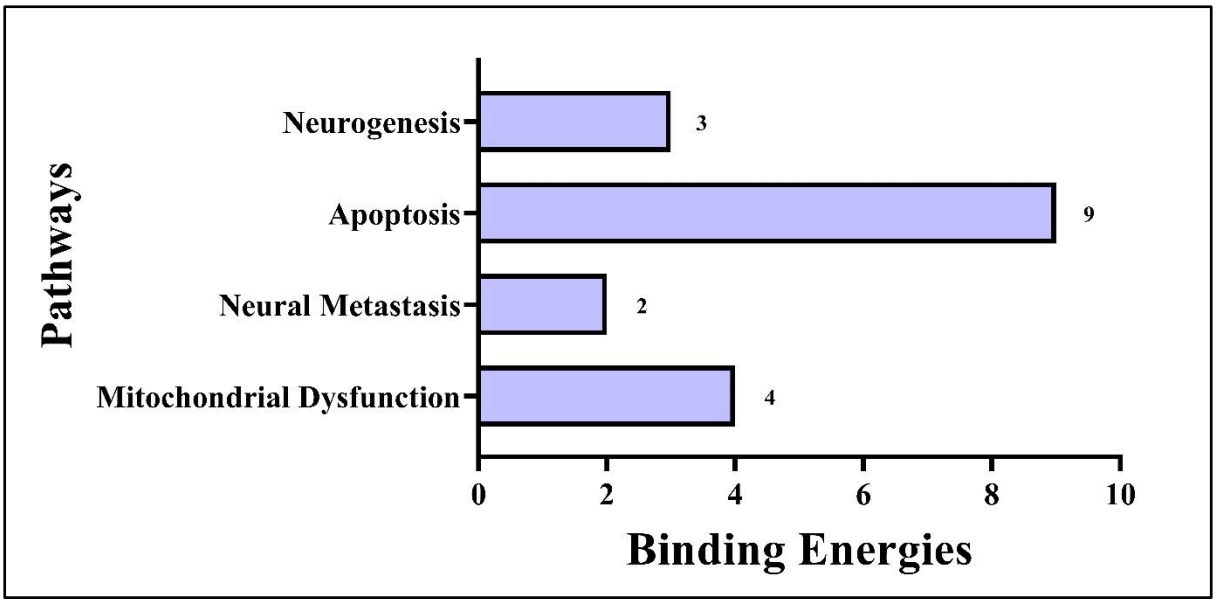


Figure 12. Proteins involved in different pathological pathways. Nine proteins have been found that are involved in apoptotic pathway, two proteins in neural metastasis, three proteins in neurogenesis, and four proteins have been found that are involved in mitochondrial dysfunction.

4.6 Drug-Protein Interaction Analysis

Drug Bank and CTD database used to find the drug compounds that are involved in the up-regulation or down-regulation of DLD protein. Three compounds were identified: ascorbic acid, methamphetamine, and acetaminophen. . All of the three drug compounds were docked against our targeted protein 5NHG in PyRx Vina Wizard according to Trott & Olson (2009). The highest binding energy (kcal/mol) that result after docking was considered as the most suitable docking model of the ligands, presented in Table 6.

Table 6. Binding energy values of Drug compounds with protein molecule. Binding energies as a result of docking of Ascorbic acid, Acetaminophen, and Methamphetamine with 5NHG evaluated by PyRx.

Drug Compound	Target	Binding Energy (kcal/mol)	Function
Ascorbic acid	5NHG	-6.1	Ascorbic acid results in increased expression of DLD protein
Acetaminophen	5NHG	-6	Acetaminophen results in increased expression of DLD protein
Methamphetamine	5NHG	-5.5	Methamphetamine results in increased expression of DLD protein

Ascorbic acid showed the highest binding energy of -6.1 kcal/mol with 5NHG. The acetaminophen also showed highest binding energy of -6 kcal/mol with 5NHG, which was comparable to the binding energy of ascorbic acid, shown in Figure 13. The interaction of these drug compounds with 5NHG yielding high binding affinity shows that these compounds can be used to block the binding sites on DLD to prevent the interaction of pathological proteins with DLD that are involved in the up-regulation or down-regulation of DLD in diseased state.

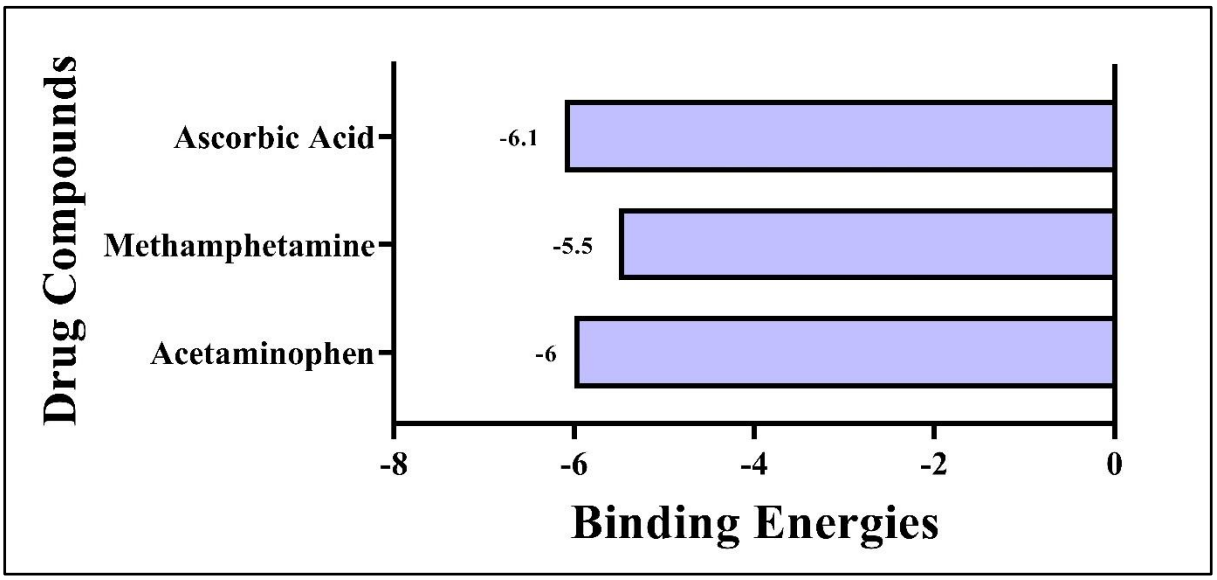


Figure 13. Binding energy values of drug compounds with 5NHG. Binding energy as a result of docking of drug Compounds with 5NHG evaluated by PyRx. The highest binding energy with 5NHG was ascorbic acid, which had a value of -6.1 kcal/mol. Acetaminophen likewise displayed the highest binding energy with 5NHG, at -6 kcal/mol, which was comparable to the ascorbic acid binding energy.

PyMol and Discovery Studio were used to visualise the 2-D structures and interactions of drug compounds into the binding pockets of protein 5NHG.

