Discovery of Heat Shock Protein90 inhibitors using structured-based virtual screening

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Dissertation submitted in partial fulfilment of the requirements for the degree of ***Magister Scientiae*** (Pharmaceutical Chemistry) School of Pharmacy, University of the Western Cape (UWC)

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Dedication

I dedicate this to my late mother Nomalungelo Ngcagawule

Declaration

I declare that Discovery *of Heat shock protein90 inhibitors using structured based virtual screening* is my work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Siyasanga Ngcagawule

Signature……. Date……..

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Keywords

Alzheimer's diseases

Neurodegenerative diseases

Heat shock proteins

Heat shock protein90

Virtual screening

HSP90 inhibitors

Molecular docking

Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid-β peptides
HSPs	Heat Shock Proteins
HSP90	Heat Shock Protein90
HSP70	Heat Shock Protein70
HSP40	Heat Shock Protein40
HSP27	Heat Shock Protein27
HSF-1	Heat Shock Factor 1
AChE	Acetylcholinesterase
ChEIs	Cholinesterase inhibitors
HSR	Heat Heat-Response
APP	Amyloid precursor protein
BBB	Blood brain barrier
DMSO	Dimethyl sulphoxide
GDA	Geldanamycin
VS	Virtual screening
SBVS	Structure based virtual screening
LBVS	Ligand-based virtual screening
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
NMDAR	*N*-methyl-D-aspartate receptor
FRED	Fast Rigid Exhaustive Docking
VIDA	Visualization & Communication of Modeling Results
P-gp	P-glycoproteins
NDs	Neurodegenerative disorders
NFTs	Neurofibrillary tangles
NTD	N-terminal domain
MD	Middle domain
CTD	C-terminal domain
HIA	Passive gastrointestinal absorption

Abstract

Alzheimer's disease (AD) is the most common irreversible dementia, caused by accumulation of protein aggregates in the central nervous system, with symptoms that include memory loss and behavioural abnormalities. Cholinesterase inhibitors (ChEIs) and *N*-methyl-D-aspartate receptor (NMDAR) antagonists are current treatment options for mild to moderate AD. These agents are not used to cure the illness, but rather as symptomatic therapy. Heat Shock Protein (HSP90), is one of the molecular chaperones that plays a role in AD pathogenesis. HSP90's main function is to regulate the heat shock factor-1 (HSF-1) transcription factor, which is the key regulator of the heat shock response. Inhibition of HSP90 activates HSF-1 and the subsequent induction of heat shock proteins such as HSP70, HSP40 and HSP27. Such protein assist the folding of newly synthesized or misfolded proteins, preventing their aggregation. Therefore, HSP90 inhibitors protect against protein toxicity and reduces brain aggregate to form in AD.

The HSP90 protein crystal structure (PDB ID; 2bz5) was used to dock a diverse structural database of about 12 000 amine containing compounds from the Maybridge Screening Collection using Fast Rigid Exhaustive Docking (FRED). The top five hundred-docked compounds were viewed using Visualization & Communication of Modeling Results (VIDA), and the top 50 compounds were analysed for important binding interactions and low Chemgausse4 scores. Ten of these compounds were selected based on their interaction profile with the desired HSP90 active site amino acids. Thereafter, the ten MayBridge compounds were drawn on Sigma-Aldrich and searched for structures that show at least 70% similarity, to the selected MayBridge compounds. Ten Sigma Aldrich compounds were then also docked using FRED within the HSP90 active site. The results were analysed using VIDA, to check their interaction profiles with the critical amino acids in the HSP90 active cite. Thereafter, six Sigma Aldrich compounds were considered for further biological-and structural evaluation because these compounds are easily purchased and imported to South Africa compared to the Maybridge compounds. Covid-19 restrictions also made it difficult to source the compounds from Maybridge. The six Sigma Aldrich compounds had Chemguass4 score ranging from -5 to -11 that were higher compared to the scores of -12 to -13 for the Maybridge compounds. High Chemguass4 score means that the compounds have weak binding affinity to the target protein. However, the six Sigma Aldrich compound should give a good indication of the accuracy of our virtual screening workflow in predicting potential inhibitors of the HSP90 enzyme.

A fluorescence polarization enzymatic inhibition assay was carried out against the HSP90 enzyme using the selected six compounds from Sigma Aldrich, the assay was performed to evaluate the inhibitory activity of the compounds against the HSP90 enzyme. The results of the assay demonstrated that compound **1, 3, 4** and **5** exhibited strong inhibition activity to HSP90 at three different concentrations. These four compounds had significant interactions with the HSP90 active site, the NH group on the indole ring of compound **1** formed a hydrogen bond with Asp93 of HSP90, and the pyrazol moiety of compound **3** also formed an H-bond with Asp93, which is one of the important residues in HSP90 ATPase. The pyrimidine attached to the triazole in compound **5** formed an H-bond with Asp93 and Thr184, while the methoxy moiety in compound **4** formed H-bond with Ans106 and hydrophobic interaction with Met98 and Ala55, which are also considered as important interactions with HSP90 ATPase. Compounds **2** and **6** exhibited weak inhibitory activity against HSP90 activity at three different concentrations. Compound **6** had the weakest Chemguass4 score of -5.4 and the *in vitro* results confirmed the weak binding affinity thereof which is in-line with the docking results.

The six compounds were subjected to computational ADME, pharmacokinetic and drug-likeness evaluation, using the web-tool Swiss-ADME. The drug-likeness predicted that the compounds did not violate more than one rule developed for identifying drug-like entities by pharmaceutical companies. In addition, the compounds were found to comply with the lipophilic criteria, therefore the compounds should be bioavailable and can be considered as oral drug-candidates. The ADME and pharmacokinetic predictions indicated that the compounds in general, could have high intestinal absorption and BBB (blood-brain barrier) permeability. Compound **5** and **6** are not BBB permeable and, compounds **1, 2** and **6** are substrates of the P-glycoproteins. All the compounds are predicted to have inhibitory activity against cytochrome p450 (CYP).

In summary, the results of this dissertation demonstrated that compounds **1, 3, 4** and **5** seem to exhibit strong inhibitory activity against the HSP90 enzyme. The compounds are also predicted to be safe for oral administration according to Lipinski's rules, and may have potential for future development or as lead compounds for the treatment of AD.

CHAPTER 1

1.1 Introduction

Alzheimer's disease (AD) is known to be the common cause of dementia that gradually destroy thinking ability, memory, and ability to perform basic tasks (Alzheimer's Association, 2010). AD is the disorder that affects people over the age of 65, accounting for approximately 60-70 percent of cases (Edhag, 2008).

AD is currently the 6th greatest cause of death in the United States, and the 5th major cause of death for individuals over 65 years old. More than 46 million individuals worldwide suffer from AD. This number is anticipated to rise to more than 100 million by the year 2050 if no new treatments are developed (Prince *et al*, 2015). Not only do patients living with dementia experience medical, psychological, social, and economic consequences as a result of AD, but their carers, families, and society as a whole also suffer. The lack of information and misunderstanding of dementia, and AD makes it difficult to diagnose and treat (Alzheimer's Association, 2010).

AD is caused by the accumulation of misfolded proteins and aggregates; these misfolded proteins have toxic effects in neuronal cells when they accumulate to a certain level. Accumulation of abnormal proteins causes neurons to lose structure and function over time. Protein misfolding happens when a protein folds inefficiently, this results in the development of a non-functional structure. Somatic mutations, transcriptional or translational mistakes, a breakdown of the folding and chaperone machinery, flaws in post-translational modifications, protein trafficking, and epigenetics are all causes of protein misfolding. (Kumar *et al*, 2016; Siddiqi *et al*, 2017; Alam *et al*, 2017).

Cholinesterase inhibitors (ChEIs) and an *N*-methyl-D-aspartate (NMDA) receptor antagonist are the only approved treatments (Lanctôt, *et al*., 2009), with the understanding that these drugs are used to only ease the symptoms of mild to moderate AD. Currently there are no effective AD treatment options to slow down or curb the progression of AD. Therefore, new targets that may be more effective at modulating AD is of current interest. The Heat Shock Proteins (HSPs) are some of the most important chaperone proteins, because they protect the cells when they are threatened by an increase in temperature or any other environmental stressor (Sgobba and Rastelli, 2009; Ou *et al*, 2014). From microbes to humans, these proteins are extremely conserved (Blagg & Kerr, 2006; Richter & Buchner, 2006; Ou *et al*, 2014). As a cell defence

mechanism, HSPs can regulate the appropriate folding of nascent polypeptides, aid in the refolding of denatured proteins, and send damaged proteins to the ubiquitin-proteasome pathway for disposal. These activities maintain cell protein homeostasis and protect the cell from intrinsic and extrinsic insults that can occur during cellular stress under normal circumstances (Taipale *et al*., 2010; Ou *et al*, 2014). Heat Shock Protein 90 (HSP90) is a molecular chaperone that helps cells survive and operate under pressure changes (Whitesell and Lindquist, 2005; Workman *et al*.., 2007; Chiosis, 2006; Ou *et al*, 2014).

So therefore, HSPs, particularly HSP90, are regarded as a potential drug target (Taldone *et al*., 2014,) of particular relevance in AD (Gammazza *et al*, 2016)

1.2 HSP90 as a Therapeutic Target for AD

Inhibition of HSP90 has the potential to repair defective heat shock response, which may be used in the treatment of AD and other neurodegenerative diseases. The use of HSP90 inhibitors as a therapy for AD and other neurodegenerative disorders has been proposed. Restoring HSP70 levels due to the inhibition of HSP90 may have a beneficial effect on a variety of aspects involved in the AD pathogenesis (Luo *et al*., 2010).

The expression of HSP is tightly regulated by the heat shock factor 1 (HSF-1), which could lead to the development of new therapeutic agents that can protect the brain from neurodegenerative diseases. When exposed to high levels of stress, cells can respond by activating the heat shock response (HSR). This is done through the synthesis of various heat shock proteins that can reduce the damage caused by the effects of the shock. These proteins are known to be useful in preventing the development of neurodegenerative diseases (Klettner, 2004; Brown, 2007; Muchowski and wacker, 2005). The regulation of the function of HSF-1 by HSP90 is done through a process known as phosphorylation. Once released, the HSF-1 is then re-positioned in the nucleus to bind to various elements that regulate the response to the heat shock. This process leads to the production of HSP27, HSP70, and HSP40. The expression of these proteins can increase the cell's buffering capacity and restore its homeostasis in stressful situations (Dickey *et al*., 2007; Zhao *et al*, 2012; Chen *et al*., 2014; Wang *et al*., 2016) (figure 1). Therefore, HSP90 inhibitors can provide a beneficial effect against multiple aspects of AD pathogenesis (Luo *et al*., 2010).

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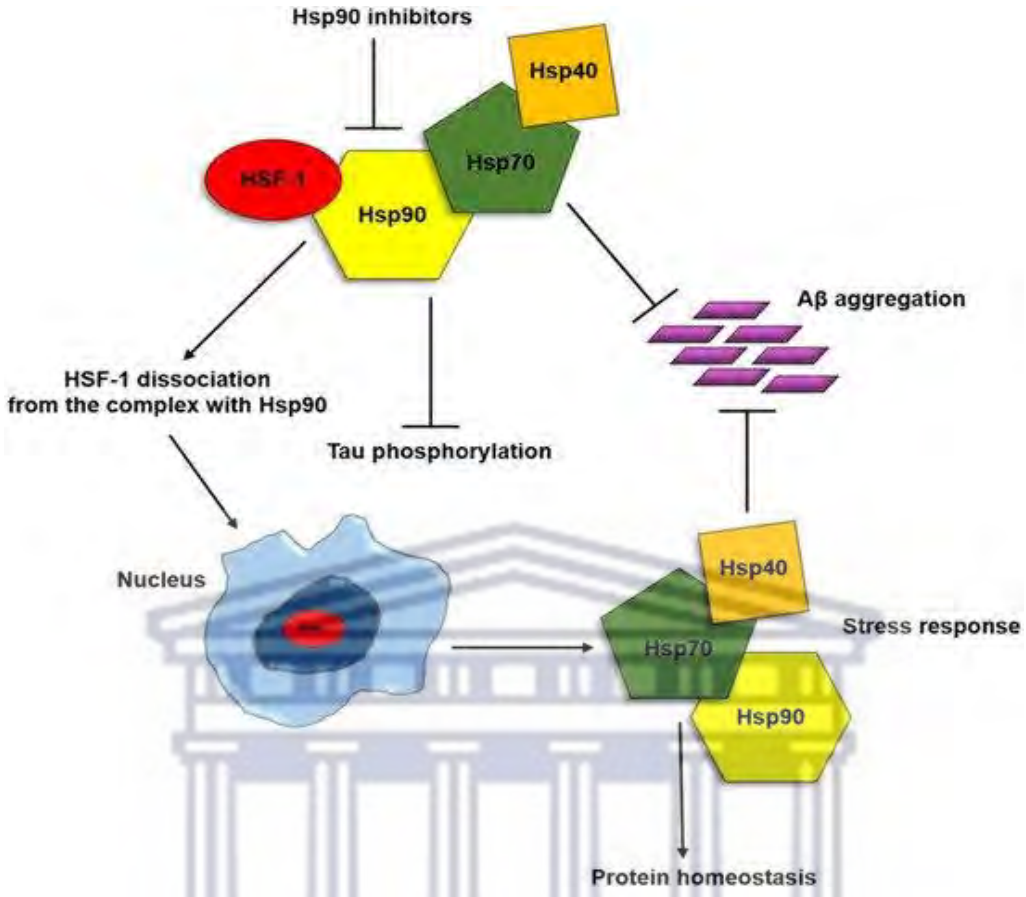


Figure 1: Illustration of HSP90 inhibition, and induction of the heat shock response (Campella *et al*., 2018).

1.3 Hypothesis

HSP90 inhibitors have the ability to induce the expression of heat-shock proteins, which leads to the solubilisation of protein aggregates and the refolding of misfolded proteins that contribute to the pathogenicity of neurodegenerative diseases such as AD.

1.4 Aim

This research aims to identify novel HSP90 inhibitors, using structure-based virtual screening. That may be beneficial in slowing down or halting the neurodegenerative process.

To achieve these aims, the following objectives have been set for this study:

- To identify the appropriate HSP90 protein crystal structure, for virtual screening which will be obtained from the Protein Data Bank (www.rcbs.org).
- To prepare a virtual ligand library of structurally diverse amine-containing compounds, obtained from the online Maybridge screening collection (https://www.maybridge.com).

- To perform structure-based virtual screening, to identify hit compounds with predicted ability to inhibit the HSP90 protein using virtual screening tools such as FRED and VIDA (www.eyeopen.com)
- To perform *in vitro* evaluation of the successfully purchased hit compounds for HSP90 inhibitory activity, using the *N*-Terminal Domain Assay Kit (www.bpsbioscience.com)
- To evaluate the drug-likeness and pharmacokinetic profiles, of the test compounds using the Swiss-ADME web tool (http://www.Swiss-ADME.ch/)

CHAPTER 2

2. Literature review

2.1 Introduction

This chapter describes AD as a neurodegenerative disorder, current treatment options and potential therapeutic effects of HSP90 inhibitors in AD. Additionally, virtual screening and recent virtual screening endeavours undertaken to identify HSP90 inhibitors for AD are also discussed.

2.2 Neurodegenerative diseases

The term, "neurodegenerative disease" is normally used to refer to a group of conditions that primarily affect the neurons in the human brain. Neurodegenerative diseases are currently a significant threat to human health. These age-related disorders are becoming more common, due to an increase in the elderly population in recent years. Neurons are the basic building blocks of the nervous system, which includes the brain and spinal cord. Because neurons do not ordinarily multiply or replace themselves, the body is unable to replace them when they are damaged or die. Neurodegenerative disorders (NDs) are a group of diseases that cause nerve cell loss and/or degeneration. This results in mobility issues (ataxias), mental functioning issues (dementias), and impairments in a person's ability to move, speak, and breathe. NDs afflict many families; these disorders are stressful for both the individual and their loved ones (Gitler *et al*, 2017).

Protein misfolding causes protein aggregation, and the accumulation of these aggregates is thought to be the root cause of NDs. As a result, neurotoxic misfolded protein aggregates accumulate in the central nervous system, causing brain damage and neurodegenerative diseases (Soto and Estrada, 2008; Shastry, 2003; Ross and Poirier, 2004). Aside from misfolded proteins, other factors linked to NDs include abnormal protein dynamics, such as defective protein degradation and aggregation, oxidative stress and free radical formation, impaired bioenergetics and mitochondrial dysfunction, and metal toxicity and pesticide exposure, among others (See figure 2). AD is now the sixth biggest cause of death in the United States, but recent estimates predict it may rank third among causes of mortality for older people, only after heart disease and cancer (Alzheimer's Disease Fact Sheet, 2021). The most recent research published by WHO indicates that in 2020 AD deaths in South Africa reached 4.676 in total deaths (World Health Organisation, 2020). As a result, therapy research has never been more crucial (Dures & Pinto, 2018).

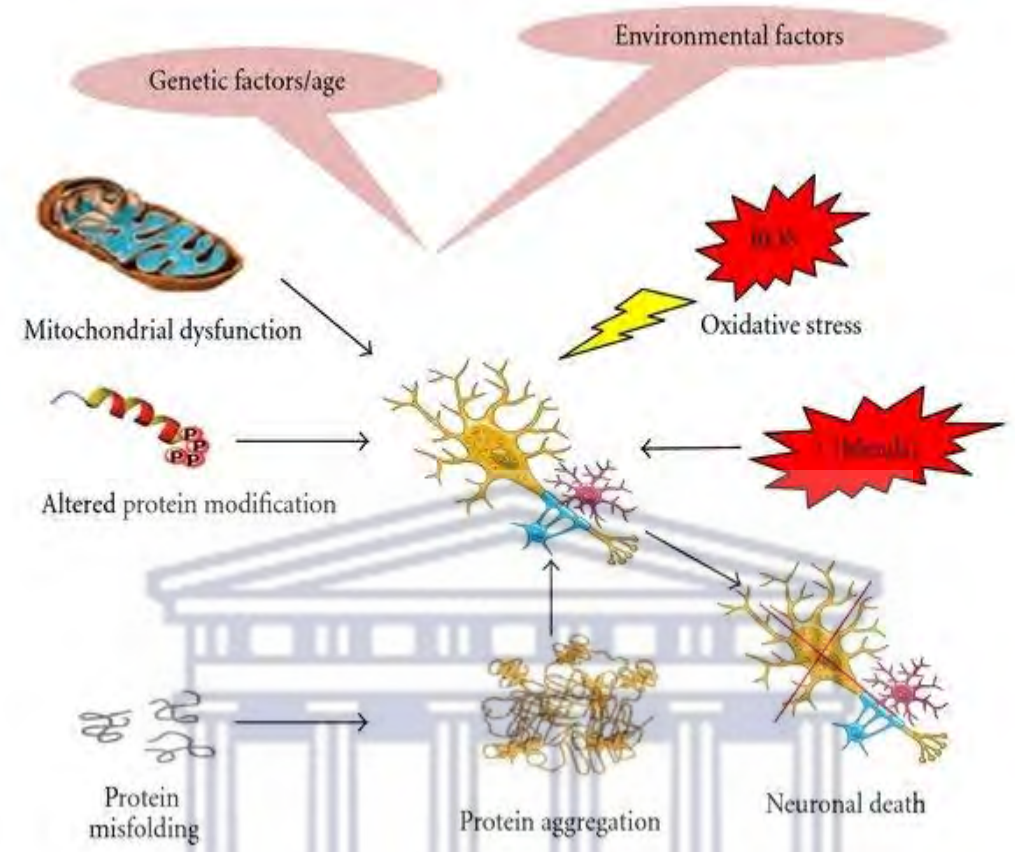


Figure 2; Factors that contribute to neurodegenerative diseases (Sheikh *et al*, 2013).

2.3 Alzheimer's disease (AD)

AD is the most common neurodegenerative illness, affecting around 50 million AD people worldwide and this number is projected to double every 5 years and will increase to reach 152 million by 2050. AD starts with moderate cognitive problems including memory loss and escalates to attention, language, and visuospatial difficulties (Wattmo *et al*., 2016; Galton *et al*., 2000).

As the disease progresses, symptoms such as decreased ability to perform daily tasks, impaired executive control and judgment, and disorientation occur (Wattmo *et al*., 2016). The prevalence of AD and other related conditions has a direct impact on people's quality of life and independence (Martyr *et al*., 2019). It is estimated that the global cost of treating this disease is around $1 trillion annually (Yiannopoulou *et al*, 2020; Livingston *et al*, 2020).

2.3.1 Pathophysiology of AD

AD is characterized by the accumulation of various deposits in the brain, such as amyloid plaques and neurofibrillary tangles (NFTs) (Selkoe, 2004; Walsh *et al*, 2002; Lambert *et al*,

1998). It can also be caused by other factors such as the loss of neurites and neuropil threads and synaptic loss. In addition, other factors such as neuroinflammation, injury of cholinergic neurons and oxidative stress can also cause neurodegeneration. These can affect cholinergic neurons (Serrano-Pozo *et al*, 2011; Spires-Jones and Hyman, 2014; Singh *et al*, 2016).

The development of senile plaques is characterized by the presence of different morphological forms of the beta-amyloid protein (Aβ). The main cause of this condition is the improper cleavage of the protein by the actions of the gamma-secretase, alpha, and beta proteases. These actions result in the formation of oligomeric plaques and fibrils (Selkoe, 1994). The accumulation of small oligomer deposits and plaques in the inner and outer spaces of neurons and synapses could lead to the destruction of the neurons (Sato *et al*, 2018; Hanseeuw *et al*, 2019). The NFTs are abnormal filaments of the tau protein that accumulate in the neural perikaryal cytoplasm, dendrites, and axons. These tangles cause the loss of tubulin-associated proteins and microtubules. The accumulation of tau protein in the NTFs has been linked to an increase in microtubule instability and tau aggregation in AD. When hyperphosphorylation occurs, tau accumulates as NFTs in neurons, which eventually lead to neurodegeneration (Mandelkow and Mandelkow, 2012; Takalo *et al*, 2013).

A synaptic loss which is described by a damage in the synapse that lead to memory impairment observed at the early stages of AD. The damage can be caused by various factors, such as the accumulation of tau and Aβ at the sites of the synaptic connections. Other processes, such as oxidative stress, can also contribute to the loss of these proteins. These tangles eventually cause the loss of various components of the synaptic network, such as the dendritic spines and pre-synaptic terminals (Overk and Masliah, 2014). The presence of synaptic protein that can be used as markers of synaptic damage can help identify individuals who are at risk of developing AD. These include neurogranin, which is a post-synaptic protein, and VILIP-1, which is a visinin-like protein. (Lleó *et al*, 2019; Tarawneh *et al*, 2016). Multiple hypotheses have been proposed as possible explanations for the disease's pathogenesis (Anand and Singh, 2013).

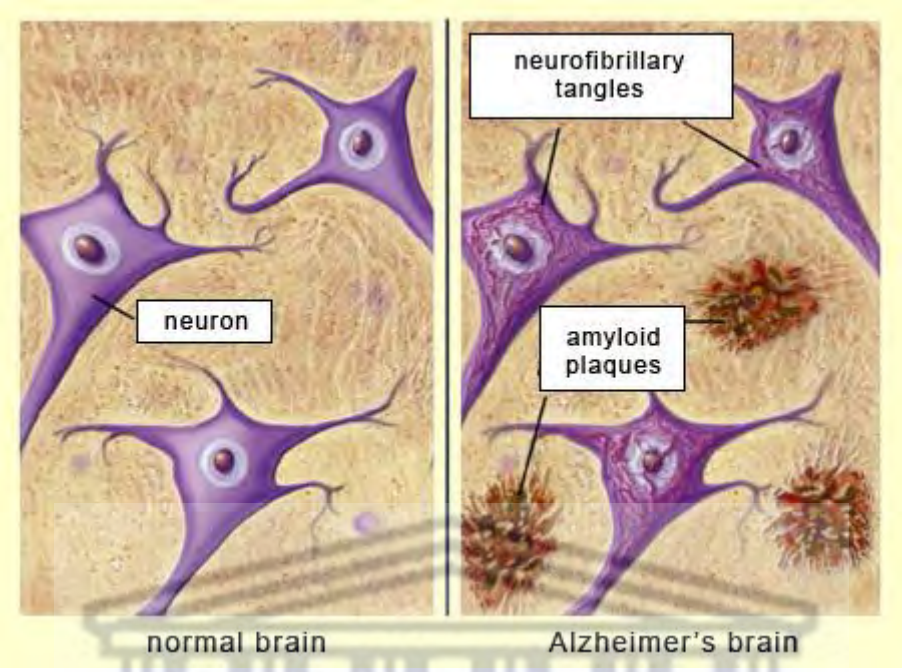


Figure 3; Major pathological hallmarks of AD are amyloid plaques and neurofibrillary tangles (De Loof, A. and Schoofs, L., 2019)

2.3.2 Alzheimer's Disease Hypotheses

2.3.2.1 Amyloid hypothesis

The amyloid hypothesis states that the accumulation of Aβ in the brain can trigger a cascade of damaging effects, such as the formation of plaques and tangles, which can lead to neurodegeneration. It is believed that Aβ accumulation is the cause of the development of AD by damaging synapses, causing the formation of NFTs, and subsequently inducing neuron loss (Morris *et al*, 2014). Under normal conditions, Aβ is removed from amyloid precursor protein (APP) by β- and γ-secretase, it is then released outside the cell, where it is rapidly degraded or removed. Under pathological conditions, the metabolism of Aβ is decreased which leads to accumulation of Aβ peptides. The two major components of the accumulation of Aβ are $A\beta_{40}$ and $A\beta_{42}$, which respectively contain 40 and 42 amino acid residues. When the levels of $A\beta_{42}$ or an increase in the ratio of $A\beta_{42}$ exceeds that of $A\beta_{40}$, the formation of amyloid fibril is triggered. These are then deposited into senile plaques, which can lead to neurodegeneration and neurotoxicity.

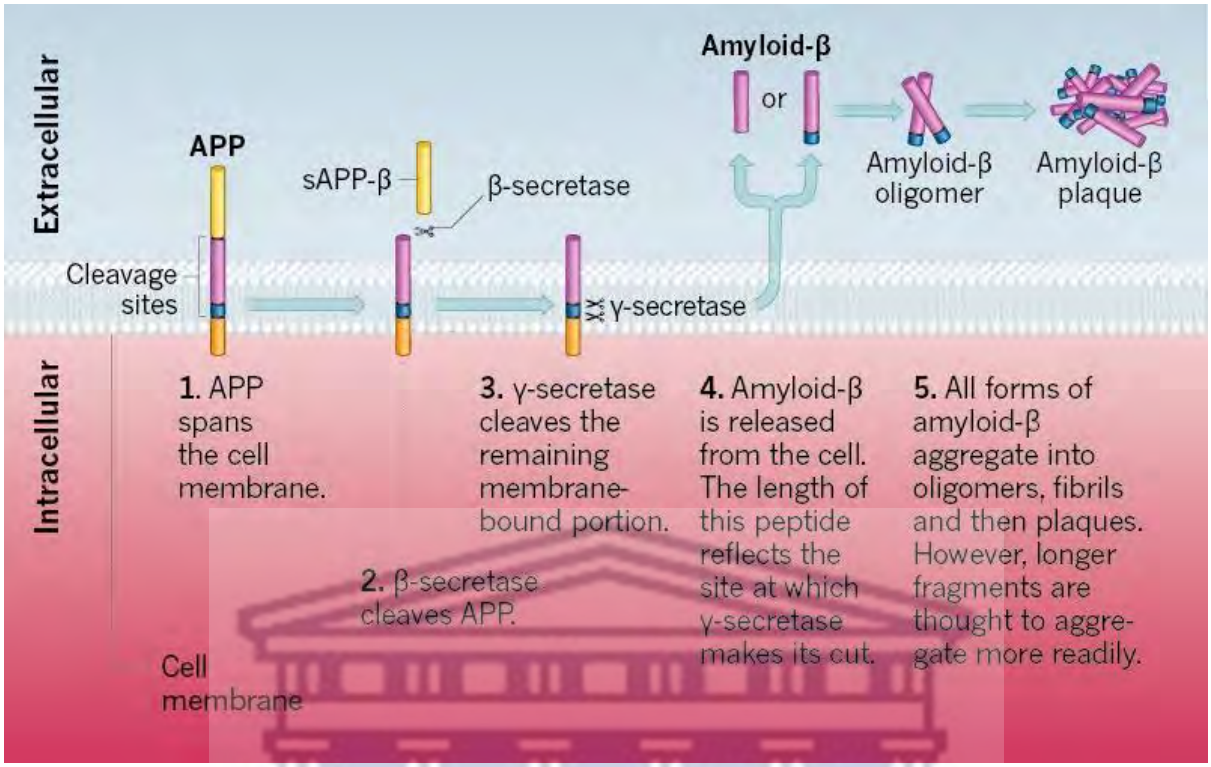


Figure 4; Representation of amyloid hypothesis (Makin, 2018)

2.3.2.2 Tau hypothesis

According to the tau hypothesis, excessive or abnormal phosphorylation of tau leads to the transformation of normal tau to a type of protein known as PHF-tau (paired helical filament) and NFTs. Tau is a highly soluble microtubule-associated protein (MAP) that interacts with tubulin to maintain the microtubule assembly. Tau proteins constitute a family of six isoforms with the range from 352-441 amino acids. The six different types of tau proteins are categorized into the long and short forms. The longest form of tau has four repeats and two inserts, while the shortest form has three repeats and no insert. All of the six tau isoforms are present in an often hyperphosphorylated state in PHFs from AD.

The presence of mutations that alter the function and expression of tau can lead to hyperphosphorylation. In the absence of these changes, the tau aggregation process can still occur. However, it is not known how this process works (Iqbal and Grundke-Iqbal, 2008). When tau is hyperphosphorylated, it breaks down microtubules and binds to the PHFs. It also sequesters normal tau and MAP 2. The insoluble structure of tau can also affect the function of the cell. It can cause cell death by interfering with the axonal transport (Mudher and Lovestone, 2002)

2.3.2.3 Metal ions hypothesis

In the brain, metal ions play a vital role. There is solid evidence supporting the homeostatic dysfunction of these ions in various neurodegenerative diseases. (Bush, 2003; Leal *et al,* 2012; Pfaender and Grabrucker, 2014). First row metals, such as iron, copper, zinc, and aluminum, are known to have important roles in the regulation of nerve function (Smaili *et al*, 2009; Egorova *et al*, 2015; Leal and Gomes, 2015). They are also involved in the pathophysiology of AD. Multiple studies have shown that these biometals, as well as non-physiological aluminum, play a significant role in the pathophysiology of AD. It is believed that the presence of Cu^{2+}, Fe^{2+}, and Zn^{2+} in the extracellular matrix of AD contributes to the development of the disease. These three compounds bind to Aβ and are known to influence the aggregation pathway of the peptide (Bush, 2003, Lovell *et al,* 1998). The aggregation process that occurs when metal ions are bound to Aβ is usually directed toward amyloid fibers or amorphous aggregates depending on the environmental conditions and the metal ion (Chen *et al,* 2011). The presence of high levels of Cu^{2+} and Zn^{2+} in the brain has been observed to shift the aggregation pathway into an oligomerization state, which is known to cause neurodegeneration. In addition, the interaction between these biometals and Aβ can lead to the production of reactive oxygen species, which can cause damage to the neurons (Rottkamp *et al*, 2001; Rival *et al*, 2009; Eskici and Axelsen, 2012).

2.3.2.4 Cholinegic hypothesis

The cholinergic hypothesis states that AD begins when the body doesn't produce enough of the vital chemical known as ACh (Shadlen and Larson, 1999). This is a neurotransmitter that has multiple functions in the central nervous system (CNS) and the peripheral nervous system, as it known to affect various cognitive functions, such as memory and learning. During the 1960s and 1970s, the study of the brains of people with AD started, with hopes to identify a neurochemical abnormality that could be treated using levodopa, similar to that of Parkinson's disease. During the 1970s, it was reported that there were significant neocortical deficits in the enzyme choline acetyltransferase (ChAT) that is responsible for the synthesis of ACh (Esiri, 1996; Bowen *et al,* 1976; Davies and Maloney, 1976) The subsequent discoveries about the reduction choline uptake (Rylett *et al*, 1983), ACh release (Nilsson *et al*, 1986) and the loss of this substance in the nucleus basalis revealed a significant presynaptic deficit. These findings led to the development of the “cholinergic hypothesis” regarding the role of ACh in memory and learning (Drachman and Leavitt, 1974).It was suggested that the reduction of cholinergic

neurotransmission and the degeneration of neurons in the basal forebrain could contribute to the development of AD (Bartus *et al*, 1982).

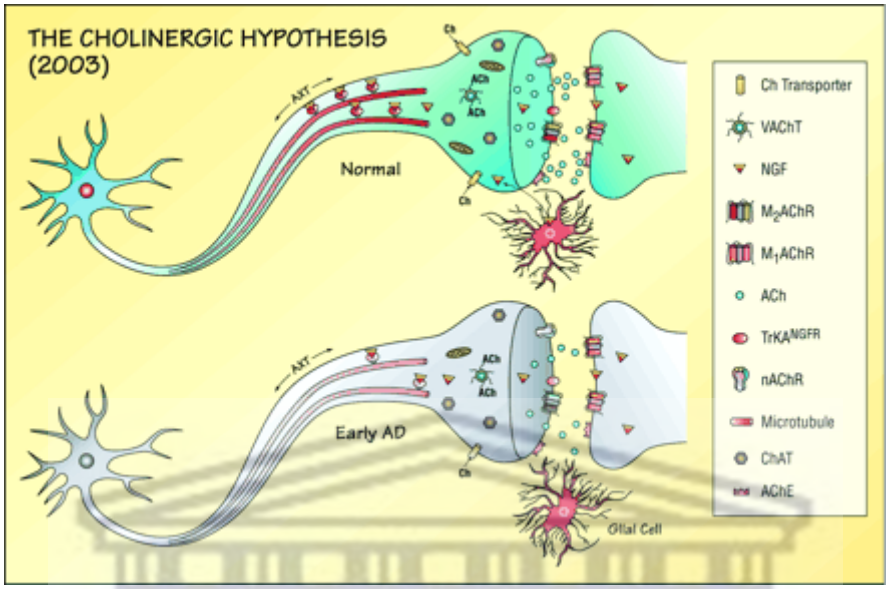


Figure 5; Representation of a normal healthy neuron versus proposed changes in cholinergic neurons that occur in the aged and early AD (Terry and Buccafusco, 2003).

2.4 Stages of AD

AD is classified into four different stages, namely mild cognitive impairment, early, moderate and severe Alzheimer stage as shown in figure 6. The mild cognitive impairment stage is characterized by early memory loss and pathological changes in the hippocampus and cortex. It does not have any functional impairments and is usually associated with a relatively low level of cognitive impairment (De-Paula *et al*, 2012; Dubois *et al*, 2016). Early or mild AD is characterized by various symptoms that start to appear in patients. These include a loss of memory and concentration, as well as a change in mood (Kumar *et al*, 2020, Wattmo *et al*, 2016). The third stage of AD is known as moderate AD, and it involves the spread of the disease to the cerebral cortex. This condition can lead to a loss of memory and other cognitive functions (Kumar *et al*, 2020). Late-stage or severe AD is when the disease has spread to the entire cortex. It can result in a buildup of NFTs and neuritic plaques, which can lead to a progressive cognitive impairment. In addition, patients may become bedridden and may not be able to recognize their relatives. These complications can eventually lead to death (De-Paula *et al*, 2012, Apostolova, 2016).

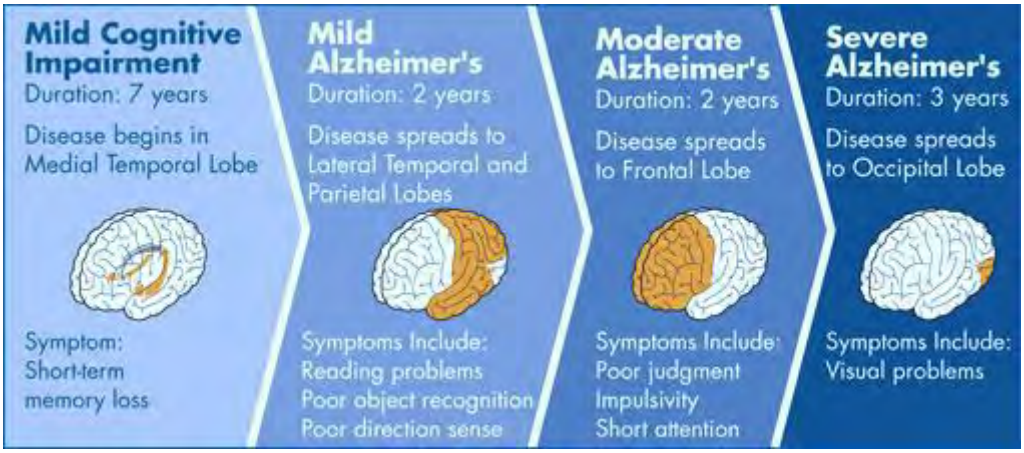


Figure 6; stages of Alzheimer's disease (Sperling *et al*., 2011).