4.6.1 Ascorbic Acid

It was observed that ascorbic acid interacted with 15 amino acids inside the binding pocket of 5NHG including, GLY13, SER14, GLY15, PRO 16, GLY 18, GLU36, LYS37, ASN38, GLY43, THR44, CYS45, ALA147, THR148, GLY149, and SER150, shown in Figure 14.

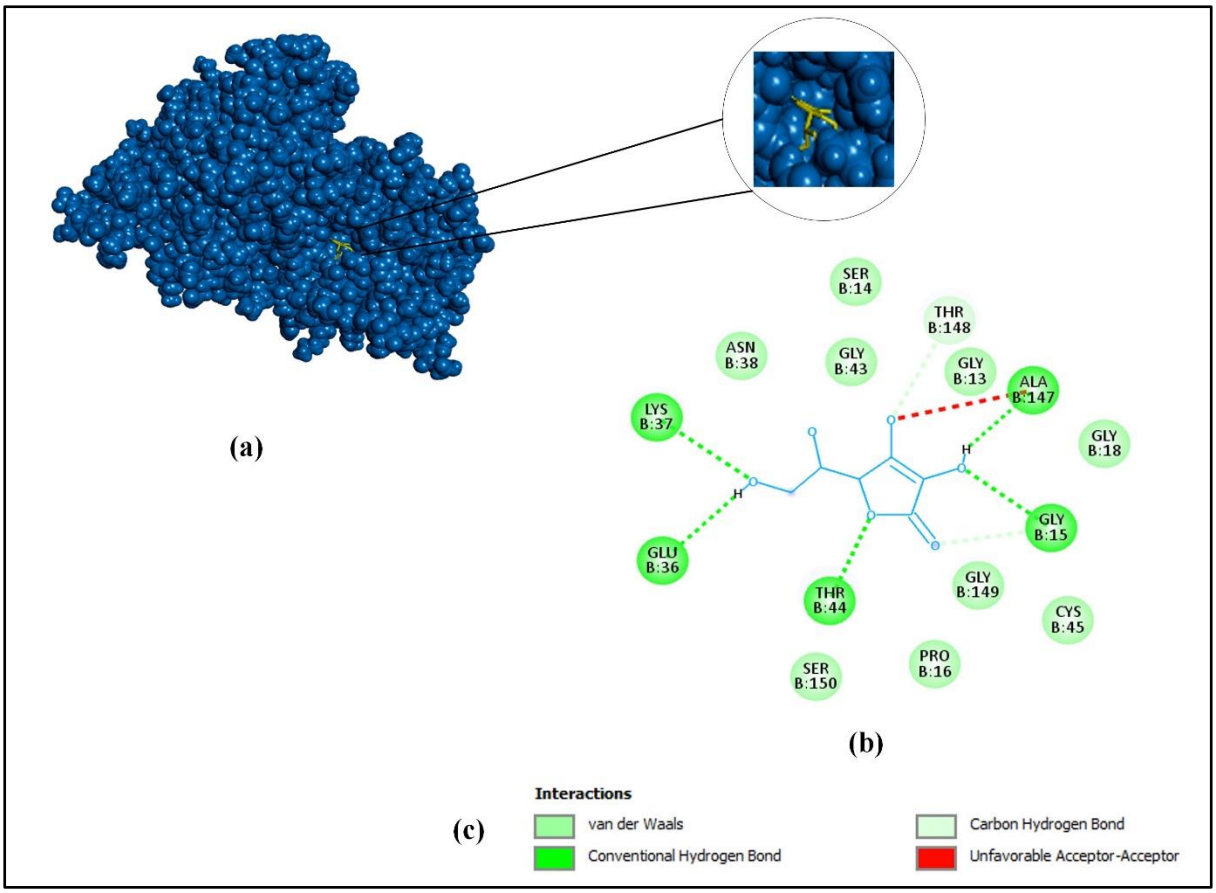


Figure 14. Molecular interaction analysis of Ascorbic acid with 5NHG. (a) Surface illustration of DLD (PDB: 5NHG) show the binding configuration of docked Ascorbic acid (Yellow) **(b)** 2-D visualisation of Protein-Ligand interactions **(c)** Interactions produced in result of docking

One Carbon Hydrogen Bond with THR148 and one Unfavourable Acceptor-Acceptor interaction with ALA147 were observed. Five Conventional Hydrogen Bonds were formed with GLY15, GLU36, LYS37, THR44, and ALA147. The remaining interactions were van der Waals with GLY13, SER14, PRO 16, GLY 18, ASN38, GLY43, CYS45, GLY149, AND SER150.

4.6.2 Acetaminophen

It was observed that acetaminophen interacted with 14 amino acids inside the binding pocket of 5NHG including, GLY15, PRO16, GLY17, GLY18, THR44, CYS45, ALA147, GLY149, ILE318, GLY319, ASP320, VAL321, ALA328, and ALA331, shown in Figure 15.