2.5 Burden of AD

AD is a devastating, irreversible disease that affects the elderly. It is estimated that 5–11 percent of people over the age of 65 have it, with up to 50% of those over the age of 85 affected (Hebert *et al*., 2013). The care required by AD patients presents considerable direct costs (such as healthcare system resources: physician services, hospital care, nursing-home care, etc.) and indirect costs (unpaid caregiver services, reduced care giver productivity, etc.) that are expected to rise as the older population grows. As a result, AD places a huge burden on not only the patient, caregivers, and physicians, but also society as a whole (Leifer. 2003)

2.6 Current AD treatments

Currently there is no effective treatment for AD, however, there are drugs that are used to slow down the symptoms and progression of the disease (Alzheimer's Association, 2010). These drugs include the cholinesterase inhibitors and *N*-methyl-D-aspartate receptor (NMDAR) antagonists (Touchon, *et al*., 2013; Frozza, Lourenco, & De Felice, 2018). Doctors divide symptoms into two groups while treating Alzheimer's patients: these groups include cognitive and behavioural. These methods allow for treatment that is specific to the symptoms being experienced. Cognitive symptoms affect memory, language, judgment, and thought processes while, behavioral symptoms alter a patient's actions and emotions (Alzheimer's Association, 2010).

2.6.1 Cholinesterase inhibitors

The main medication for AD is acetylcholinesterase (AChE) inhibitors or anticholinesterases. These inhibitors block the normal breakdown of ACh, which is the main neurotransmitter found in the body and has functions in both the peripheral nervous system and the central

nervous system (Rollinger *et al*., 2004). Researchers think that reduced levels of ACh in the brain causes some of the symptoms of AD. Blocking the enzyme that destroys ACh with a cholinesterase inhibitor (anticholinesterase) increases the concentration of ACh in the brain, and this increase may improve memory and cognitive function (Lane *et al*., 2006). Patients suffering from AD have reduced levels of ACh in the brain (Ali *et al*, 2016). Cholinesterase inhibitors have been shown to have a modest effect on dementia symptoms such as cognition.

This medication is mostly prescribed symptomatic medication for mild to moderate AD, this therapy is believed to increase the amount of neurotransmitter ACh in the brain's neuronal synaptic clefts (Cummings, 2000). Depending on the stage of the disease, short-term therapeutic response and long-term outcome can differ, patients experiencing mild to moderate or severe stages of dementia have a stronger 6-month cognitive response to all three ChEIs, according to several studies (Kurz *et al*, 2004; Lilienfeld and Parys, 2000; Wattmo *et al*, 2008). It has been reported that long-term benefits of ChEI care were more pronounced in patients with milder AD, the benefits were less pronounced in those with more severe AD (Wattmo *et al*, 2011).

There are currently approved ChEIs inhibitors include donepezil, rivastigmine, and galantamine (Figure4, Ali *et al*., 2016). All three drugs have been approved and accepted as the first line treatment of AD. Systemic studies including multiple double-blind, randomized, placebo-controlled trials of these three ChEIs all showed positive effects on executive functions, daily living activities and overall function. Individual ChEIs were not significantly different in terms of efficacy (Farlow, 2002; Birks, 2006). Furthermore, donepezil is approved in the United States for the treatment of serious AD (Cummings *et al*., 2010).

Rivastigmine- is used to treat AD by inhibiting the activity of the various cholinergic enzyme in the affected region of the brain. Its side effects include vomiting and nusea (Corey-Bloom *et al*., 1998). According to Ballard, this compound also inhibits butyrylcholinesterase (BuChE) and acetylcholinesterase (AChE) (Ballard, 2002), which play a vital role in the transmission of cholinergic signals (Giacobini *et al*, 2002).

Galanthamine- galanthamine has a competitive, reversible, and selective inhibition of AChE, this makes it very effective in treating AD. In addition, it has a nicotinergic effect in the CNS, which makes it more effective in treating the condition, this compound is a type of alkaloid,

alkaloid galanthamine (Reminyl [R)] was also approved and licensed in many European countries for the treatment of early stages of Alzheimer's disease) (Sramek *et al*., 2000).

Donepezil- is used to treat the cognitive and behavioral effects of AD and other types of dementia (Seltzer, 2005). This drug has shown to reduce the activity of AChE in the cerebral cortex and other parts of the brain (Small, 1998). It was approved in 1997 (Nordberg and Svensson, 1998) for the treating mild to moderate AD, due to its effectiveness it became the main-line treatment in mild to moderate AD. Complications associated with the use of this drug include nausea, vomiting, anorexia, fatigue and, dizziness (Rogers *et al*., 1998).

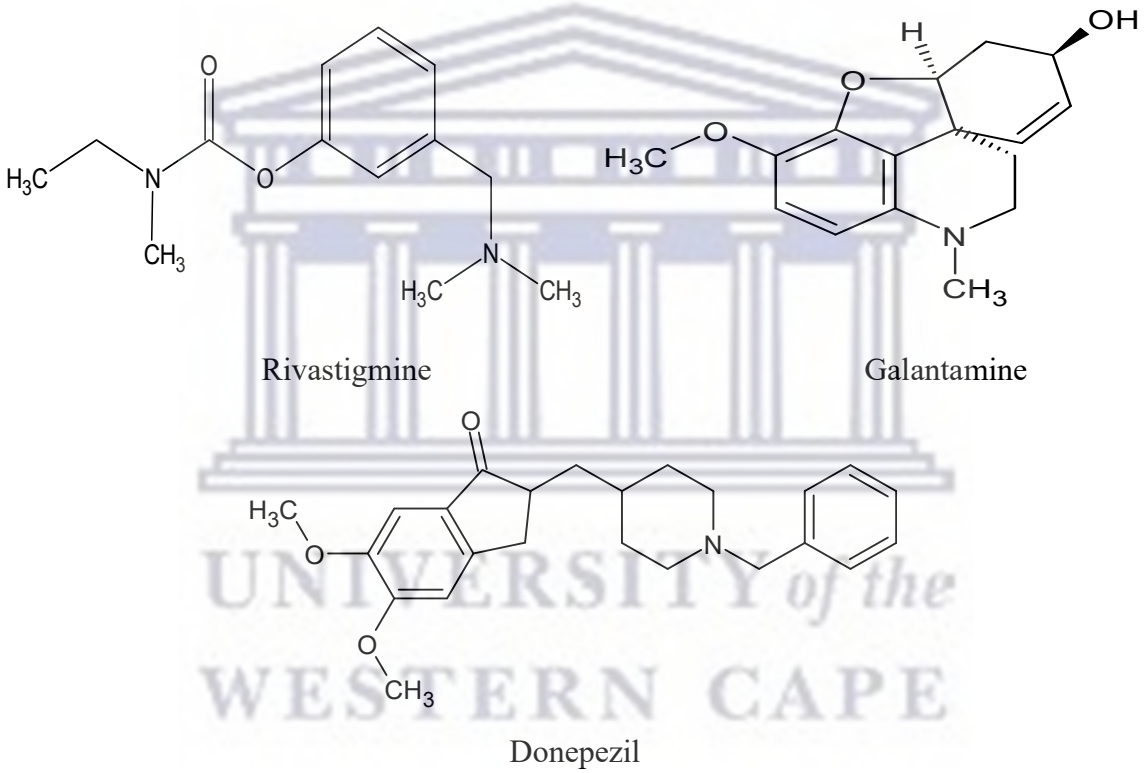


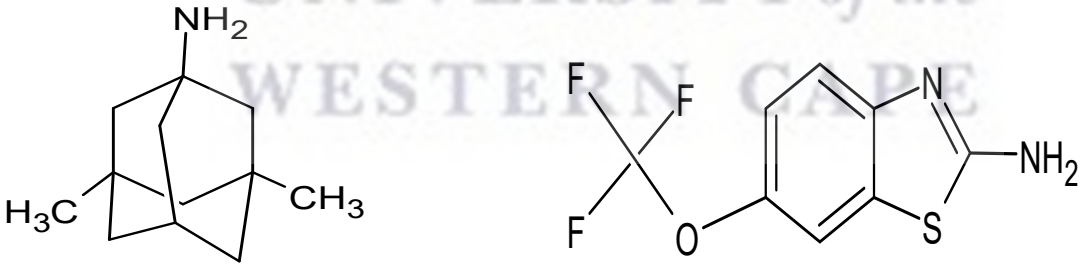
Figure 7; Acetylcholinesterase (AChE) inhibitors in AD.

2.6.2 *N*-methyl-D-aspartate (NMDAR) antagonists

NMDAR has proven to be crucial in the control of synaptic plasticity and memory function (Li and Tsien, 2009). Glutamate plays an essential role in learning and memory by triggering NMDA receptors to let a controlled amount of calcium into a nerve cell. The calcium helps create the chemical environment required for information storage (Alzheimer's association, 2017). According to studies, over-activation of the receptor, triggered by excessive release glutamate causes an excessive amount of Ca^{2+} to enter a neuron, triggering a number of

processes that can result in necrosis or apoptosis (Zipfel *et al*, 2000). As a result, inhibiting NMDAR would improve the condition of AD patients, which is why NMDAR antagonists were developed for treating AD. For moderate to severe AD, memantine, an uncompetitive NMDAR partial antagonist, has been approved as an anti-dementia medication (Reisberg *et al*, 2003; Lipton, 2006). Memantine may protect cells against excess glutamate by partially blocking MNDA receptors and was prescribed to improve memory, attention, reason, language and the ability to perform simple tasks. It was the first Alzheimer's drug of the NMDA receptor antagonist type approved in the United States (Alzheimer's association, 2017).

Riluzole also known as a glutame release inhibitor, is a promising drug for the treatment of other neurodegenerative conditions. Over-expression of glutamate in the brain leads to the activation of MNDAR, which causes excessive Ca^{2+} accumulation (Hardingham and Bading, 2010). In addition to reducing the release of presynaptic glutamate (Martin *et al*., 1993), riluzole can also help facilitate the reuptake of glutamate by astrocytes (Frizzo *et al*., 2004). It can also improve the metabolism of nutrients by increasing the number of mitochondria (Mu X *et al*., 2000). Riluzole has been known to create a behavioral profile that suggests it is a competitive antagonist of the *N*-methyl-D-aspartate (NMD) receptor. However, studies conducted on the drug with other receptor ligands revealed that this mechanism is not correct. In fact, riluzole actually acts indirectly by binding to the glutamate receptors (Kretschmer *et al*, 1998).



Memantine Riluzole

Figure 8; Structures of memantine and riluzole explored in the treatment of AD.

2.7 HSP90 function and structure

2.7.1 Function

HSP90 a molecular chaperone of adenosine triphosphate (ATP) involved in various cellular processes, such as cell cycle control, hormone secretion, and cell survival (Jackson, 2013). It is a highly conserved protein, and it accounts for approximately 2 percent of the cell protein

mass (Finka and Goloubinoff, 2013; Finka *et al*, 2015). HSP90 is found in the cytoplasm (HSP90α and HSP90β), the endoplasmic reticulum (Grp94), and the mitochondria (TRAP1) of most mammals (Frey *et al*, 2007; Leskovar *et al*, 2008). It can also be found on the surface of cancer cells. In 2004, it was discovered that this protein can be produced in the extracellular matrix (Eustace, and Jay, 2004, Sidera and Patsavoudi, 2008). It has been suggested that HSP90 can play various roles in the development of complex protein structures in the different cellular compartments.

There are two types of HSP90 found in the cytoplasm which are the inducible HSP90α and the constitutively expressed HSP90β. HSP90β is ubiquitously and highly expressed throughout all cell types (Sreedhar *et al*, 2004), while HSP90α is expressed upon a stressor and is differentially expressed depending on tissue type (Grad *et al*, 2010). The HSP90 enzyme is a protein folding enzyme that helps to protect proteins from heat stress and helps in protein degradation. It is also important in maintaining the viability and functional stability of cells during pressure transformation (Whitesell and Lindquist, 2005; Workman *et al*, 2007; Chiosis, 2006). Over the past decade, the role of the HSP90 enzyme in cancer therapy has been acknowledged (Neckers and Workman, 2012), and it has also been shown to be useful in treating neurological conditions (Miyata *et al*, 2011; Kalia *et al*, 2010). The interaction between the enzyme and various types of proteins in signal transmission is mainly due to the co-chaperones. The co-chaperones play a vital role in the regulation of the activities of the HSP90 enzyme. This process allows the protein to maintain its diverse physiological activities (Xu *et al*, 2012).

The co-chaperones are known to play a significant role in the regulation of the activity of HSP90s in the cytoplasm and in the interactions between the two substrates (Xu *et al,* 2012; Zuehlke and Johnson, 2010; Johnson and Brown, 2009). More than 20 of these have been identified, they are capable of inhibiting the activity of the HSP90 enzyme and recruiting the various client proteins to the cycle (Buchner and Li, 2013; Taipale *et al*, 2010; Li *et al*, 2012). In addition to the co-chaperones, HSP90 also plays a role in the activation and folding of various substrates. These include the transcription factors and kinases that are involved in the regulation of signal transmission (Xu *et al*, 2012; Krukenberg *et al*, 2011). HSP90 functions also include the formation of complex chaperone proteins, such as the HSP70, Hop, p23, and CDC37 complexes. One of these complexes directs the degradation of HSP90 client proteins by binding to HSP70 and Hop (Pratt and Toft, 2003). The other one, which is responsible for stabilization of HSP90 client proteins, is composed of p23 and CDC37 (Adachi *et al,*

2009, McClellan *et al,* 2005). HSP90's client proteins can be recognized by the different variants of the HSP40/HSP70 complex (Adachi *et al*, 2009).

2.7.2 Structure of HSP90

The HSP90 protein is composed of three highly conserved domains, N-terminal domain (NTD), middle domain (MD) and the C-terminal domain (CTD). A 25 kDa highly conserved NTD, a 35 kDa flexible MD, and a 12 kDa CTD as shown figure 9.

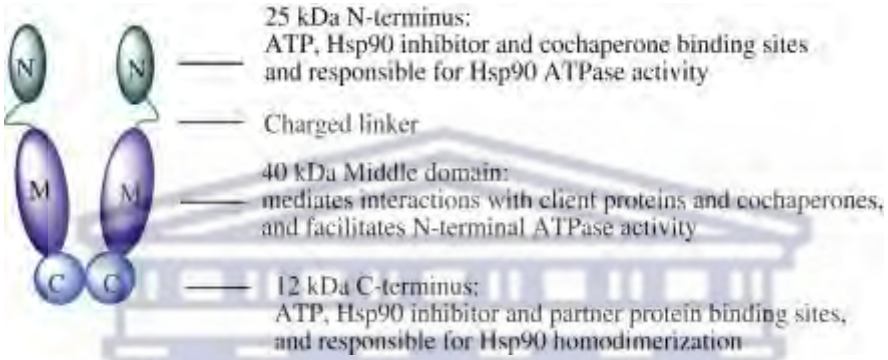


Figure 9; HSP90 structural domains and their functions (Zhao *et al*., 2012).

The NTD domain of the HSP90 enzyme contains the ATP binding site where the activity of the enzyme is required for binding the client proteins (Dutta and Inourye, 2010; Panaretou *et al*, 1998). It is also a major target of HSP90 inhibitors, such as radicicol (RD) and geldanamycin (GDA). These natural product inhibitors compete with the ATP for binding the NTD of HSP90. The MD of the HSP90 enzyme modulates its activity by binding the γ-phosphate of the ATP, which is required for the NTD (Meyer *et al*, 2003). In addition, studies have shown that this domain is involved in the interaction between client proteins and co-chaperones like Aha1 (Huai *et al*, 2005). The MEEVD motif at the C-terminus allows the interaction between the HSP90 and the co-chaperone tetratricopeptide repeat domains (Buchner, 1999).

The long, flexible, and highly charged linker between the NTD and the MD allows the protein to influence the activity of the HSP90 by regulating the interaction between NTD and MD (Jahn *et al*., 2014; Tsutsumi *et al*., 2012). This domain has variable amino acid and length composition and is characterized by its unique structure (Tsutsumi *et al*, 2009). The ability of the charged linker to regulate the activity of the HSP90 is believed to be a contributing factor to the development of a flexible and dynamic environment in eukaryotic cells (Tsutsumi *et al*, 2009; Shiau *et al*, 2006). However, conflicting reports have been presented regarding the role of this region in the regulation of the HSP90's chaperone activity. Some studies suggest that the charged linker plays a role in the regulation of the chaperone activity (Scheibel *et al*, 1999).

Others claim that it does not play a significant role in the development of the HSP90's functions (Louvion *et al*, 1996).

The CTD of the HSP90 enzyme contains two key sites, one for calmodulin binding and the other for homo-dimerization (Meng *et al*, 1996). In addition, it has a nucleotide-binding site that opens following the NTD site's occupancy. This region is known to play an important role in the regulation of the N-terminal ATPase activity (So″ ti *et al*, 2003). The binding sites of the N- and C-terminal nucleotides exhibited different specificities against their respective compounds. For instance, the N-terminal site was specifically focused on adenosine nucleotides, which are known to contain an intact adenine ring. On the other hand, the C-terminal site was more unspecific when it interacted with pyrimidine and purine nucleotides (So″ ti *et al*, 2003).

2.7.2.1 ATP-binding N-terminal domain of HSP90

The NTD is mostly conserved part among both the HSP90 domains, with sequence identity of 69% from human species to yeast. The tertiary structures of the two domains are very similar, with eight β-sheets sandwich a face, while nine α-helices and loops cover the other side. Stebbins and co-workers described the pocket to flat-bottomed cone-like pocket is about 15 deep, 12 in diameter, and has a width of 8 at its midpoint. It can hold three water molecules. The bottom portion of the pocket is formed by the antiparallel sheet's central portion, the H2, H4, H7, and H7 helices, and the L1 loop (Stebbins *et al*, 1997). The H7 helix is the deepest part of the pocket, and it is shallowest by the L1 loop. This is because it packs at an angle perpendicular to the sheet's bottom.

The HSP90 ATP-binding site is located in a deep pocket on the NTD' helical face. This region features a mixed polar and hydrophobic character. Approximately half of the site's 17 amino acids are composed of hydrophobic and polar compounds. These are Leu48, Asn51, Asp54, Ala55, Lys58, Ile91, Asp93, Ile96, Gly97, Met98, Asn106, Leu107, Lys112, Gly135, Phe138, Val150, Thr184, and Val186. Although its surface becomes hydrophobic toward the bottom, the pocket still has one charged and one polar residue in its deep portion (Asp93 and Thr184 from the β sheet, Stebbins *et al*, 1997). This region is mainly polar at its entrance, as shown in figure 10. On the other hand, it becomes hydrophobic near the bottom except for the conserved amino acid Asp93.

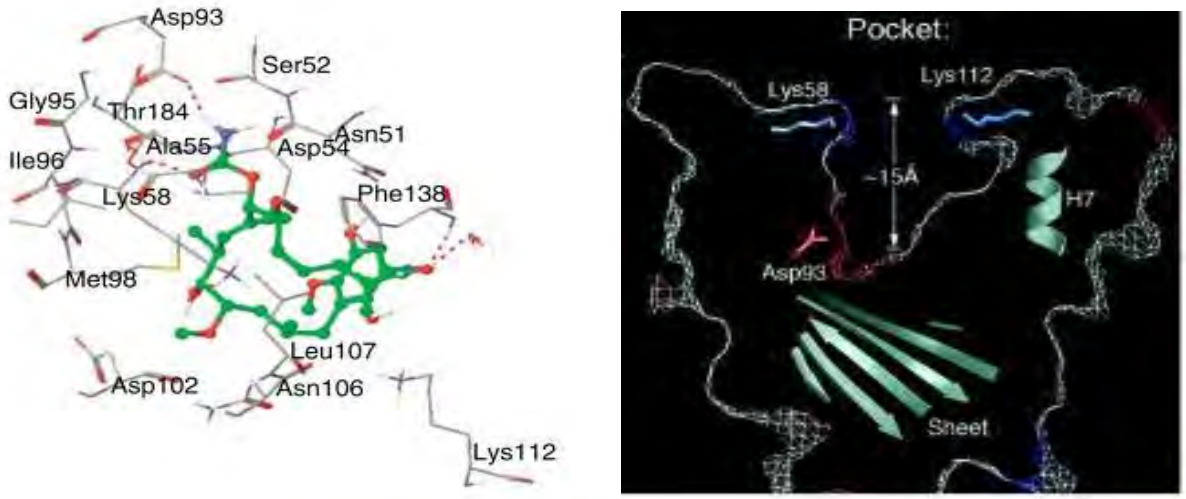


Figure 10; (A) Representation of HSP90 active site bound to GDA (PDB ID; 1YET, Patel *et al*, 2011), (B) HSP90 conserved pocket that is 15 Å deep (Stebbins *et al*, 1997).

2.8 Potential Therapeutic Effects of HSP90 Inhibitors in AD

As mentioned in Chapter 1, inhibiting HSP90 is a promising strategy for the treatment of AD, where the inhibitors of this protein disrupt the interaction between the heat shock factor 1 and HSP90. This causes the activation of a heat shock response by increasing the levels of various chaperones, such as HSP70 and HSP40 (figure 11, Zou *et al*., 1998). These proteins are induced in response to HSF-1 transcriptional activation, and the expressions of these proteins in unfavourable condition restores protein homeostasis (Zhao *et al*, 2012). By activating HSF-1 and producing heat shock proteins like HSP70, HSP90 inhibitors protect against protein toxicity and reduces brain aggregate to form in AD.

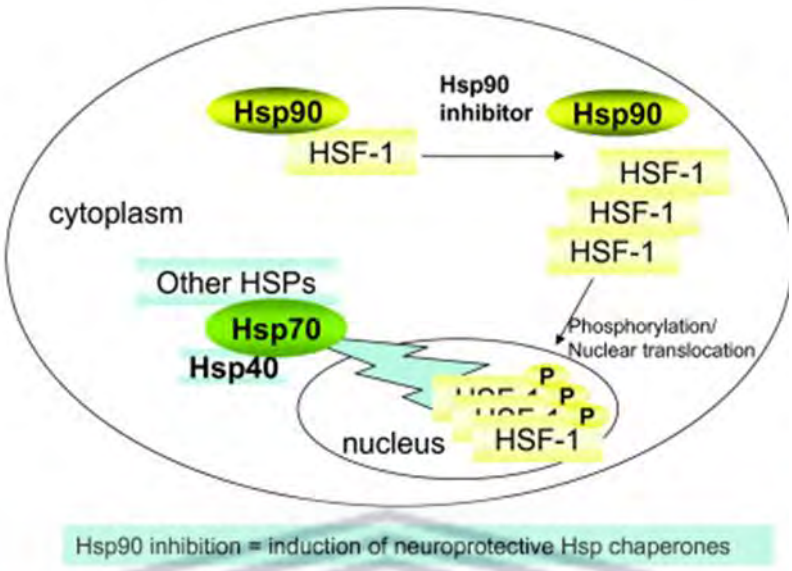


Figure 11; Representation of HSF-1 regulation by HSP90 inhibitors (Luo *et al,* 2010).

In numerous animal models of neurodegenerative disorders, protein overexpression of a few heat shock proteins such as HSP70, HSP40, and HSP27 has demonstrated a certain level of neuroprotection. In an in vitro AD model, for instance, HSP70 overexpression has been shown to protect neurons against Aβ (Magrané *et al*., 2004). The aforementioned neuroprotective observations were due to elevation of heat shock response upon the administration of GDA an HSP90 inhibitor. This compound significantly decreased the formation of NFTs and decreased the levels of tau in the brains of patients with AD. This compound was also demonstrated to increase the levels of soluble tau in a patient suffering from AD (Dou *et al*, 2003). This was also proven by Petrucelli and co-workers that GDA significantly reduced 80% the levels of insoluble tau aggregates in cultured cell cultures that were transfected with tau constructs. It also increased soluble tau levels, which is associated with an increase in the MT-bound tau (Petrucelli *et al*., 2004). In normal condition tau enhance the stability of microtubule structure. However, in AD, the hyperphosphorylation of tau can cause the dissociation of these structures, which allows for the incorporation of NFTs into the brain (Muchowski and Wacker, 2005; Dou *et al*, 2003).

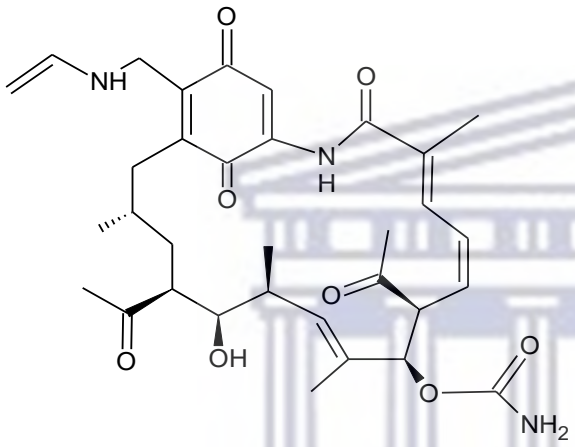
Another HSP90 known inhibitor called 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG, was introduced in treating neurodegenerative diseases by Waza *et al*. in 2005. 17-AAG is a semisynthetic version of GDA that is less toxic. It has been shown that it can induce a heat shock response in various cell types through the HSF-1 pathway (Chen *et al,* 2014). In 2013, it was demonstrated that high doses of 17-AAG can decrease the NFTs in a mouse model (Ho

et al, 2013). This compound has shown to decrease tau pathology in vitro (Lou *et al*, 2007) and in vivo in a transgenic mouse model of frontotemporal dementia (Ho *et al*, 2013). In addition to inhibiting multiple HSPs, 17-AAG can also up-regulate the expression of synaptic proteins such as synapsin I, synaptophysin and PSD95 (Chen *et al*., 2014). This compound is known to work through the Raf-MEK-ERK pathway, which is a component of the HSP90 client protein Raf. It is believed that the presence of ERK inhibitors can help in the stabilization and activation of p-tau (Waza *et al*, 2006; Pratt. and Toft, 2003).

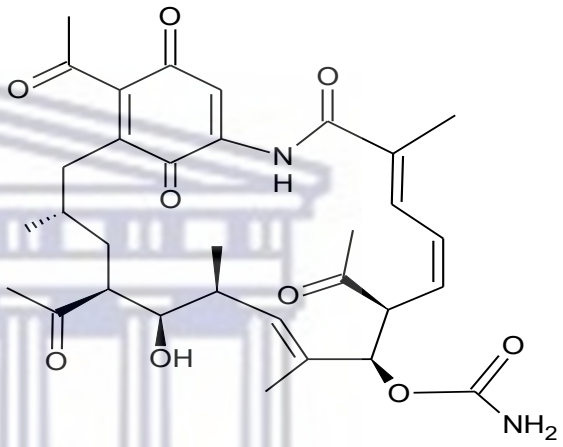
Synthetic N-terminal ATPase inhibitors of HSP90 have been developed based on the purine scaffold, including highly BBB permeable EC102 (Dickey *et al*, 2005) and water-soluble PU24FCl and PU- DZ8 derivates, all these inhibitors has shown to reduce tau in transgenic mouse models of tauopathy and led to the induction of HSP70 (Luo *et al*, 2007). In studies on the effects of HSP90 inhibitors on the accumulation of hyperphosphorylated tau in Tg mice, it has been shown that HSP90 inhibitor PUDZ8 can reduce both the soluble and hyperphosphorylated insoluble tau (Luo *et al*, 2007). Also the administration EC102 has shown to be able to reduce the levels of tau and induce the expression of HSP70 in a mouse brain and this compound is promising because it was able to cross blood brain barrier (BBB) and reduce tau levels without causing toxicity (Dickey *et al*, 2005). The inhibition by the PU24FCl inhibitor led to a reduction in the number of m-tau and p35 and an increase in the induction of HSP70 (Luo *et al*, 2007). In addition, HSP90 inhibitors has shown to prevent the formation of aggregates and trigger apoptosis, which can lead to neurodegeneration. In studies conducted by Salehi and colleagues, they were able to show that an inhibitor of HSP90, known as AEG3482, activates the heat-shock response and prevents the activation of JNK. Furthermore, increasing the levels of HSP70 could prevent the development of neurotoxic effects (Koren *et al*, 2009; Ansar *et al*, 2007). The findings of these studies suggest that the use of HSP90 inhibitors could be a potential treatment for AD.

The relationship between HSP90 inhibitors, HSR and tau levels was investigated using human H4 neuroglioma cells (Dickey *et al*, 2005). It was revealed that several of these synthetic HSP90 inhibitors had a significant effect on the cell's HSR, as evidenced by elevated levels of HSP70, HSP40 and HSP27. The results of the study revealed that the cells that were treated with these compounds exhibited a reduction in tau levels. The reduction in tau levels was also linked to the induction of HSPs (Dickey *et al*, 2005). In 2017, it was revealed that OS47720 can also induce a heat shock-like response and provide synaptic protection in a mouse model

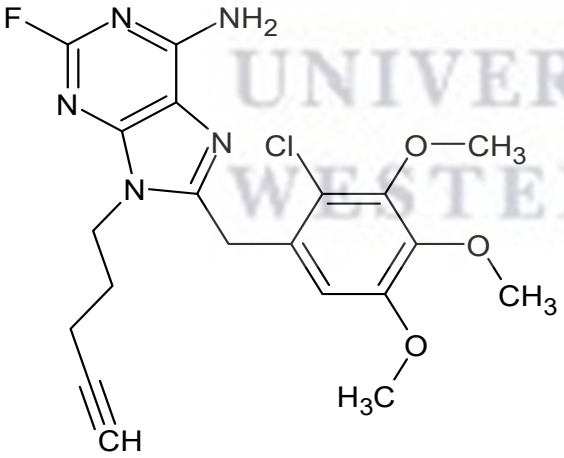
of Tg2576. Reduction of Aβ was observed after chronic OS47720 administration this is due to the modest induction of stress responsive proteins HSP70 and HSP40 by this drug. OS47720 was also found to induce heat-shock-like response and also offers synaptic protection in symptomatic Tg2576 mice (Wang *et al*, 2017). Dickey *et al* demonstrated that treatment with an HSP90 inhibitor resulted in reduction of aberrant p-tau species, including a substantial decrease in tau phosphorylated, and increased HSP70 levels demonstrated drug efficacy in treated mice (Dickey *et al*, 2007).



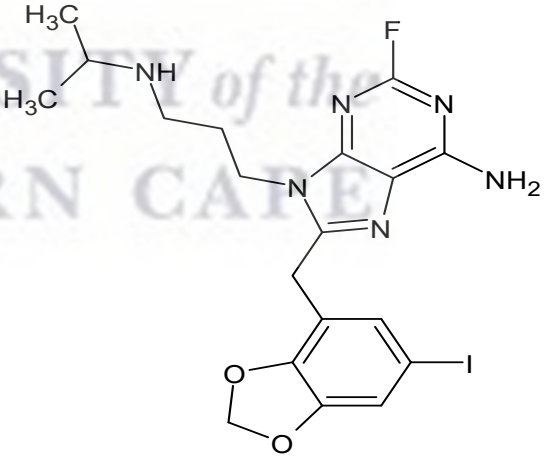
17AAG



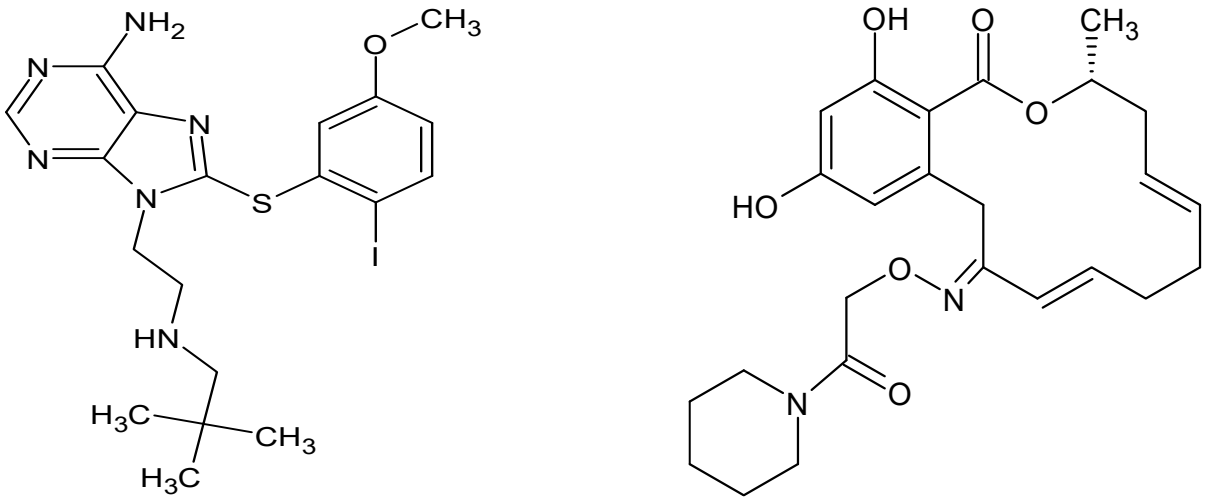
Geldanamycin



PU24FCl



PU-DZ8



EC102 OS47720

Figure 12; Six HSP90 *N*-terminal inhibitors.

2.9 Virtual screening

An *in-silico* technique known as virtual screening is used in the drug discovery process to analyze large databases of 3D structures. It allows for the selection of promising compounds for *in vitro* research. This method is becoming very popular in the drug development industry as it allows for the quick and cost-effective discovery of new drugs (Maia *et al*., 2017). This technique aids in the discovery of bioactive molecules by allowing the collection of molecules in a structure database which are most likely to exhibit biological activity against a protein of interest. These bioactive molecules are subjected to biological tests after they are discovered. VS have other techniques that involve machine learning to anticipate molecules with certain pharmacodynamic, pharmacokinetic, or toxicological effects based on structural and physicochemical parameters that are derived from ligand structure (Martin and Jasen, 2020). As a result, VS is currently the most widely employed methodology for discovering novel bioactive compounds (Maithri and Narendra, 2016).

VS approach can be divided into two classes based on the structural knowledge available: structure based virtual screening (SBVS) and ligand based virtual screening (LBVS). The molecular similarity theory is used to explore the structural features and physicochemical properties of the chemical scaffolds of known active and inactive compounds in ligand based virtual screening (LBVS) (Johnson and Maggiora., 1990). As a consequence, similarity measurements using relevant molecular descriptors are used to investigate the relationships between compounds in a given library and one or more recognized actives. These measurements can be made using 1D and 2D descriptors (Jørgensen and Pedersen., 2001;

Ivanciuc *et al*.,2000; Duan *et al*., 2010) as well as 3D descriptors related to molecular fields, shape and volume (Cross *et al*., 2010; Metress *et al*., 1997; Cheeseright *et al*., 2008; Vázquez *et al.,*2018; Hawkins *et al.,* 2007, Sastry *et al.,* 2011), and pharmacophores (Cross *et al.,* 2010; Abrahamian *et al.,*2003).

On the other hand, SBVS also referred to as target-based virtual screening, refers to methods that take advantage of the target's three-dimensional (3D) structure. Molecular docking is the most used SBVS technique, this technique uses structural and chemical complementarity to its advantage, that occurs when a drug-like compound interacts with its target protein, predicting the desired pose of ligands in the binding site using scoring functions, which are often supplemented with pharmacophoric constraints (Taylor *et al*., 2002; Li J *et al*., 2019; Torres *et al*., 2019; Pagadala *et al*., 2017).

A variety of tasks related to the discovery of drugs are involved in structure-based drug discovery. These include virtual screening, predicting the strength of a compound's binding activity, predicting the binding site's interaction with a compound, and predicting how a protein or compound will alter its binding pose (Bohacek *et al*, 1996). Unlike ligand-based approaches, which rely on the binding site's atomic locations to predict the strength of a compound's binding activity, structure-based approaches consider the compound's similarity to a known target of interest. This method is very promising as it allows for the identification of truly novel compounds (Drwal and Griffith, 2013).