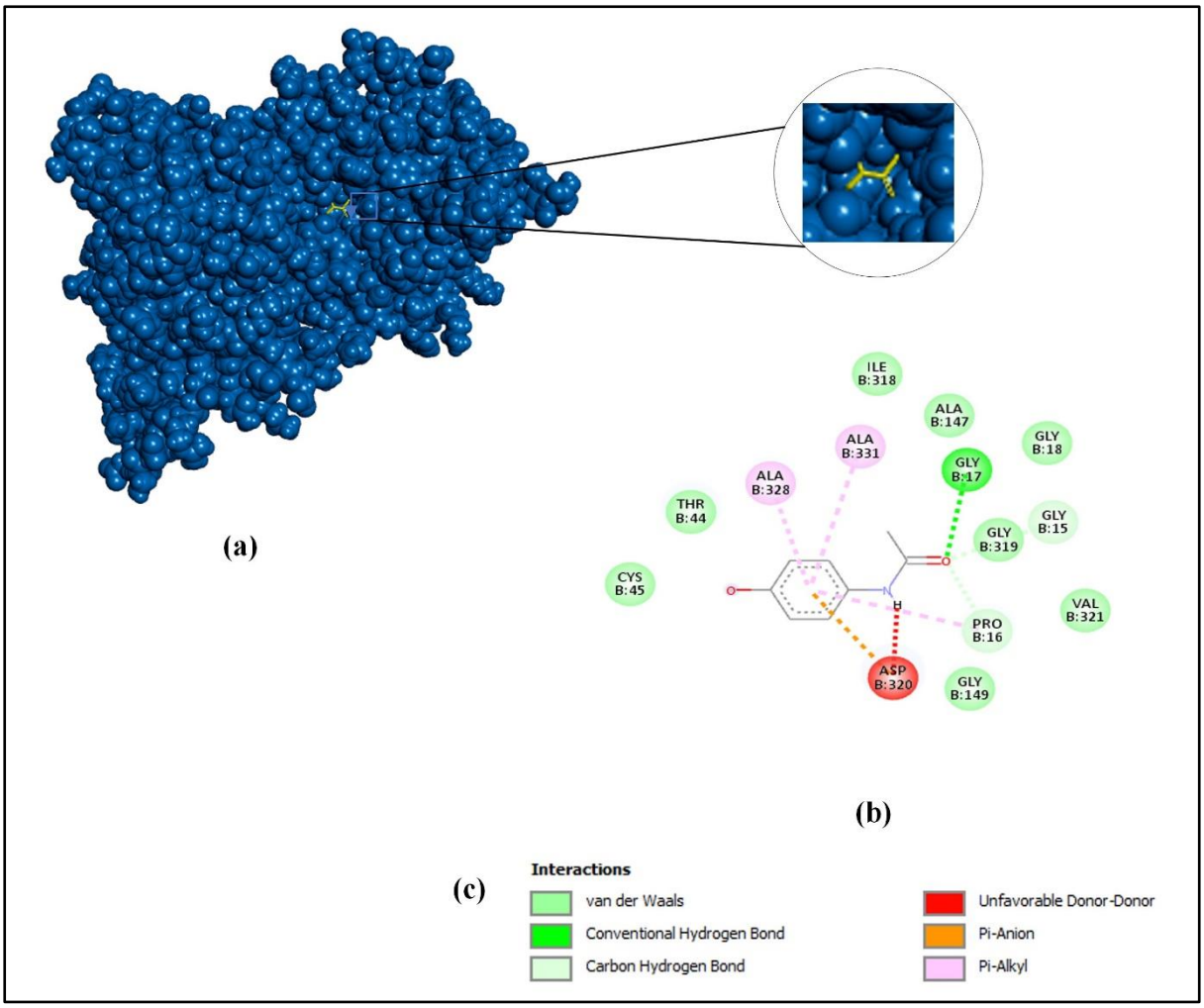


Figure 15. Molecular interaction analysis of Acetaminophen with 5NHG. (a) Surface illustration of DLD (PDB: 5NHG) show the binding configuration of docked Acetaminophen (Yellow) **(b)** 2-D visualisation of Protein-Ligand interactions **(c)** Interactions produced in result of docking

Three Pi-Alkyl interactions with PRO16, ALA328, and ALA331, one Pi-Anion and one Unfavourable Donor-Donor interactions with ASP320 were observed. Two Carbon Hydrogen Bonds with GLY15, PRO16, and one Conventional Hydrogen Bond with GLY17 were formed. The remaining interactions were van der Waals with GLY18, THR44, CYS45, ALA147, GLY149, ILE318, GLY319, and VAL321.

4.6.3 2-D Drug-Protein Complex Interaction Analysis

Ligplot software was used for the evaluation of the 2-D protein-drug compound complex interaction, shown in Figure 16. 5NHG-Ascorbic acid complex was able to form four hydrogen bonds at a distance of 3.10 Å with Gly15, 2.85 Å and 2.92 Å with Glu36, 2.79 Å with Thr44, and 2.79 Å and 2.98 Å with Ala147. Seven hydrophobic interactions were also observed with Gly13, Pro16, Lys37, Asn38, Gly43, Thr148, and Gly149.

5NHG-Acetaminophen complex was able to form only one hydrogen bond at a distance of 3.00 Å with Gly17. Ten hydrophobic bonds were also observed with Gly15, Pro16, Thr44, Cys45, Ala147, Ile318, Gly319, Asp320, Ala328, and Ala331.

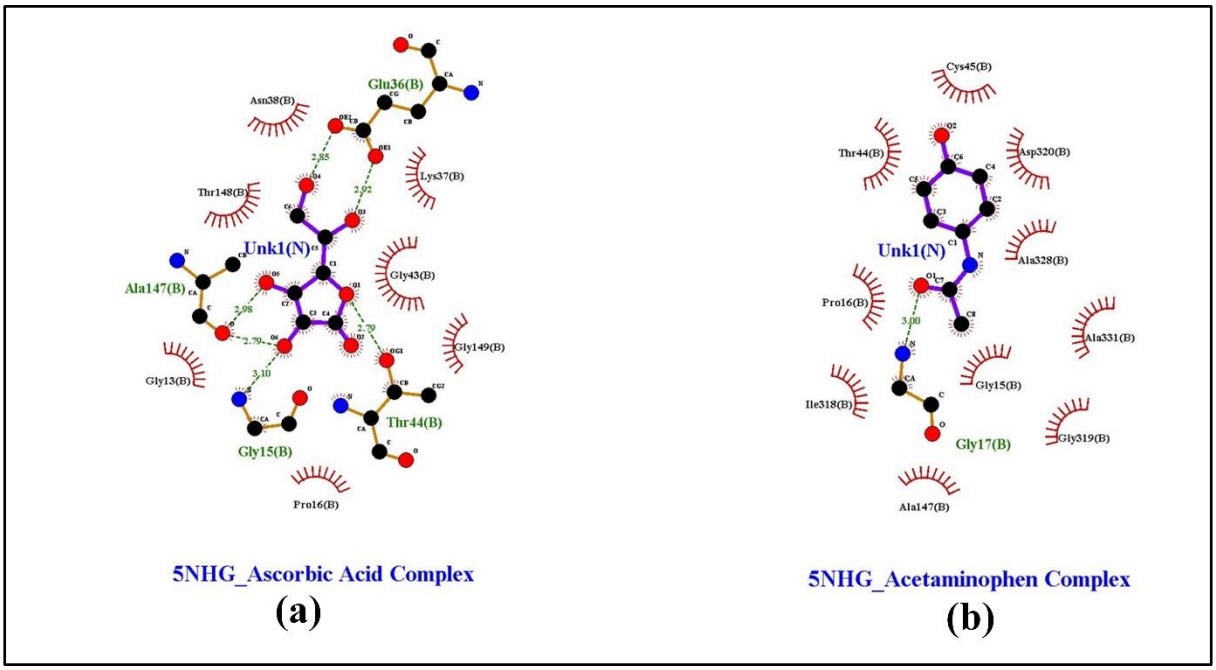


Figure 16. Protein-Drug Compounds complex interaction analysis. 2-D Diagrammatic sketch illustrating the interactions between **(a)** 5NHG-Ascorbic acid complex, **(b**) 5NHG-Acetaminophen complex. Green dashed lines indicate hydrogen bonds with distance in angstrom (Å), spoked red arcs indicate hydrophobic interactions

These Insilco studies show that these compounds can be used to block the binding sites on DLD to prevent the interaction of pathological proteins with DLD that are involved in the up-regulation or down-regulation of DLD in diseased state.

4.7 Interaction Analysis of Protein-Ligand Complex with Drug Compounds

All of the three drug compounds were docked against our protein and ligand complex in PyRx Vina Wizard according to Trott & Olson (2009). The highest binding energy (kcal/mol) that result after docking was considered as the most suitable docking model of the ligands, shown in Table 7. Both ascorbic acid and acetaminophen showed highest binding energies with all three protein-ligand complexes, 5NHG-CAND1 complex, 5NHG-LAMP1 complex, and 5NHG-TPP1 complex, shown in Figure 17. The 2-D analysis of all three drugs with protein-ligand complex interactions was observed using Discovery Studio, as shown in Table 9.