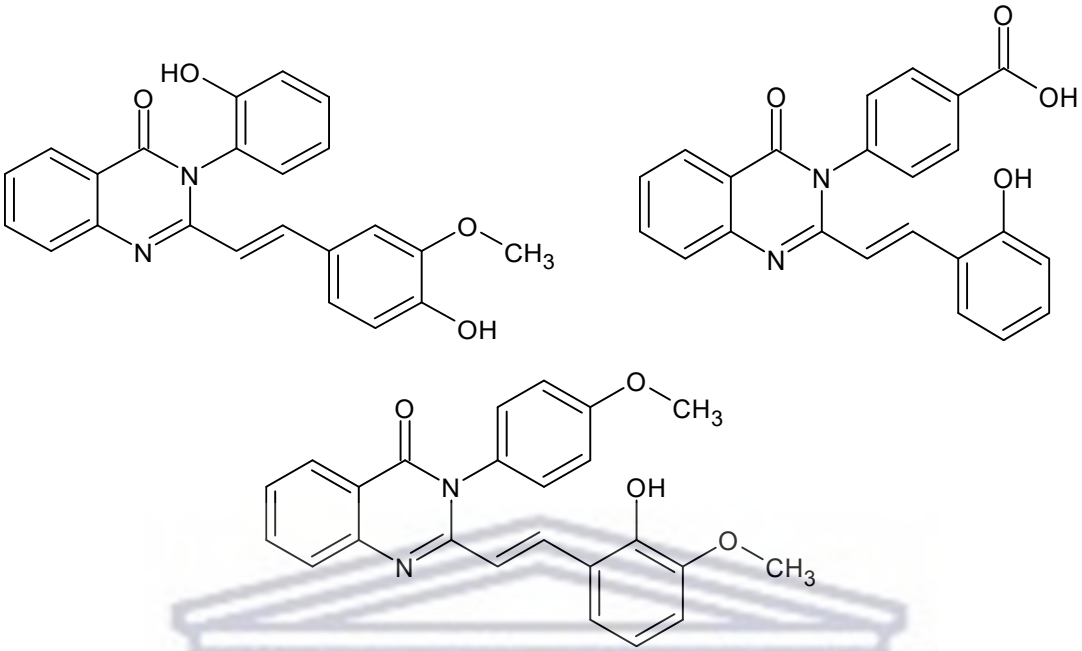
Fast Rigid Exhaustive Docking (FRED) (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com) is a docking tool that is used to dock a large number of molecules into the active site of a target protein (McGann, 2012). FRED is a command-line program with a powerful Graphical User Interface (GUI) for setting up the active site and adding custom restraints (www.eyesopen.com). FRED's docking technique is to score every potential position in the active site exhaustively. The exhaustive search is based on each conformer's rigid rotation and translation. This unique solution eliminates the sample difficulties that are common with stochastic docking methods (Miteva *et al*, 2005). As a result, when used in combination with the Chemgauss score function, this application provides a good method for structure-based virtual screening (Xia *et al*, 2018). FRED has a very excellent way for assessing the shape of an active site, allowing it to reject poses that are in the wrong position quite effectively (www.eyesopen.com).Considering its high speed, FREDs docking time is few seconds per ligand (Lakshmanan *et al*, 2019).

2.10 Structure based virtual screening for HSP90 inhibitors.

VS has been used is many *in silico* attempts to identify HSP90 inhibitors, and many of these studies led to new HSP90 inhibitors. All of these studies used SBVS technique in the discovery of HSP90 inhibitor as shown in table and the chemical structures of the inhibitors is represented in figure 13.

Table 1; Summary of the methods and results of HSP90 inhibitors that used virtual screening to discover technique

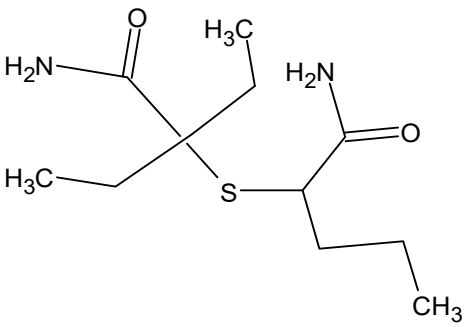
Virtual screening technique	**Software**	**Chemical database**	**HSP90 PDB**	**Compound name/s**	**Author/s**
SBVS	RDock	rCat	1YET and 1UY6	1-(2-Phenol)-2-naphthol inhibitors	Barril *et al,* 2005
SBVS	AutoDock	InterBioScreen	2BZ5	3-Phenyl-2-styryl 3Hquinazolin-4-one inhibitors	Park *et al*, 2007
SBVS	AutoDock	InterBioScreen	2BZ5	Pyrimidine-2,4,6-trione and 4H-1,2,4-triazole-3-thiol	Hong *et al,* 2009
SBVS	Gold	NCI and Maybridge	1YET	ZINCO1748069 and ZINCO1752263	Saxena *et al*, 2010
SBVS	AutoDock	ZINC	3OWD	Ethyl 2-amino-1H-benzoimidazole-1-carboxylate	Abbasi *et al*, 2021
SBVS	LibDock	Specs	4CE1	1,3-Dibenzyl-2-aryl imidazolidine scaffold	Liu *et al*, 2019



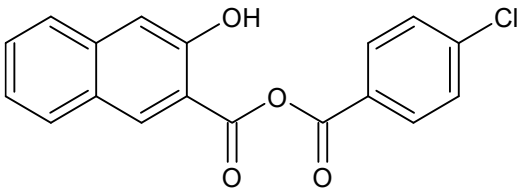
Three 3-phenyl-2-styryl-3H-quinazolin-4-one inhibitors

Ethyl 2-amino-1H-benzoimidazole-1-carboxylate

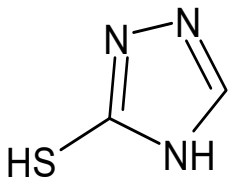
1,3-dibenzyl-2-aryl imidazolidine scaffold



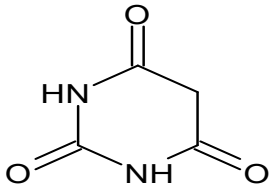
ZINCO1748069



ZINCO1752263



4H-1,2,4-triazole-3-thiol



pyrimidine-2,4,6-trione

Figure 13; Chemical structure of HSP90 inhibitors discovered through SBVS

2.11 Conclusion

AD is a neurodegenerative disease that is characterized by a gain of toxic function of misfolded proteins, mainly β-Amyloid and neurofibrillary tangles (NFTs), with symptoms of memory loss and behavioural abnormalities. The prevalence and effects of AD are expected to rise in the future as the population ages. Therefore, HSP90 inhibitors have the ability to induce the expression of heat-shock proteins, which leads to the solubilisation of protein aggregates and the refolding of misfolded proteins that contribute to the pathogenicity of neurodegenerative diseases such as AD.

Chapter 3

3. Computational procedure

3.1 Instrumentation

In this chapter different software tools were used to perform virtual screening, the software tools include FRED, OMEGA, VIDA and MakeReceptor, all of these software are programs from OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com. FRED was used to dock the ligands collection into the active site of the target protein. Omega was used to generate a 3D conformation of the molecules which is the initial step of docking. Makereceptor was used create a receptor which is understood by FRED, and VIDA was used to visualize ligand-protein interactions. ACD/ChemSketch (Freeware) software was used to draw and save the compounds and the Sigma Aldrich website was used to search for compounds that show similarity to the Maybridge compounds.

3.2 Protein preparation

The 3D protein crystal structure of Human HSP90 (PDB code- 2bz5) in complex with a benzenesulfonamide inhibitor was obtained from the protein data bank (www.rcbs.org) and was saved in PDB format. The reason for this selection lies in that the crystal structure was determined at high resolution (1.9 Å). Resolution is a measure of the quality of the data that has been collected, and high-resolution structures means the structure is of good quality and it is easy to see every atom in the electron density map (Zardecki *et al*, 2022), which makes the structure good for molecular docking. This protein structure has been used for *in silico* studies (Barril *et al*, 2005; Park *et al*, 2007; Hong *et al*, 2009). Molecular Operating Environment (MOE) (www.moe.com) was used to prepare the protein for docking using the following protocol**:** (i) the enzyme protein structure was checked for missing atoms, bonds and contacts, (ii) removal of water molecules, 3D protonation of the protein-ligand complex. The file was saved as a PDB file for the purpose of preparing the receptor with MakeReceptor.

3.2.1 Make Receptor

MakeReceptor is a graphical utility for creating or modifying a receptor. Receptor are specialized files used by FRED and Ligand Guided Docking (HYBRID) that contain the structure of a target protein, the location and shape of the active site and optionally the structure of a bound and docking constraints (eyesopen.com). The MakeReceptor is process used the following method.

First, the optimized PBD protein ligand was visualized in VIDA and saved as an .oeb file. The molecule was then classified using the checkboxes mark ligand and mark protein, with the macromolecule labelled as the protein and the target ligand labelled as the ligand. The active site was defined or marked by circling a box in the active site location, allowing atoms that do not fit in the active site to be discriminated and subsequently rejected. Custom constraints were created to ensure efficient protein-ligand interactions. The custom constraints selected was Asp93 and Thr184, then receptor was then saved.

3.3 Library preparation

Ligands that were used in virtual screening analysis were collected from the Maybridge database (www.maybridge.com) and saved as sdf file, under the correct pathway where OpenEye is installed with the license file. The Maybridge database contains a large number of structurally diverse drug-like compounds that are well suited for screening programmes. These compounds obey the Lipiski's rule and demonstrate good ADME profiles 52 160 drug-like compounds were collected from this database. Then the database was narrowed down to only compounds containing amine moieties, this step was taken to ensure that the database contained only amine-containing compounds. Based on literature many known HSP90 inhibitors contain amine moiety (Biamonte *et al*, 2006; Magwenyane *et al*, 2022). This step was done using OGREP, a command-line tool of OpenBabel 2.2.3 (O'Boyle *et al*, 2011), to a focused database of 10 904 amine-containing compounds. Then OMEGA 2.4.3 software (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com) was used to generate the 3D conformations of the molecules, to prepare the molecules for docking.

3.4 Molecular docking

Virtual screening was performed to identify novel inhibitors of HSP90, using FRED 2.2.5 docking software (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com). FRED was used to dock the amine-containing Maybridge database collection into the active site of HSP90. The maybridge screening collection contains 10904 amine containing compounds that were docked in HSP90 active site, 7824 molecules were successfully docked out of 10 904 amine containing compounds. Amine containing compounds were selected, as mention in **3.3** that many known HSP90 inhibitors contain amine moiety (Biamonte *et al*, 2006; Magwenyane *et al*, 2022). The software then ranks the top 500 compounds according to their Chemgauss4 score which can be viewed on VIDA. Subsequently, the top 50 were analysed using VIDA to view hydrogen-bond interaction and

using the Protein Plus Server (https://proteins.plus/) to check for additional non-hydrogen bond interactions, such as π-π and/or lipophilic interactions. Ten compounds were selected from the top 50 analysed compounds based on hydrogen bonds with one or both of the custom constraints, the Chemgauss4 score, and finally binding interactions with any other desired amino acids in the active site of HSP90 protein (Figure 14).

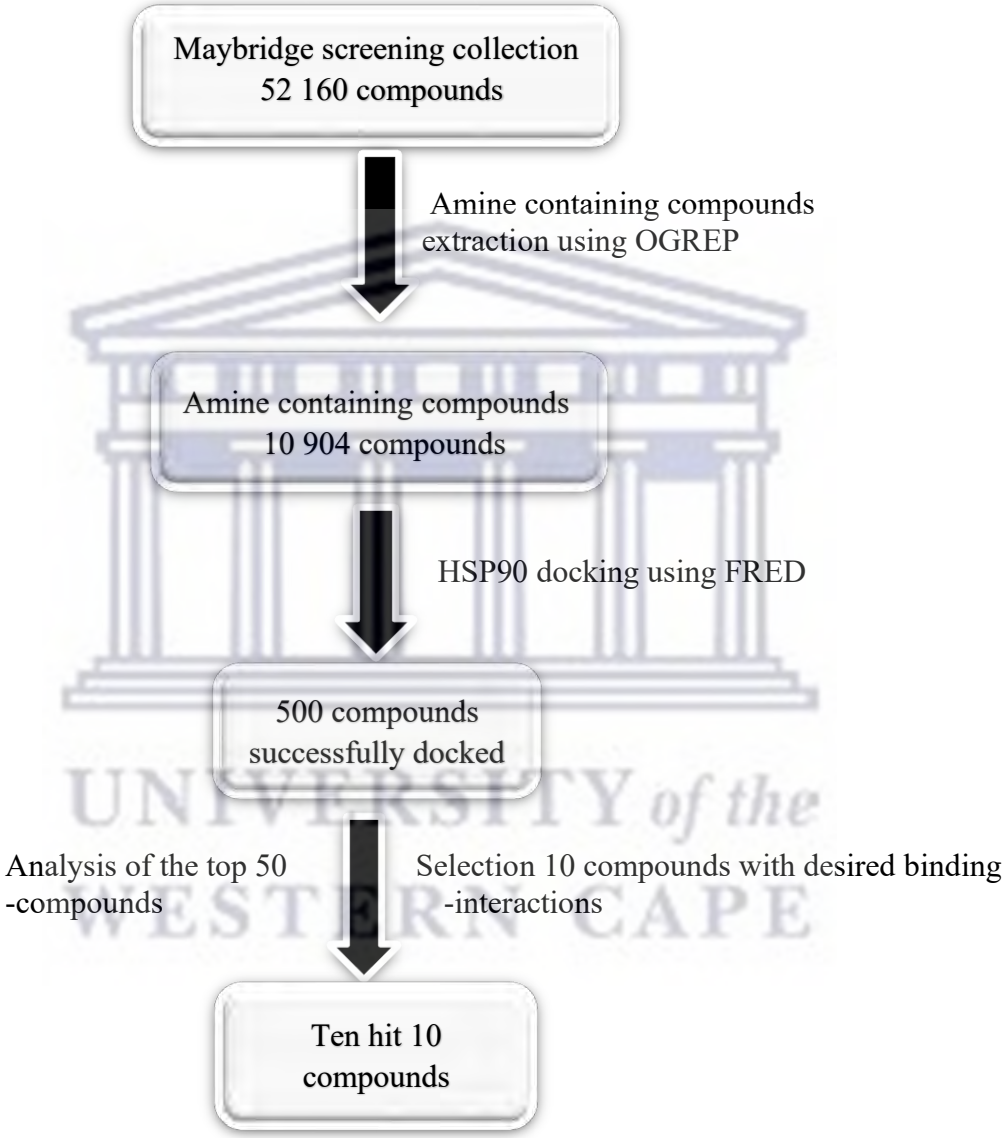


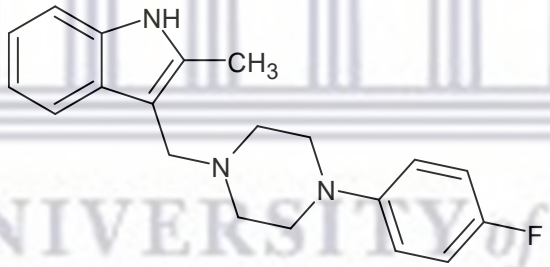
Figure 14; Flow diagram that represents virtual screening protocol.

3.5 Molecular docking results analysis

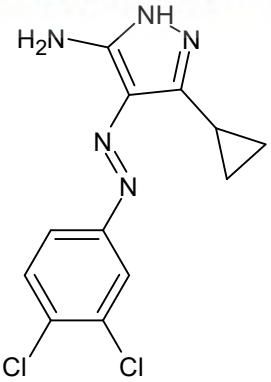
The extracted amine-containing compounds were successfully docked in the active site of the HSP90 protein, and the selected compounds were analysed on VIDA and on the protein plus server. In the process of selecting the 10 hit compounds, and the compounds that had a binding interaction with Asp93 and Thr184 were mostly considered in this study and this is because it

has been reported in previous studies that molecules that bind the ATP pocket of HSP90 are known to exhibit key H-bonding contacts with Thr184 and Asp93 (Knox *et al*, 2009; Rampogu *et al*, 2019), with Asp93 mentioned as the most important amino acid in the active site of the HSP90 protein (Abbasi *et al*, 2017). The selected top 10 compounds exhibited a hydrogen bond with both of the two important amino acids or one of amino acid, and as well as other residues including Asn51, Ala55, Lys58, Gly97, Met98 and Phe138 at the ATP-binding site of HSP90. The results of ligand-protein interactions for each compound is summarised in table 2.

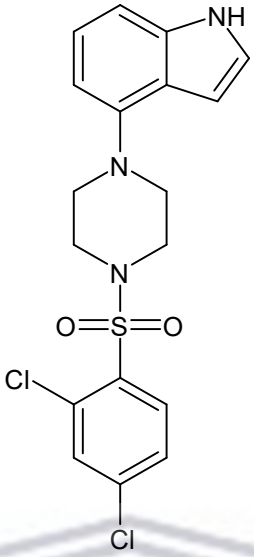
Structurally the top 10 compounds contain an indole ring or a similar moiety in their structure, indole and indazoles are two of the most important classes of nitrogen-containing heterocyclic compounds bearing a bicyclic ring structure made up of a pyrazole ring and a benzene ring (Teixeira *et al*, 2006). There are also compounds that contain a triazole ring, which is five membered heterocyclic scaffolds. Triazole are suggested to penetrate the HSP90 ATP binding site (Taddei *et al*, 2014), and several triazole derivatives are claimed to be another class of highly potent HSP90 inhibitors (Messaoudi *et al*, 2011; Messaoudi *et al*, 2008).



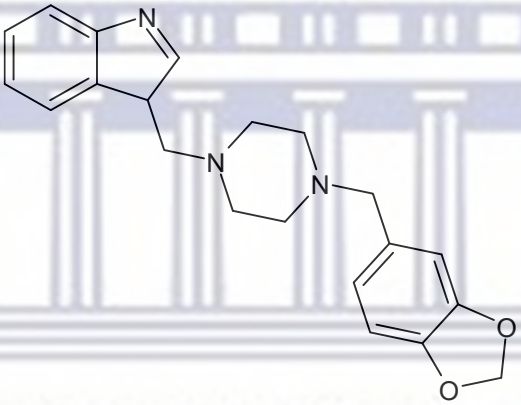
3-{[4-(4-fluorophenyl) piperazin-1-yl]methyl}-2-methyl-3*H*-indole



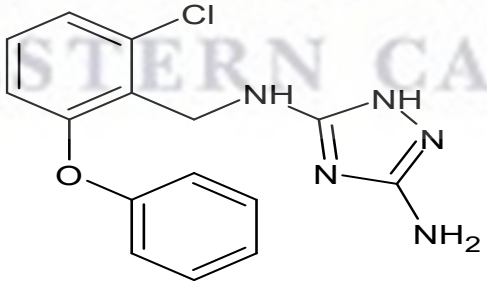
3-cyclopropyl-4-[(*E*)-(3, 4-dichlorophenyl) diazenyl]-1*H*-pyrazol-5-amine



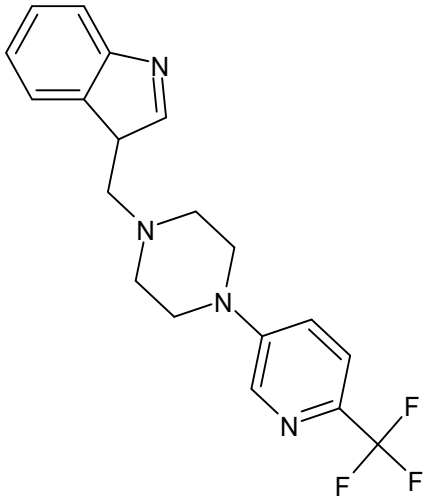
4-[4-(2, 4-dichlorobenzene-1-sulfonyl) piperazin-1-yl]-1H-indole



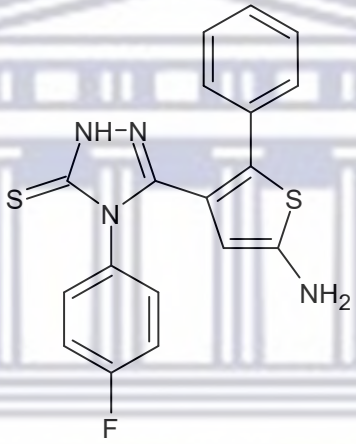
3-({4-[(2*H*-1, 3-benzodioxol-5-yl)methyl]piperazin-1-yl}methyl)-3*H*-indole



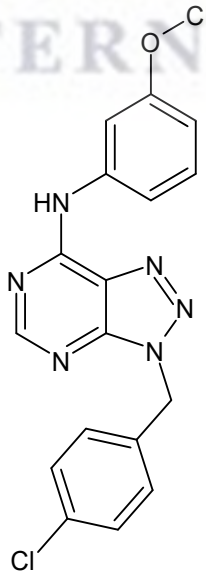
N^5-[(2-chloro-6-phenoxyphenyl) methyl]-1*H*-1, 2, 4-triazole-3, 5-diamine



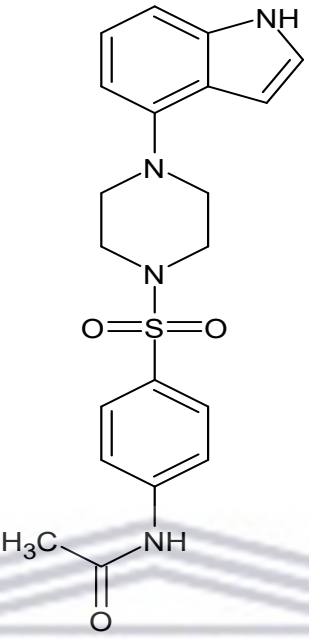
3-({4-[6-(trifluoromethyl)pyridin-3-yl]piperazin-1-yl}methyl)-3*H*-indole



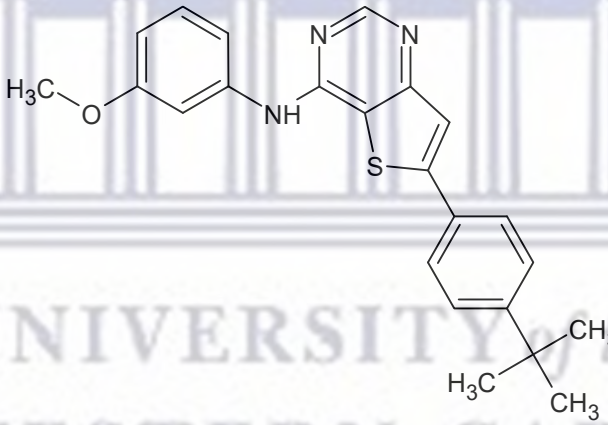
5-(5-amino-2-phenylthiophen-3-yl)-4-(4-fluorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione



3-[(4-chlorophenyl) methyl]-*N*-(3-methoxyphenyl)-3*H*-[1, 2, 3]triazolo[4,5-*d*]pyrimidin-7-amine



N-{4-[4-(1*H*-indol-4-yl) piperazine-1-sulfonyl]phenyl}acetamide



6-[4-(tert-butyl)phenyl]-N-(3-methoxyphenyl)thieno[3,2-d]pyrimidin-4-amine

Figure 15; Maybridge top 10 hit compounds.

Table 2; Binding interaction of the 10 hit compounds in HSP90 active site along with their Chemguass4 score.

Name	**H-Bond**	**Hydrophobic bonds**	**Chemguass4 score**	**Numbering system**
3-{[4-(4-fluorophenyl) piperazin-1-yl]methyl}-2-methyl-3*H*-indole	Asp93 and Ans51	Thr184, Leu48, Ans51 and Val186	-12,9419	A

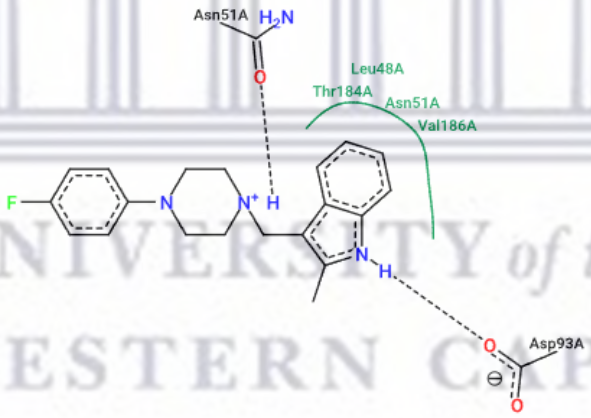
3-cyclopropyl-4-[(*E*)-(3, 4-dichlorophenyl) diazenyl]-1*H*-pyrazol-5-amine	Asp93 and Thr184	Met98	-12,8261	B
4-[4-(2,4-dichlorobenzene-1-sulfonyl)piperazin-1-yl]-1*H*-indole	Asp93	Met98, Thr184 and Ans51	-12,6636	C
3-({4-[(2*H*-1,3-benzodioxol-5-yl)methyl]piperazin-1-yl}methyl)-3*H*-indole	Asp93	Thr184, Met98, Ans51	-12,6240	D
N^5-[(2-chloro-6-phenoxyphenyl) methyl]-1*H*-1, 2, 4-triazole-3, 5-diamine	Asp93, Thr184, Ser52 and Leu48	Ala55, Asp54 and Ans51	-12,6201	E
3-({4-[6-(trifluoromethyl)pyridin-3-yl]piperazin-1-yl}methyl)-3*H*-indole	Asp93	Thr184, Met98, Ans51 and Val186	-12,6005	F
5-(5-amino-2-phenylthiophen-3-yl)-4-(4-fluorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione	Asp93, Thr184 and Gly97	Ans51, Leu48 and Val186	-12,5824	G
3-[(4-chlorophenyl) methyl]-*N*-(3-methoxyphenyl)-3*H*-[1, 2, 3]triazolo[4,5-*d*]pyrimidin-7-amine	Asp93, Thr184, Ser52 and Ans51	Met98	-12,5685	H
N-{4-[4-(1*H*-indol-4-yl)piperazine-1-sulfonyl]phenyl}acetamide	Asp93 and Gly137	Thr184 and Ans51	-12,4826	I
6-[4-(tert-butyl)phenyl]-N-(3methoxyphenyl)thieno[3,2-d]pyrimidin-4-amine	Thr184 and Ans51	Gly135, Val186, Ans51, Ala55 and Met86	-12,4207	J

3.5.1 2D protein-ligand prediction of Maybridge compounds

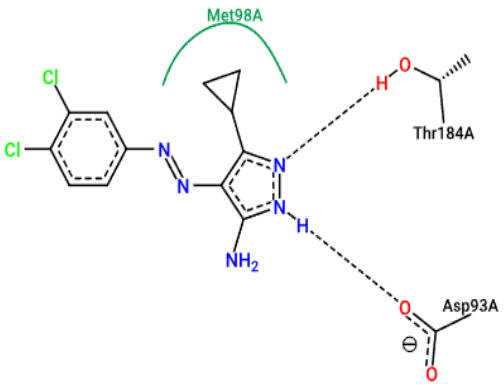
The 2D protein-ligand prediction interaction was evaluated using pose-view, a tool in Protein Plus Server (https://proteins.plus/). This tool allows automatic creation of 2D diagrams of complexes with known 3D structure according to chemical drawing conventions (Stierand *et al*, 2006). The hydrogen bonds between the ligand and protein are presented by dotted lines and the hydrophobic interactions are represented more indirectly through spline sections highlighting the hydrophobic parts of the ligand and the label of the contacting residue (Fricker *et al*, 2004). The 2D prediction of the interaction between the 10 compounds within the HSP90 active site is represented in figure 16, all of the compounds interacted with important amino

acid which are Asp93 and Thr184. Also, studies have demonstrated the presence of Ala55 rendered by hydrophobic π-π/π-alkyl interactions between the ligands and the protein, this interaction is previously reported effective in the inhibition of HSP90 active site (Abbasi *et al*, 2017)

The compounds that contain an indole ring in their structures, the NH in the indole ring forms a hydrogen bond with Asp93, and also observing compound 1 that has an indole ring, the piperazine moiety formed an H-bond with Ans51. The compounds that contains the triazole moiety such as compound 5 formed H-bonds with Asp93, Thr184, Ser52 and Leu48. Looking at compound 8 the pyrimidine ring formed an H-bong with Thr184 and hydrophobic interaction with Ala55 and the benzyloxy moiety formed an H-bond with Asn51 along with hydrophobic interactions with Val186 and Asn51. Compound 2 contains a pyrazole moiety which formed H-bond with Asp93, Thr184 and a hydrophobic interaction with Met98. The thiolane ring in compound 7 formed an H-bond with Asp93, and this structure also contains a triazole ring that formed H-bonds with Gly97 and Thr184.



A



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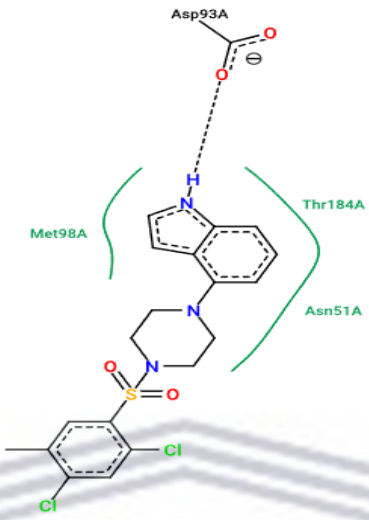
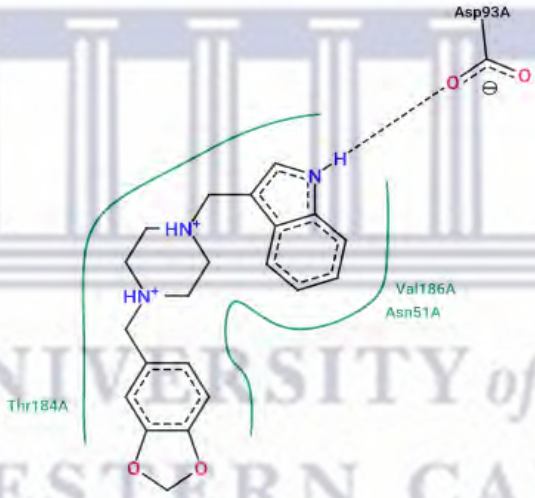
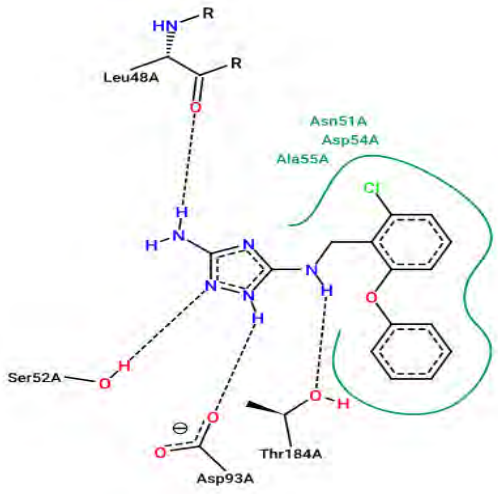


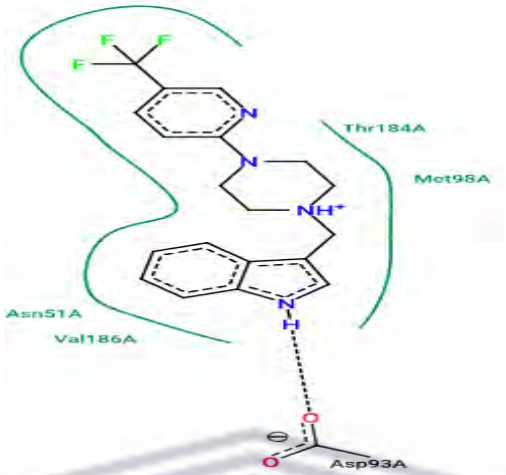
C



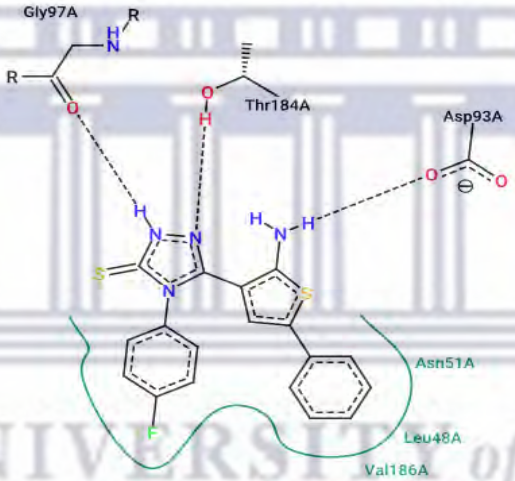
D



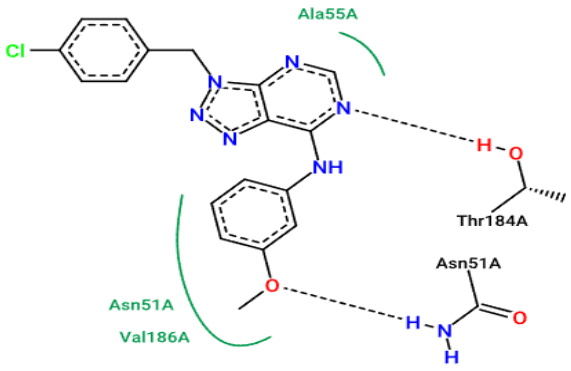
E



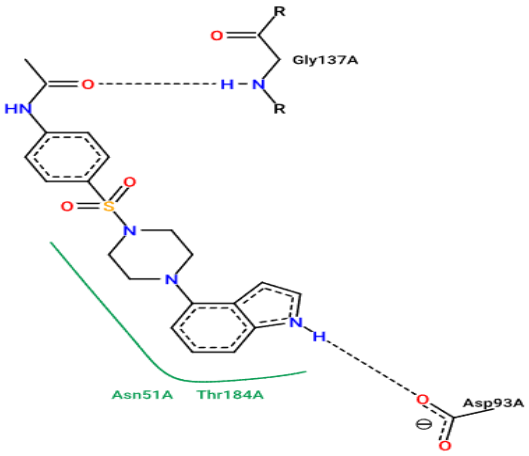
F



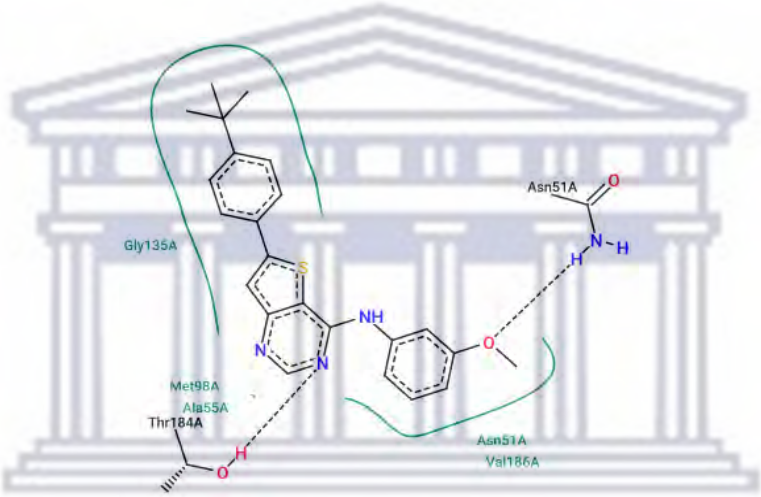
G



H



I



J

Figure 16; 2D prediction of the binding interaction between HSP90 and the 10 hit compounds

3.6 Virtual screening of Sigma Aldrich compounds

3.6.1 Sigma Aldrich compound preparation

Since the 10 Maybridge compound could not be easily imported to South Africa because of Covid-19 restrictions, these compounds were then drawn on Sigma-Aldrich website to search for structures that resemble the structure of the selected MayBridge compounds or show at least 70% similarity. The 10 Maybridge compounds were then drawn on the Sigma-Aldrich website (https://www.sigmaaldrich.com/catalog/search/substructure/SubstructureSearchPage) to search for structures that resemble the structure of the selected MayBridge compounds or show at least 60% similarity. Selected one structure that showed resemblance for each selected MayBridge compounds on Sigma Aldrich website, results shown on table 3 and figure 18. The

selected Sigma Aldrich compounds were drawn using ACD/ChemSketch (Freeware) software, Product Version: 12.01 and saved as a mol file. The analogs were then opened in MOE and saved as sdf files. Then, VIDA was used to put the analog compounds as a collective to form a small compound library of 10 compounds. Omega was used to optimize the ligands into their 3D confirmations. The optimized 3D compounds were then ready to be docked into the active site of receptors.

3.7 Molecular docking protocol

The 10 Sigma Aldrich compounds were docked into the active site of HSP90 using the method stated under section 3.2. The successfully docked the compounds using FRED, were ranked according to the Chemguass4 score and out of 10 compound that were docked in the active site of HSP90 protein, 9 compounds were read on FRED and 8 compounds were successfully docked. The 8 compounds were evaluated for desired interaction in the active site of HSP90 protein, and these compounds demonstrated to interact with the important amino acids. Additionally, 6 compounds were successfully purchased from Sigma-Aldrich® (Steinheim, Germany).