Table 7. Binding energy values of drug compounds with protein-ligand complex. Binding energy values of Ascorbic acid, Acetaminophen, and Methamphetamine with Protein-Ligand Complex evaluated by PyRx docking

Drug Compound	Target	Binding Energy (kcal/mol)
Ascorbic acid	5NHG_CAND1 (1191-1200)	-5.9
	5NHG_LAMP1 (216-225)	-5.8
	5NHG_TPP1 (546-555)	-6.1
Acetaminophen	5NHG_CAND1 (1191-1200)	-5.3
	5NHG_LAMP1 (216-225)	-6.1
	5NHG_TPP1 (546-555)	-5.5
Methamphetamine	5NHG_CAND1 (1191-1200)	-5.1
	5NHG_LAMP1 (216-225)	-5.2
	5NHG_TPP1 (546-555)	-5.3

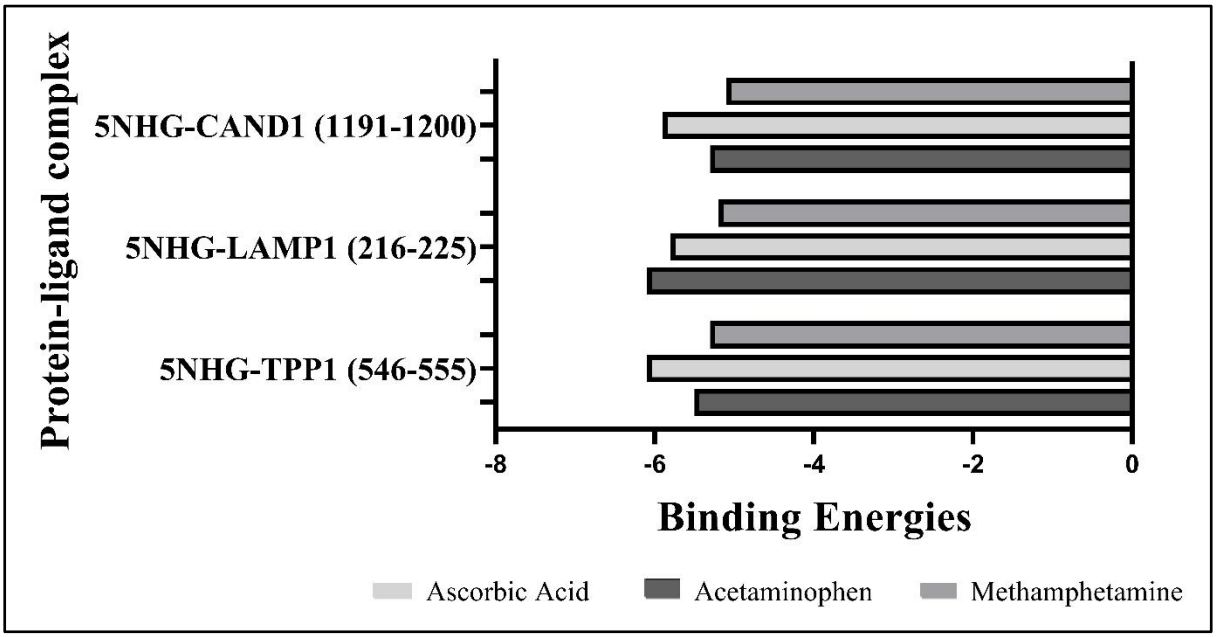


Figure 17. Binding energy values of Protein-ligand complex with drug compounds. Binding energies as a result of docking results of Drug Compounds with protein-ligand complexes evaluated by PyRx. Both ascorbic acid and acetaminophen showed highest binding energies with all three protein-ligand complexes

CHAPTER 5

DISCUSSION

In recent years, numerous studies have revealed that *in silico* techniques are useful in finding newer and more effective therapeutics in the development and discovery of drugs. The development of fast and precise target identification and prediction methods for the drug discovery depended on *in silico* approaches (Rao & Srinivas, 2011). Drug discovery processes often begin with the selection of appropriate drug targets, which are typically proteins including receptors, enzymes, and ion channels. It is estimated that 24 million people worldwide are affected by AD, which accounts for 60% to 80% of all dementia cases. Age is regarded as one of the most prevalent risk factors for AD and cognitive impairment, according to various epidemiological research. (dos Santos et al., 2018). Intra-neuronal NFTs of tau and extracellular amyloid plaques are two pathogenic hallmarks of AD. One of the most common and main pathogenic features of AD that causes neuronal dysfunction, damage to nerve cells, and dementia is the extracellular deposition of Aβ plaques caused by protein misfolding (Huang & Mucke, 2012).

Additionally, neuroimaging studies showed that AD brains in later stages of the disease had impaired glucose metabolism and decreased mitochondrial enzyme activity (Mosconi, 2012). It is unclear how these discoveries are connected in terms of cause and effect because, while poor energy metabolism may lead to protein misfolding that results in the development of Aβ plaques (Calkins & Reddy, 2011). AD is linked to the increased risk of late-onset AD and genetically linked to the human dld locus. DLD that has been found linked to late-onset AD further highlights the significance of energy metabolism in AD (Ahmad & Ebert, 2021). There is a link between AD and decreased energy metabolism and mitochondrial enzymes. It is known that DLD and two of its enzyme complexes namely, PDH and KGDH play a significant role in energy metabolism and are associated with AD (Ahmad, 2017).

To understand how energy metabolism affects the development of AD, *in silico* methods are used in this study. During the past decade, computational approaches have shown their success and power in assisting interactive studies, drug development and disease control (Shaker et al., 2021). As a result, a molecular docking approach could be a viable alternative for the advancement of novel drugs for the management of/treatment of patients (Umar et al., 2021).

In our study, seven proteins were identified through immunoprecipitation that are interacting DLD and playing a role in the progression of AD, out of these three proteins were selected,

CAND1, LAMP1, and TPP1, on the basis of their role in mitochondrial dysfunction and their activated binding domains that are involved in aggregation.

The effects of Cullin-associated NEDD8-dissociated 1 (CAND1) on neddylation demonstrates how delicately the biological balance of the cell is preserved. The activity of Cullin-RING ubiquitin ligases (CRLs), which is typically dysregulated in cancer, is tightly controlled by CAND1. CRLs activities are impaired by decreased or increased levels of this regulatory molecule (Chen et al., 2012). Functional studies showed that the activation of mitochondrial apoptosis by CAND1 knockdown effectively inhibited the growth of liver cancer cells (Che et al., 2018). Lysosomal membranes have a high expression of the glycoprotein LAMP-1. In AD cases, LAMP-1 immunoreactivity has been increased in neurones, as well as in glial cells surrounding senile plaques. In addition, immunoreactivity to LAMP-1 in neurones with NFTs has little correlation with tau phosphorylation or neurofibrillary tangle formation (Barrachina et al., 2006). Tripeptidyl peptidase 1 (TPP1) is responsible for the development of late infantile neuronal ceroid lipofuscinosis (CLN2), where mutations in tripeptidyl-peptidase I remove tripeptides from peptide N-termini. There is no clear understanding of the nature of the physiological substrates as well as the specificity of the enzyme (Bernardini & Warburton, 2001). TPP1 has been found involved in mitochondrial dysfunction and dysregulated mitochondrial axonal trafficking that linked to the neurodegenerative disorders (Shlevkov et al., 2019).