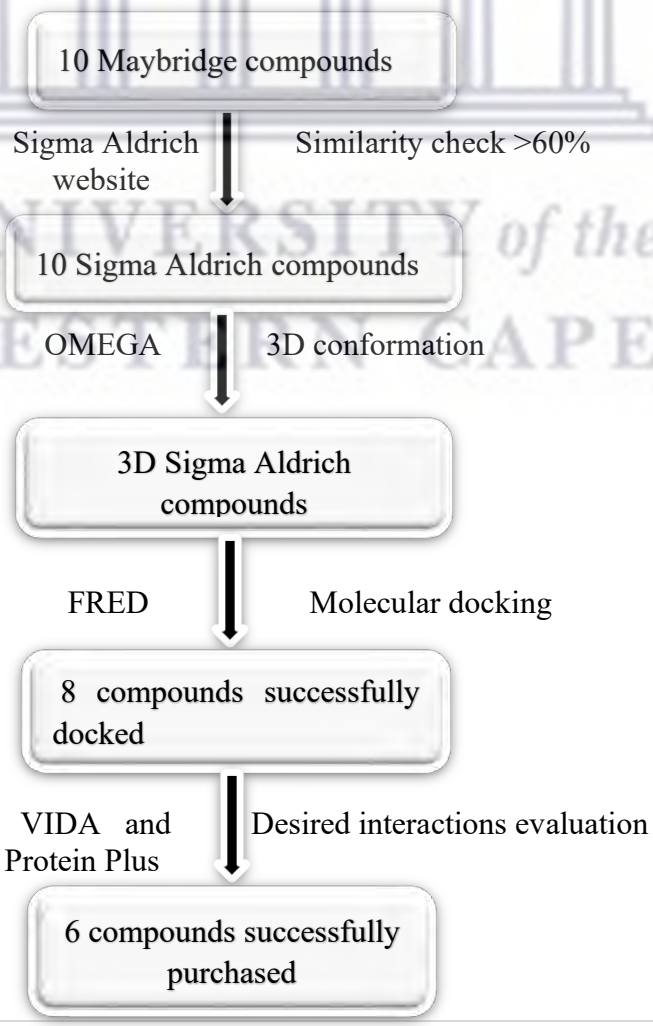
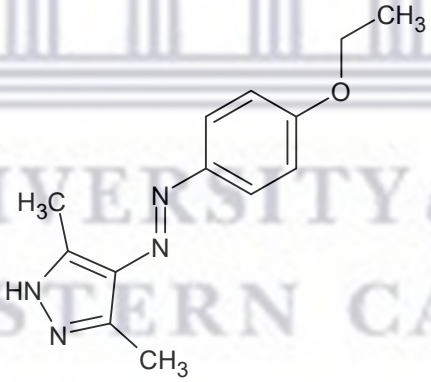


Figure 17; Flow diagram that represents virtual screening protocol

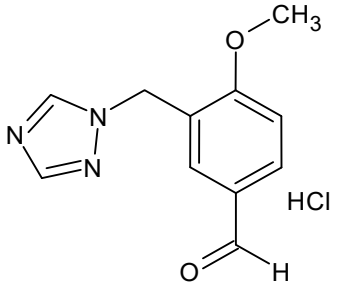
Table 3: Shows Sigma Aldrich percentage similarity of Maybridge compounds

Maybrige compounds	**Sigma Aldrich percentage similarity**
Omega – 13078	60%
Omega – 5210	63%
Omega- 9524	74%
Omega 11500	68%
Omega 9589	65%
Omega 12259	66%
Omega – 8368	65%
Omega -9853	63%
Omega – 4090	65%
Omega 9853	62%

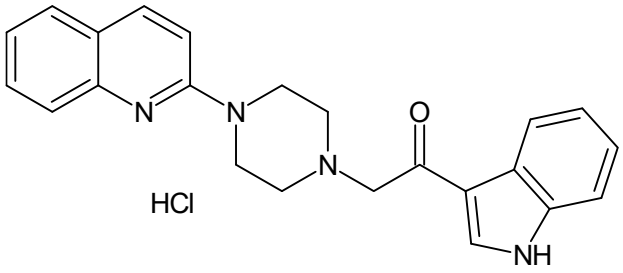
The 10 Sigma Aldrich compounds identified include the following:-



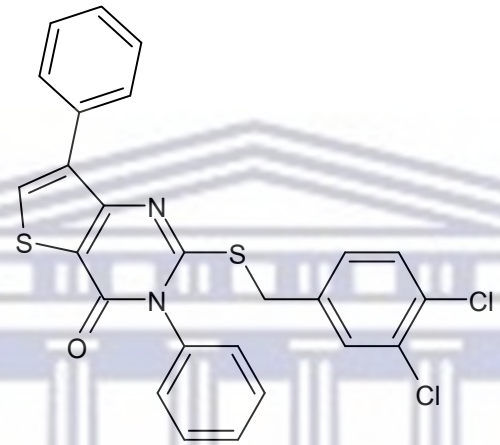
(3,5-Dimethyl-1h-pyrazol-4-yl)-(4-ethoxy-phenyl)-diazene



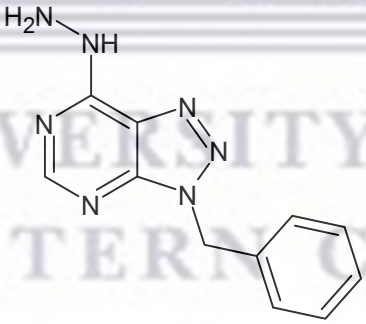
4-Methoxy-3-(1H-1,2,4-triazol-1-ylmethyl)benzaldehyde hydrochloride



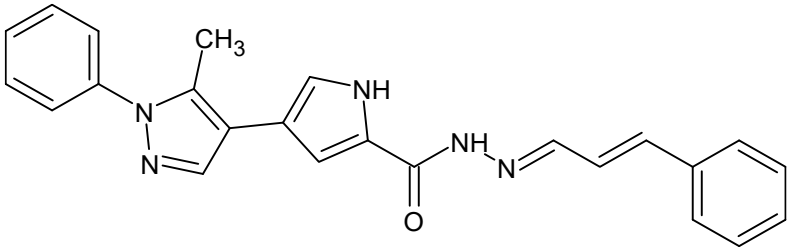
1-(1h-Indol-3-yl)-2-(4-(2-quinolinyl)-1-piperazinyl)ethanone dihydrochloride



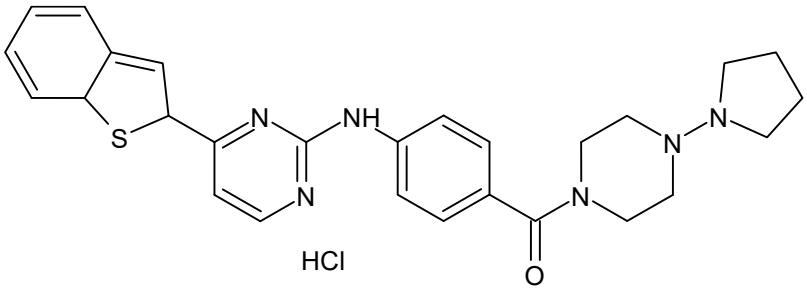
2-((3,4-Dichlorobenzyl)thio)-3,6-diphenylthieno(2,3-d)pyrimidin-4(3h)-one



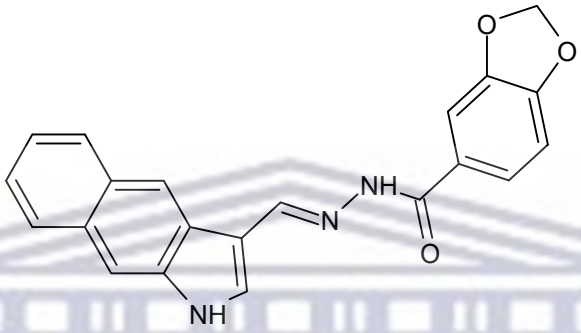
3-Benzyl-7-hydrazino-3H-[1,2,3]triazolo[4,5-d]pyrimidine



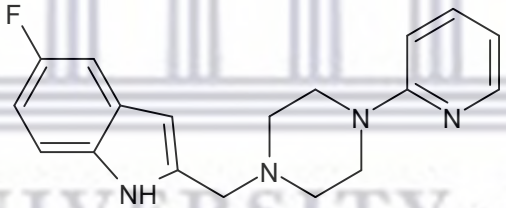
(E)-3',5'-Dimethyl-1'-phenyl-n'-((e)-3-phenylallylidene)-1h,1'h-3,4'-bipyrazole-5-carbohydrazide



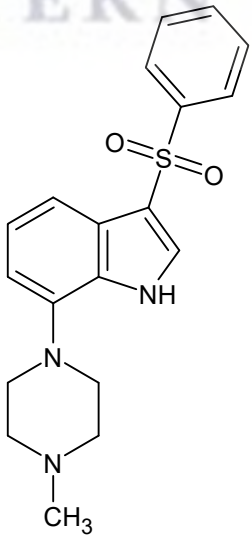
IKK-16 dihydrochloride



Benzo(1,3)dioxole-5-carboxylic acid (1h-benzo(g)indol-3-yl methylene)-hydrazide



5-Fluoro-2-{[4-(2-pyridinyl)-1-piperazinyl]methyl}-1H-indole



3-Benzenesulfonyl-7-(4-methyl-piperazin-1-yl)-1H-indole

Figure 18; Ten Sigma Aldrich compounds

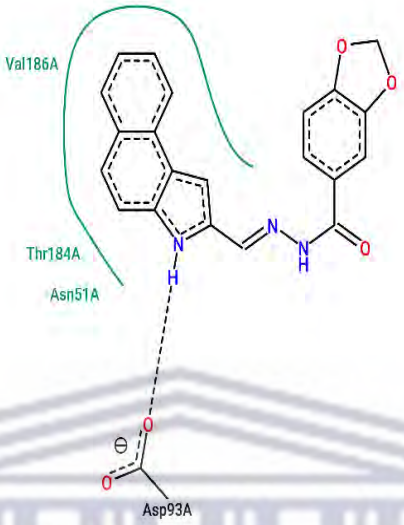
Table 4; Binding interaction of the 10 hit compounds in HSP90 active site along with their Chemguass4 score

Name	H-Bond	Hydrophobic bonds	Chemguass4 score	Numbering system
Benzo(1,3)dioxole-5-carboxylicacid(1hbenzo(g)indol-3-yl methylene)-hydrazide	Asp93	Val186, Thr184 and Asn51	-4.7622	Compound 1
1-(1h-Indole-3-yl)-2-(4-(2-quinolinyl)-1-piperazinyl)ethanone dihydrocloride	Asp93	Val186, Gly135, Thr184 and Asn51	-11.3880	Compound 2
(3,5-Dimethyl-1h-pyrazol-4-yl)-(4-ethoxy-phenyl)-diazene	Asp93	Ans106	-11.3901	Compound 3
4-Methoxy-3-(1H-1,2,4-triazol-1-ylmethyl)benzaldehyde hydrochloride	Ans106	Ala55 and Met98	-8.3751	Compound 4
3-Benzyl-7-hydrazino-3H-[1,2,3]triazolo[4,5-d]pyrimidine	Asp93 and Thr184	Met98	-8.9052	Compound 5
IKK-16 dihydrochloride	Asp93, Ser52 and Lys58	Lys58, Ala55, Asp54 and Leu48	-5.4322	Compound 6
5-Fluoro-2-{[4-(2-pyridinyl)-1-piperazinyl]methyl}-1H-indole	Asp93 and Ans106	Thr184, Met98, Ans51, Val186, Ala55 and Ile96	-8.8076	Compound 7
3-Benzenesulfonyl-7-(4-methyl-piperazin-1-yl)-1H-indole	Ser52	Thr184, Met98, Ans51, Val186 and Leu48	-7.0361	Compound 8

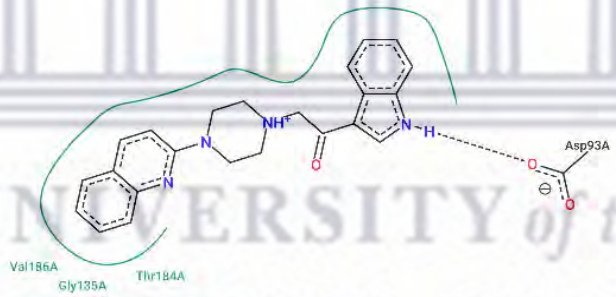
3.7.1 2D protein-ligand prediction of Sigma Aldrich compounds

The 2D protein-ligand prediction interaction was also used to check for check for addition non-hydrogen bond interactions, such as π-π and/or lipophilic interaction on Protein Plus Server (https://proteins.plus/). The 2D prediction of the interaction of the 8 Sigma Aldrich compounds

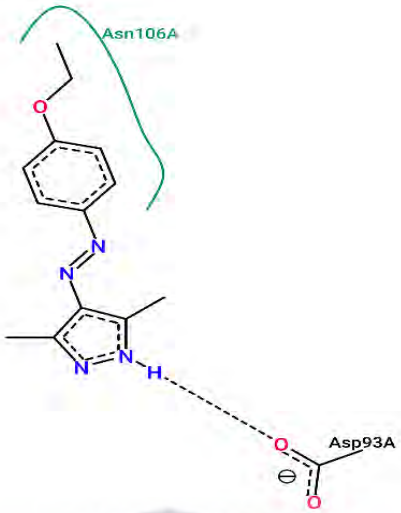
within the HSP90 active site is represented in figure 19. All the 8 compounds interacted with important amino acid which are Asp93 and Thr184, and other desired interactions.



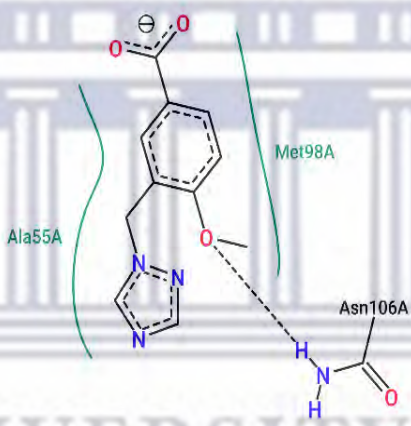
Compound 1



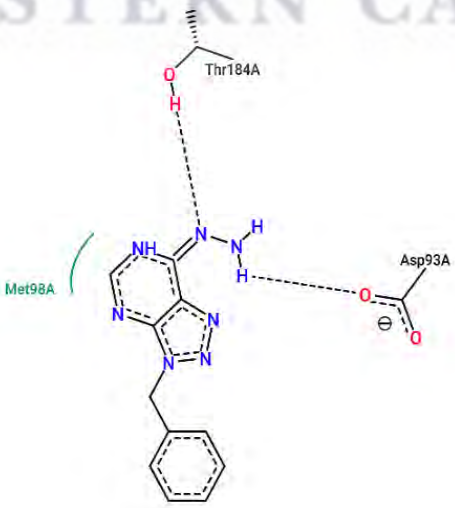
Compound 2



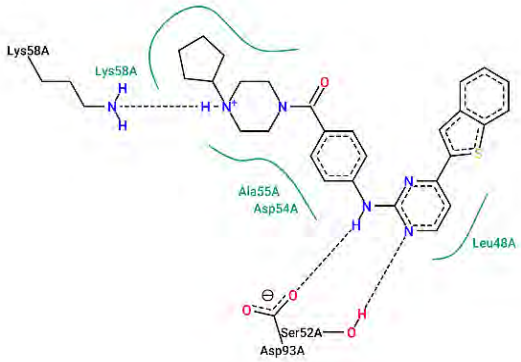
Compound 3



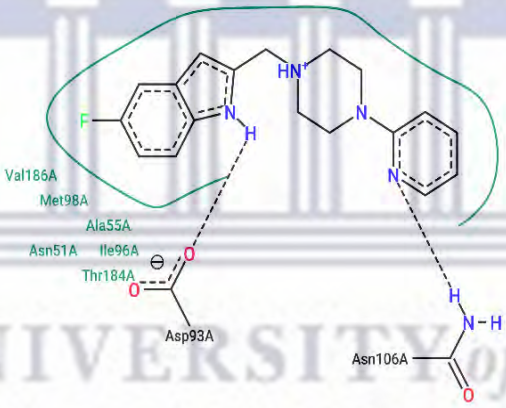
Compound 4



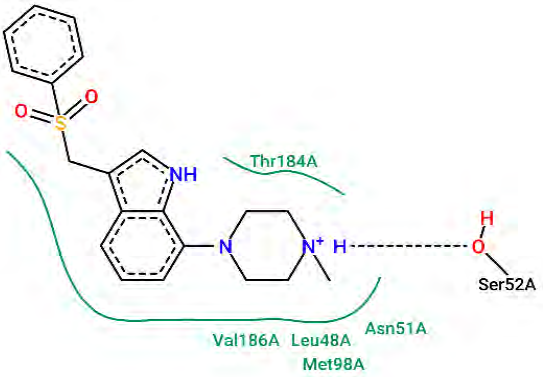
Compound 5



Compound 6



Compound 7



Compound 8

Figure 19; 2D prediction of the binding interaction between HSP90 and eight Sigma Aldrich compounds

Conclusion

Both Maybridge and Sigma Aldrich compounds showed potential to inhibit the HSP90 enzyme, all the compounds showed significant interactions with HSP90 active site, but only the Sigma Aldrich compounds were further purchased from Sigma Aldrich, this is because the Sigma Aldrich compounds would be easily purchased and imported to South Africa compared to Maybridge compounds. Next, the compounds were further evaluated for the inhibition of HSP90 and the compounds were subjected to computational ADME, pharmacokinetic and drug-likeness evaluation, using the web tool Swiss-ADME the results are discussed in Chapter 4.

CHAPTER 4

4. Biological materials and methods

4.1 Introduction

Following the successful purchase of the six Sigma Aldrich test compounds described in the last chapter, the compounds were evaluated using the HSP90 enzyme assay. As the compounds are expected to show inhibitory activity towards HSP90 enzyme, the compounds were also evaluated for pharmacokinetics and drug-likeness. The results are described in detail in this chapter.

4.2 Fluorescence Polarization (FP) Enzymatic Assay

The HSP90α molecular chaperone is known to play a role in protein folding and disease progression. Its function is also implicated in the progression of diseases. The development of an efficient and effective screening tool such as the HSP90α Assay Kit for identifying HSP90 inhibitors is very important. The design of the HSP90 inhibitor kit is based on protein binding of geldanamycin which is the common component to a recombinant form of the protein. This allows the determination of the overall polarization of the protein. This is done by measuring the change in its fluorescence intensity using a fluorescence reader.

4.2.1 Consumables and instrumentation

All test compounds were purchased Sigma-Aldrich® (Steinheim, Germany), and the HSP90α *N*- Terminal Domain Assay Kit is from Bioscience (San Diego, United States). Fluorescence intensity was measured at 485 nm and 520 nm using a Synergy H1 multimodal plate reader (BioTek Instruments, Inc.). The data obtained was analysed using GraphPad Prism 5 (GraphPad Software, Inc) and Microsoft excel.