In this study, we used *in silico* methods to assess the stability and binding of these proteins to the parent protein DLD), as well as their relevance to the development of AD. The complexes were studied and analysed on the basis of their binding energies and the formation of bonds between the molecules. In our study, the interactors CAND1, LAMP1, and TPP1 were docked with DLD (PDB ID: 5NHG) active sites by using computational tools: PyRx, PyMOL, Discovery Studio and LigPlot++. CAND1 (1191-1200), LAMP1 (216-225), and TPP1 (546-555) showed highest binding energies with 5NHG. However, CAND1 (1191-1200) yields the highest binding energy of -7.6 kcal/mol when docked with 5NHG. LAMP1 (216-225) shows highest binding energy of -8.6 kcal/mol and TPP1 (546-555) produces the highest binding energy of -10.8 kcal/mol when docked with 5NHG. Using Discover Studio, it was observed that the binding sites of 5NHG are surrounded by 68 amino acids and CAND1 (1191-1200) was able to interact with 32 amino acids, LAMP1 formed interactions with 33 amino acids and TPP1 produced interactions with 31 amino acids within in the binding pocket of 5NHG. These interactions included carbon hydrogen bonds, Conventional Hydrogen bonds, unfavourable donor-donor and acceptor-acceptor, and van der Waals interactions. LigPlott++ was used to

visualise the 2-D interactions of protein-ligand complexes. Hydrogen bonds and hydrophobic interactions of ligands with 5NHG were observed. The interaction of ligand proteins with 5NHG yielding high binding affinity, producing different interactions within the binding pocket of DLD (5NHG) shows how AD can be caused by the apoptotic regulation of these interacting proteins. Protein-Protein network analysis was carried out to analyse the common interacting protein between DLD, CAND1, LAMP1, and TPP1. All the interactive proteins were analyzed, and maximum number of common proteins were identified in between the proteins. Identified proteins were further analyzed for different pathways they were involved in such as apoptosis, mitochondrial dysfunction, neural metastasis, and neurogenesis which may play a role in the progression of AD.

In order to develop drugs, the binding of small molecules to proteins is predicted by virtual libraries of drug-like molecules (Sousa et al., 2006). Due to their potential to reduce time, labor, and costs associated with drug discovery, *in silico* approaches have recently attracted significant interest. The use of computational methods has led to the development of many new drugs (Shaker et al., 2021). In this study, we evaluated three drug compounds, ascorbic acid, acetaminophen, and methamphetamine as a potential drug target and to find a potential inhibitor for our against three interacting proteins, molecular docking was used.

With antioxidant and anticancer properties, ascorbic acid is a crucial vitamin that our bodies need for regular physiological function. In the development of anticancer drugs, ascorbic acid has been a key player by inhibiting cancer growth through a variety of mechanisms, such as, producing ROS with specificity and promoting cytotoxicity against tumour cells, preventing glucose metabolism, and acting as an epigenetic regulator, in tumour cells (Reang et al., 2021). One of the most widely used medications in the world to relieve pain and fever is acetaminophen. Acetaminophen has been used as an analgesic and antipyretic in medicine for more than a century (Muramatsu et al., 2016). Acetaminophen, also known as paracetamol, is a medication that reduces temperature and relieves mild to moderate discomfort. Examples of well-known brand names are Tylenol and Panadol. A powerful stimulant of the central nervous system, methamphetamine is more frequently used recreationally than as a second-line treatment for obesity and attention deficit hyperactivity disorder (Methamphetamine, 2022). Ascorbic acid and acetaminophen gave the highest binding energy with 5NHG -6.1 (kcal/mol) and -6 (kcal/mol). The drugs were also docked against a protein-ligand complex and evaluated similarly. The binding energies for a drug-protein-ligand complex were similar to the binding energies observed without the ligand. These Insilco studies show that these compounds can be

used to block the binding sites on DLD to prevent the interaction of pathological proteins with DLD that are involved in the down-regulation of DLD in diseased state.

The process of finding new drugs and developing them is time-consuming and expensive. It begins with target identification, follows with target validation, and then determines drug candidates. Any brand-new drug must pass rigorous preclinical and clinical testing and receive FDA approval before it can be sold. Because experimental procedures cannot be used widely due to limitations in throughput, accuracy, and expense, drug development has recently switched to *in silico* methods including homology modelling, protein-ligand interactions, microarray analysis, etc (Rao & Srinivas, 2011). The development of quick and precise target identification and prediction methods for the discovery relied heavily on *in silico* approaches.

Although computer-aided drug design appears to offer promising results, it is important to recognize its limitations. Further, it is important to note that machine learning or deep learning models have many challenges and potential pitfalls, it is particularly important since large, high-quality datasets exist for the drug target (Meqbil et al., 2022). There is limited structural information available in the RCSB PDB for protein-protein or -peptide complexes. However, the vast majority of the available fragment libraries have aromatic compounds, which may still restrict the study of chemical space (Macalino et al., 2018). Therefore, future initiatives would need to concentrate on standardising experimental data collection as well as computational data management and modelling. Furthermore, this study is based on virtual computational methods that would be verified in cell-based and animal-based methods to validate the drug efficiency.

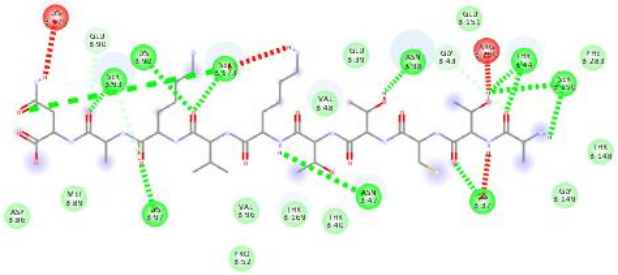
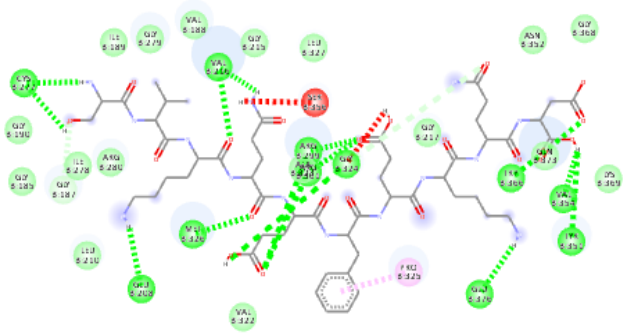
CHAPTER 6

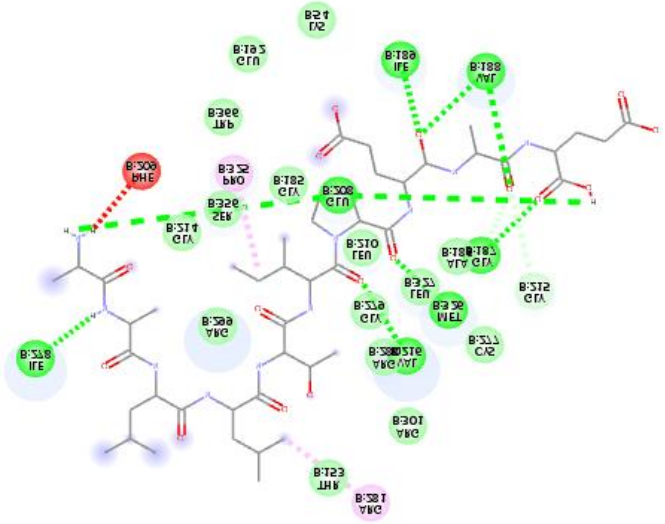
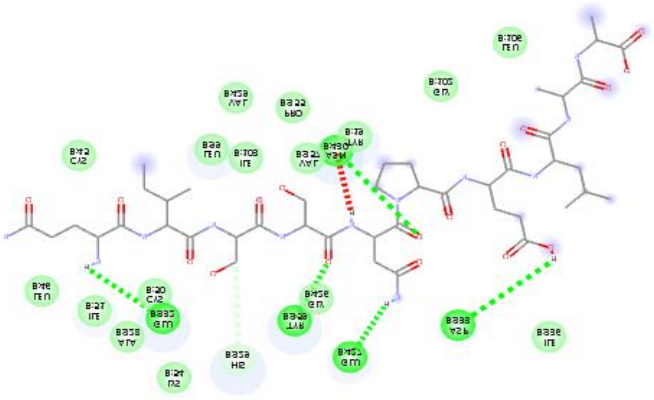
CONCLUSION

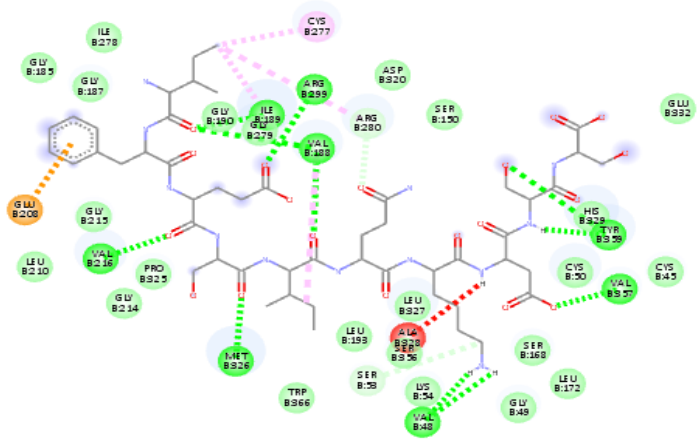
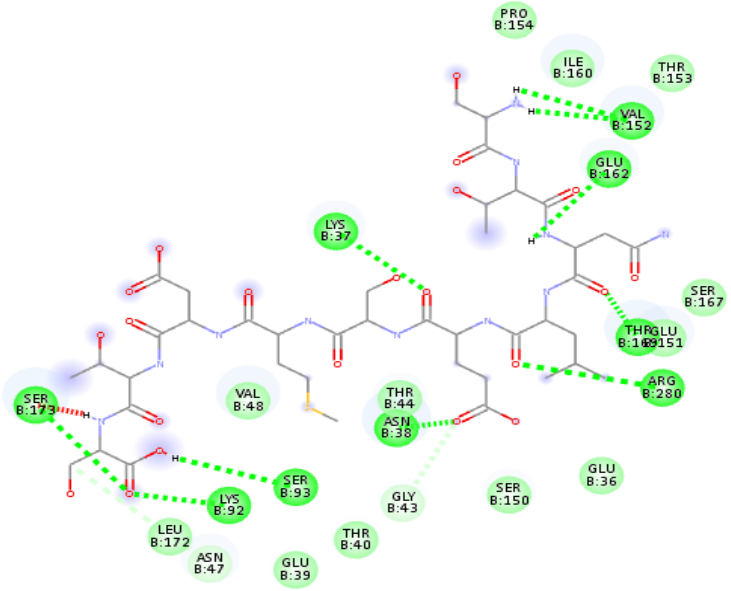
The study evaluated that the identified interactor proteins interact with DLD during the pathology of disease to cause the down-regulation of DLD, leading to AD. The associative interactions in this study were analysed using the LigPlot+ software. All of the protein ligands exhibit both hydrophobic and hydrogen bonding interactions. In this study, interaction between the DLD and its interactors CAND1, LAMP1, and TPP1 were examined for AD through *in silico* methods using molecular docking and visualisation tools like PyRx, PyMOL, Discovery Studio, and Ligplot++. It was observed that The binding sites of 5NHG are surrounded by 68 amino acids, and CAND1 (1191-1200), LAMP1 (216-225), and TPP1 (546-555) were able to interact with amino acids inside the binding pocket of 5NHG. When CAND1 domain sequences are docked with 5NHG, CAND1 (1191-1200) produces the highest binding energy of -7.6 kcal/mol when docked with 5NHG. Similarly, when LAMP1 sequences are docked, LAMP1 (216-225) yields the highest binding energy of -8.6 kcal/mol with 5NHG. Also, TPP1 (546-555) produces the highest binding energy of -10.8 kcal/mol when docked with 5NHG respectively. The interaction of sequences of ligand proteins with 5NHG yielding high binding affinity shows how AD can be caused by the apoptotic regulation of these interacting proteins. The protein-protein interaction analysis revealed the interaction of proteins contributing to a pathological pathway. These common interacting proteins between DLD, CAND1, LAMP1, and TPP1 have been found in different molecular pathways that are involved in inhibition of cell proliferation, apoptotic cell-death pathway, mitochondrial apoptosis and dysfunction, neurogenesis, and neural metastasis. Many new drug compounds have been successfully developed using computational methods. In our study, we also evaluated the binding of different drug compounds such as ascorbic acid, acetaminophen, and methamphetamine after docking experiment *in silico*. Ascorbic acid showed the highest binding energy of -6.1 kcal/mol with 5NHG. The acetaminophen also showed the highest binding energy of -6 kcal/mol with 5NHG, closed to the ascorbic acid binding energy. The interaction of these drug compounds with 5NHG yielding high binding affinity shows that these compounds can be used to block the binding sites on DLD to prevent the interaction of pathological proteins with DLD that are involved in the up-regulation or down-regulation of DLD in diseased state.

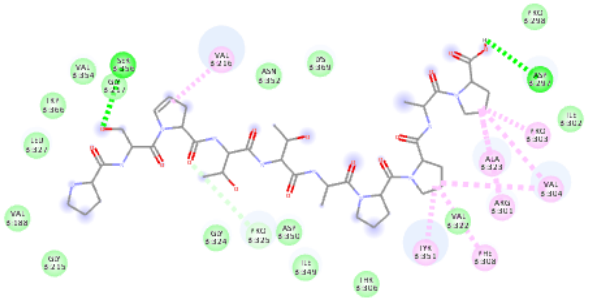
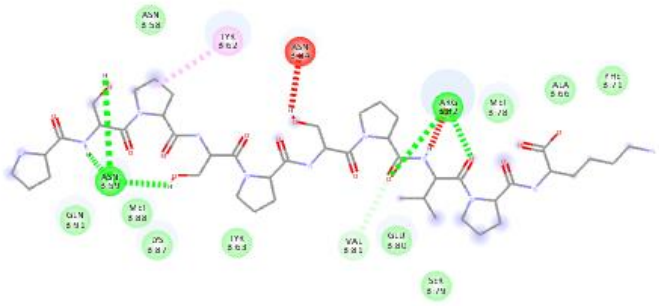
7. APPENDIX A

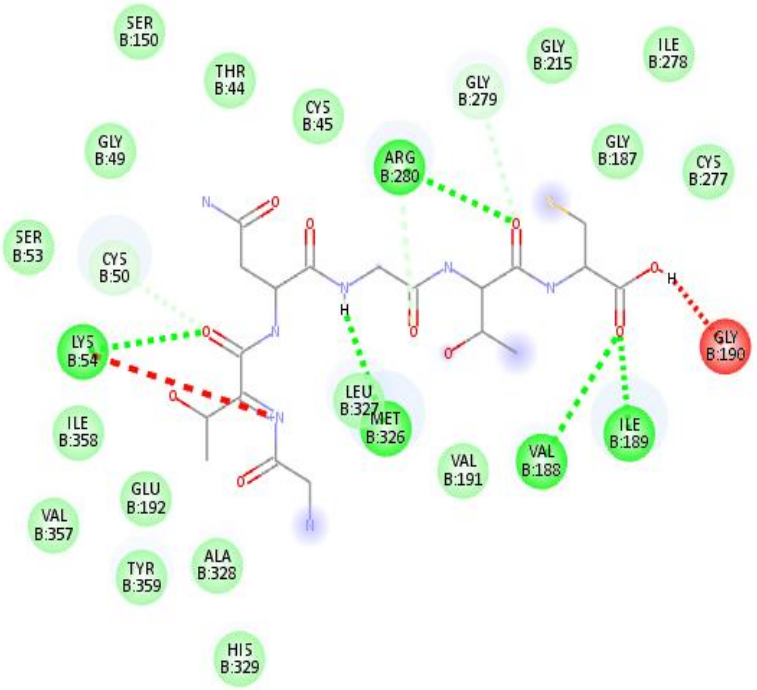
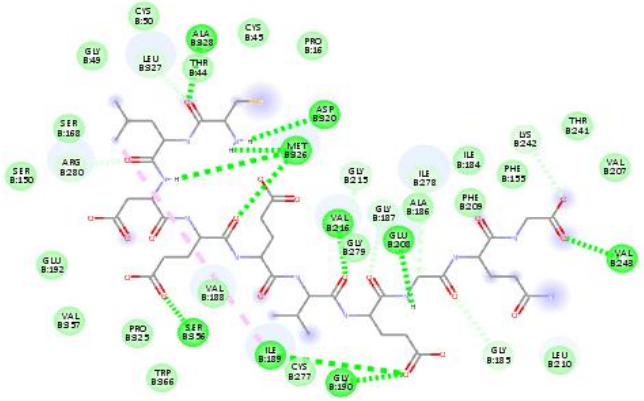
Table 8. Molecular interaction analysis of ligands with protein molecule. 2-D interaction analysis of CAND1, LAMP1, and TPP1 ligands with DLD (5NHG) using Discovery Studio. Different interactions were observed including carbon hydrogen bonds, conventional hydrogen bonds, unfavourable donor-donor and acceptor-acceptor bonds, van der Waal forces, etc.

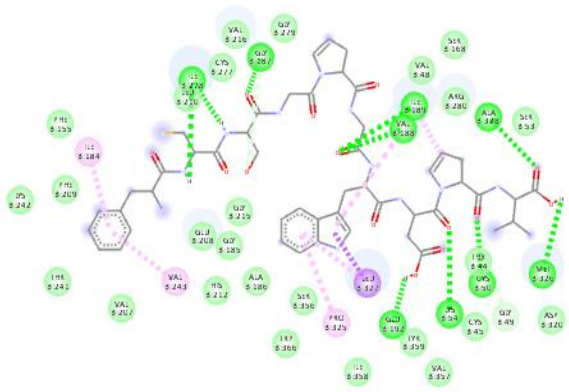
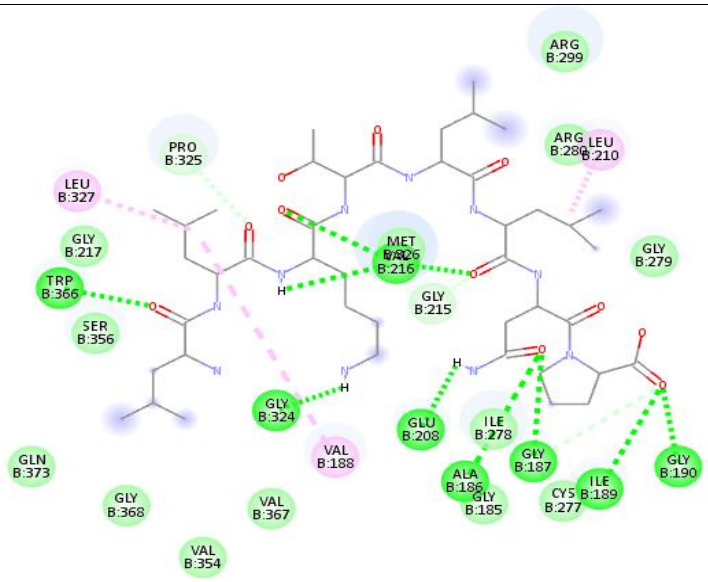
Ligands	Binding Domains Sequence	2-D Structure
Cullin-associated NEDD8-dissociated protein 1 (CAND1)	ATCTTKVKAN (1151-1160)	
	SVKQEFEKQD (1161-1170)	

	AALLTIPEA E (1181-1190)	
	QISSNPELA A (1201-1210)	

	IFESIQKDSS (1211-1220)	
	STNLESMDTS (1221-1230)	

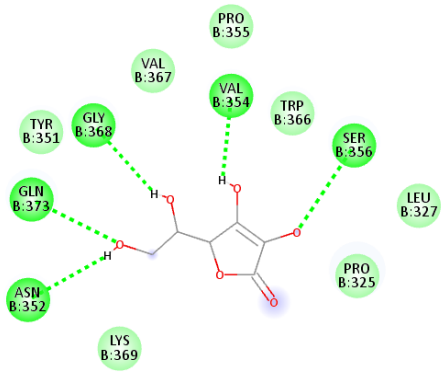
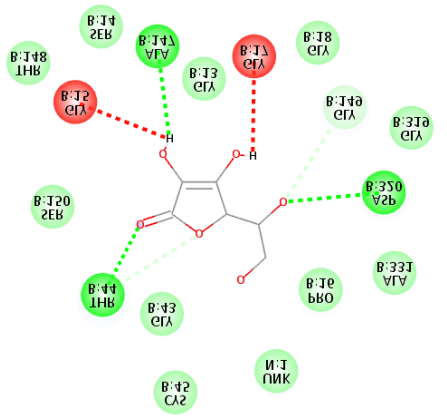
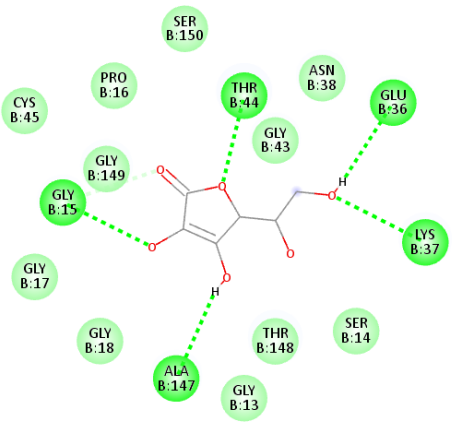
Lysosome-associated membrane glycoprotein 1 (LAMP1)	PSPTTAPPAP (196-205)	
	PSPSPSPVPK (206-215)	

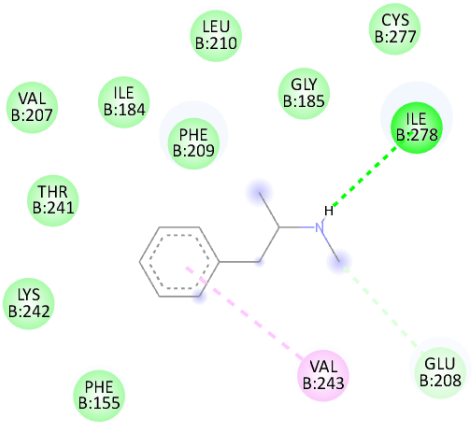
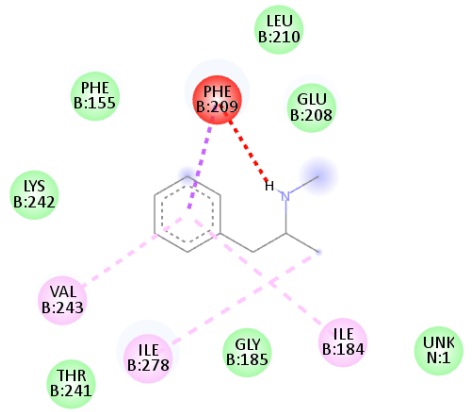
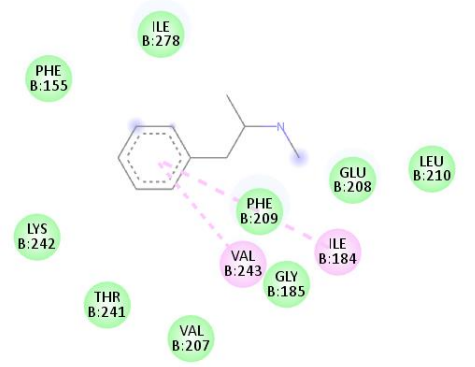
	GTNGTC (226-231)	
Tripeptidyl-peptidase 1 (TPP1)	CLDEEVEGQG (526-535)	

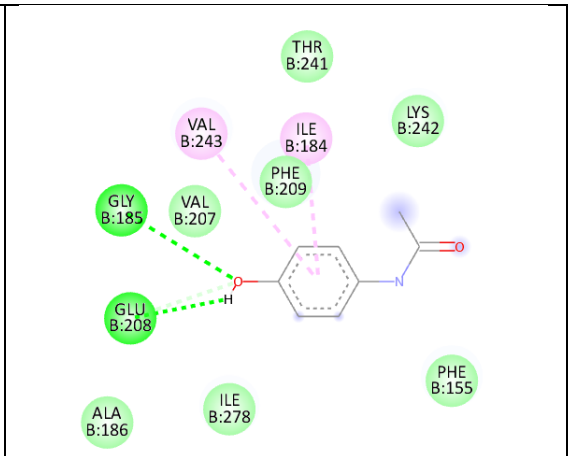
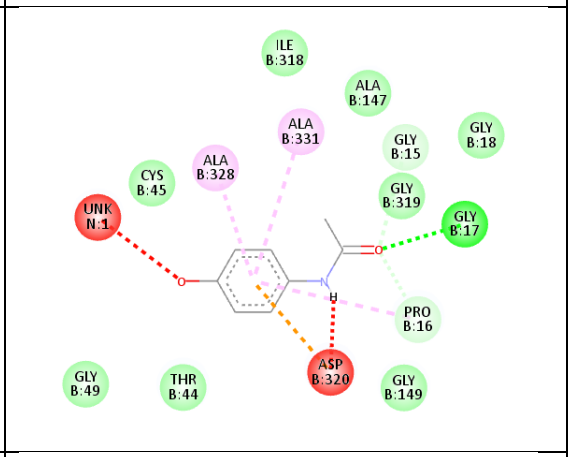
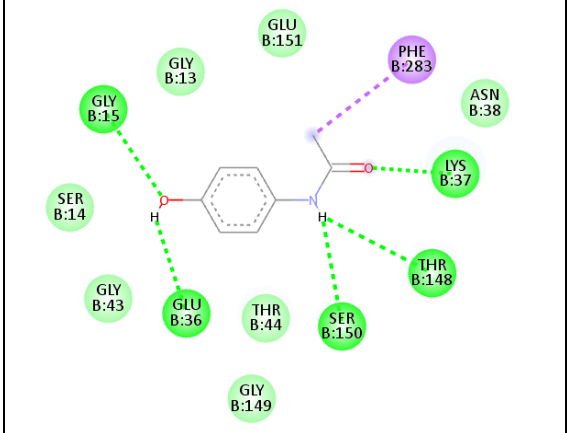
	FCSGPGWDPV (536-545)	
	LLKTLLNP (556-563)	

8. APPENDIX B

Table 9. Molecular Interaction analysis of protein-ligand complex with drug compounds. 2-D interactions analysis between protein-ligand complexes and drug compounds using Discovery Studio. Different interactions were observed including carbon hydrogen bonds, conventional hydrogen bonds, unfavourable donor-donor and acceptor-acceptor bonds, van der Waal forces, etc.

Drug Compound	Complex	2-D Structure
Ascorbic Acid	5NHG-CAND1	
	5NHG-LAMP1	
	5NHG-TPP1	

Methamphetamine	5NHG-CAND1	
	5NHG-LAMP1	
	5NHG-TPP1	

Acetaminophen,	5NHG-CAND1	
	5NHG-LAMP1	
	5NHG-TPP1	

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