4.2.2 Experimental procedures

The test compounds were dissolved in dimethyl sulphoxide (DMSO) to prepare 10 000 µM stock solutions. The stock was then further diluted by factors of ten to produce solutions of 100 µM, 10 µM and 1 µM as the final concentration.

Below is an illustration of the serial dilution.

$DF = V_f / V_I$

$= 100/10$

$= 10$-time dilution

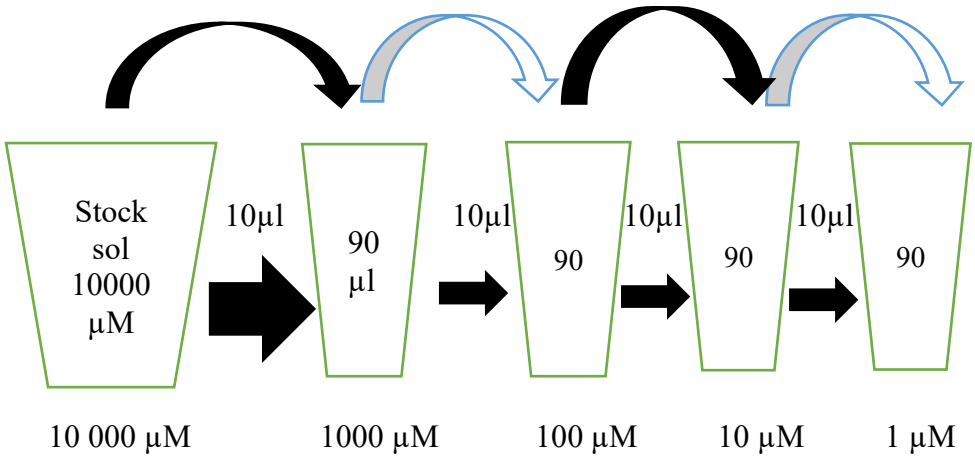


Figure 20; Schematic representation of the serial dilutions of the stock solution.

The assay was carried out in a 96 well plate fluorescence intensity, which measures the competitive binding of fluorescently labelled geldanamycin to recombinant HSP90α as a function of check compound concentration. This assay was not done in triplicates/duplicates due to the high cost of the assay kit made it impossible to perform the assay in triplicates/duplicates. Therefore, this assay was done using HSP90α N-Terminal Domain Assay Kit (BPS Bioscience, Catalogue #50293, CA, USA) following the manufacturer's instructions. In brief, HSP90 assay buffer (15 µl), 40 mM dithiothreitol (DTT) (5 µl), 2 mg/mL BSA (5 µl), and H_2O (40 µl) was added to the plate. In each well, also as described in table 5 below, 100 nM fluorescein isothiocyanate (FITC)-labelled geldanamycin (5 µl) and compounds in different concentrations (100, 10 and 1 µM) was added in an orderly manner. The reactions were initiated by adding 20 µL of HSP90α (17 ng/µL), then after 2.5 h of incubation with slow shaking at room temperature, the fluorescence intensity was measured at 485/520 nm using Synergy H1 multimodal plate reader. The blank reading was subtracted from all other values. Results were analysed using GraphPad Prism 5 (GraphPad Software, Inc) (Liu *et al*, 2019).

Table 5: Representation of components used in each well.

	Blank	Enzyme Positive Control	Enzyme Negative Control	Test Inhibitor
5x HSP90 assay buffer 1	15 µl	15 µl	15 µl	15 µl
40 mM DTT	5 µl	5 µl	5 µl	5 µl
2 mg/ml BSA	5 µl	5 µl	5 µl	5 µl
H2O	40 µl	40 µl	40 µl	40 µl
FITC-Labeled geldanamycin (100 Nm	-	5 µl	5 µl	5 µl
Inhibitor				10µl
Inhibitor Buffer (no inhibitor)	10 µl	10 µl	10 µl	-
1x HSP90 assay buffer	25 µl	-	20 µl	-
HSP90α (17 ng/µl)	-	20 µl	-	20 µl

4.2.3 Results and discussion

In fluorescence intensity assay, the change of total light output is monitored and used to quantify a biochemical reaction or binding event. Normally used to measure enzyme activity, therefore the expectations of this assay are to get a low fluorescence intensity value when fluorescence intensity is measure. This is because these compounds are expected to have a good inhibitory capacity as predicted by the docking studies. Figure 21 below demonstrate the inhibition of HSP90 observed at the highest concentration (100 µM) used for this assay. The results demonstrates that compound **6** and **2** showed high intensity levels which means these compounds seem to exhibit weak inhibitory activity on HSP90 enzyme. Whereas compound **1**, **3**, **4** and **5** showed less intensity which means less enzyme activity, that shows that these compounds where able to inhibit the activity of HSP90.

Whereas, in figure 22 the results observed at 10 µM, shows that compound **1**, **3** and **4** has the same fluorescence intensity as the positive control and the positive control contains GDA which is the known HSP90 inhibitor, which implies that these compounds have the same activity as GDA at this concentration. However, compound **5** and **6** show less intensity which means less enzyme activity that implies that these compounds where able to inhibit the activity of HSP90, whereas compound 2 showed high intensity levels which means this compound seem to exhibit weak inhibitory activity at this concentration. The results of the lowest concentration (1 µM) demonstrated that (figure 23), compound **1**, **3**, **5** at this concentration has the same inhibitory activity as the GDA, whereas compound **2** and **6** have no inhibitory effect

on HSP90 activity at this concentration. However, compound **4** seem to have good inhibitory activity against HSP90 activity.

As mentioned in Chapter 3, the *N*-terminal ATP-binding domain of HSP90 was discovered to contain a critical Asp93 residue at the bottom of the pocket for ligand binding (Stebbins *et al*, 1997; Jez *et al*, 2003; Dymock *et al*, 2004). Compounds **1**, **3**, **4**, and **5** appear to be good inhibitors of HSP90 *N*-terminal binding site, and this is in-line with the docking results in Chapter 3. These compounds are predicted to make an interaction with the important amino acids in HSP90 ATPase and these biological results confirm that the test compound are good inhibitors of HSP90 ATPase. However, the assay will have to be repeated to get more data points and a wider concentration range needs to be explored. Additionally, the high cost of the assay kit made it impossible to repeat the experiment in this study. This is a limitation and follow up studies on cells that overexpress HSP90 could be useful in future studies to confirm these results and potentially make the assay more cost-effective.

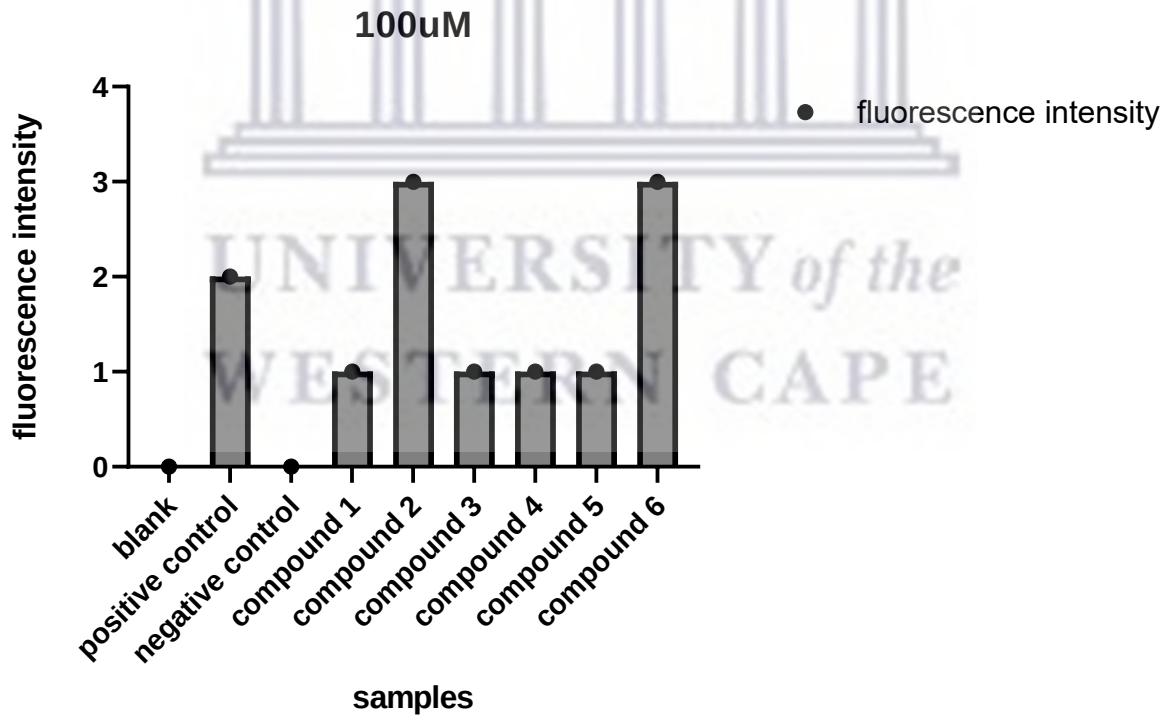


Figure 21: HSP90 inhibition for each test compound at 100 µM.

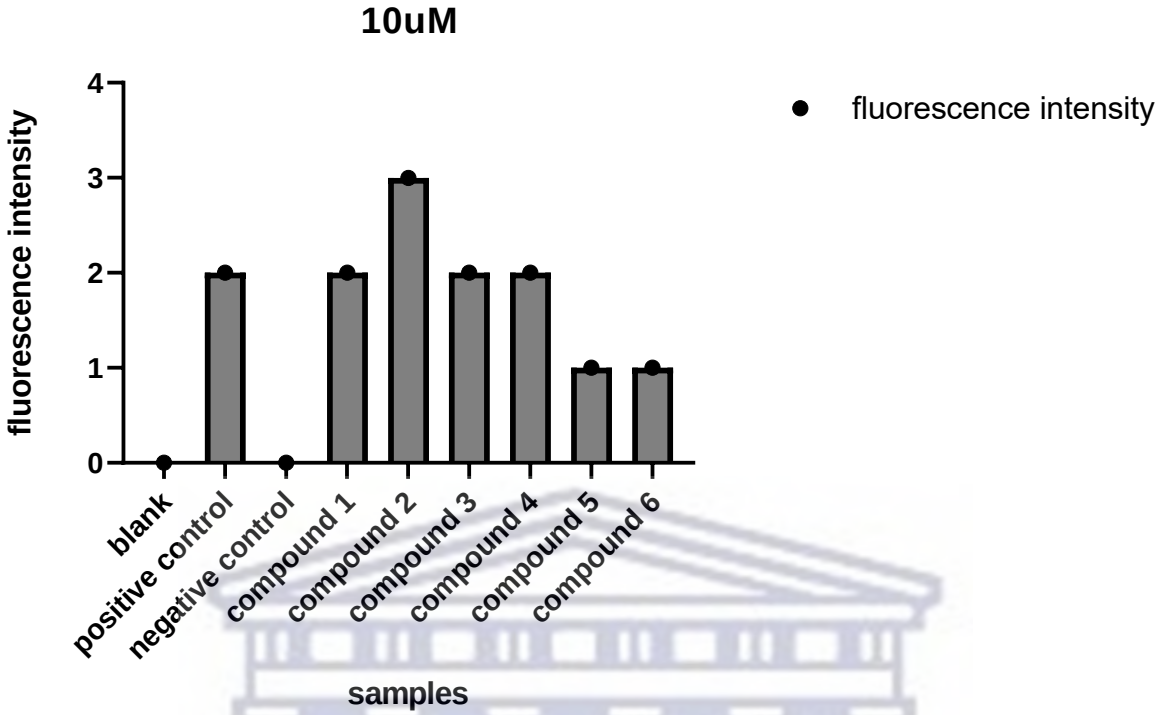


Figure 22: HSP90 inhibition for each test compound at 10 μM.

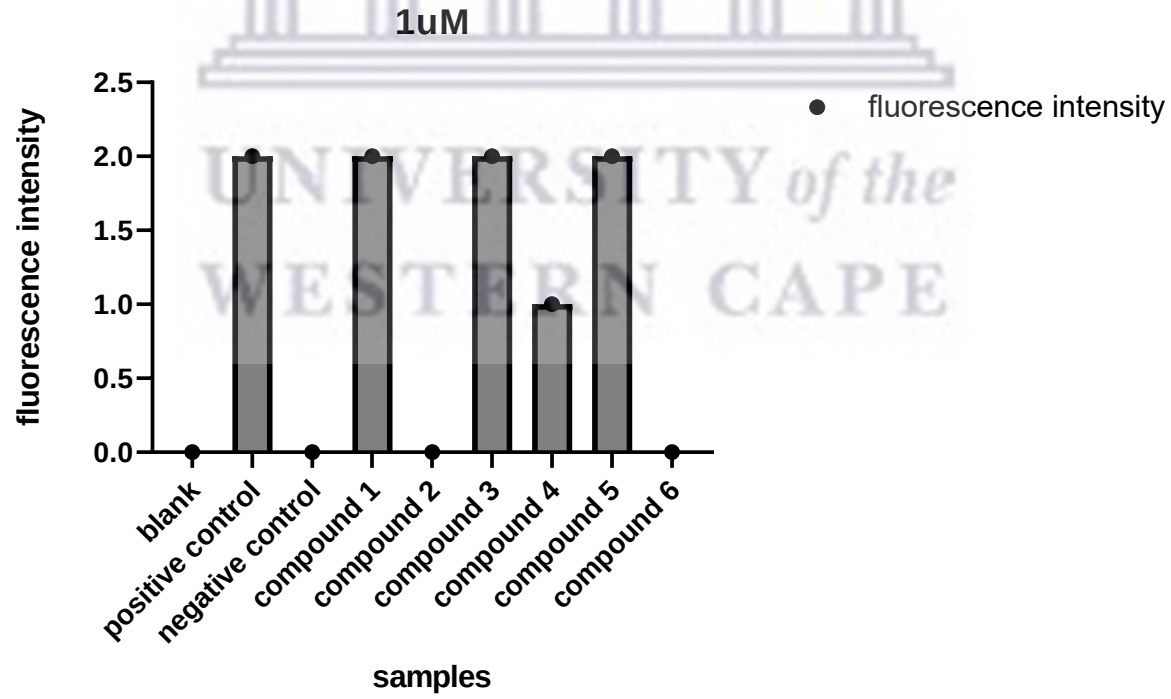


Figure 23: HSP90 inhibition for each test compound at 1 μM. Compounds 2 and 6 were not tested at the lowest concentration as only minimal activity was observed at the higher concentrations.

4.3 *In silico* pharmacokinetic and drug-likeness evaluation

The drug-likeness and pharmacokinetic profiles of the six Sigma Aldrich compounds, were evaluated using the Swiss-ADME web tool (http://www.Swiss-ADME.ch/). Swiss-ADME is very reliable, fast tool used predict physicochemical properties, pharmacokinetics, drug-likeness and medicinal chemistry of small molecules. The structures were drawn directly on Swiss-ADME then converted SMILES (simplified molecular-input line-entry system) format which were then input into the web tool and the pharmacokinetic parameters were generated (Mzezewa *et al*, 2021)

4.3.1 Results and discussion

A drug's biological activity and low toxicity are important factors that determine its effectiveness in treating diseases. Early in its development, it is important to analyze the various aspects of its distribution, absorption, metabolism, and excretion (ADME). This process can be challenging because of the number of compounds being studied and the limited access to samples (Dahlin *et al*, 2015). In order to minimize the time and effort involved in the development of a drug, many of the procedures related to the drug's biological activity and toxicity are carried out using ADME tools. One of these tools is the Swiss-ADME web tool, which allows users to visualize the various aspects of a drug's pharmacokinetics (Daina *et al*, 2017).

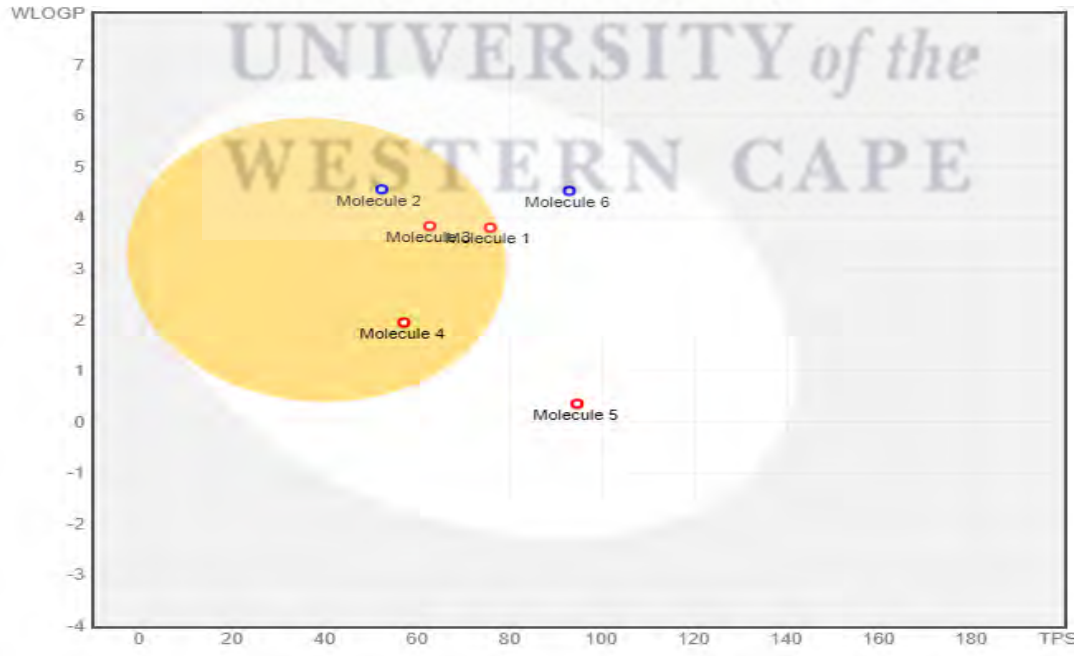


Figure 24; Representation of perceptive evaluation of passive gastrointestinal absorption (HIA) and Blood-Brain Barrier (BBB) penetration of the six test compounds using the BOILED-Egg method.

BOILED-Egg is a method that is used to predict both passive gastrointestinal absorption (HIA) and BBB permeability of small molecule, useful in drug development. Where the yolk represents brain penetration (BBB) and the white represents HIA absorption. Molecules with low absorption and limited brain penetration are represented in the outer grey region (Daina *et al*, 2017). Based on the results shown on figure 23, compound **1**, **2**, **3** and **4** are inside the york, these compounds have a high probability of brain penetration, whereas compound **5** and **6** in the white part of the egg, this means that these compounds have a high probability of passive absorption by gastrointestinal track (HIA).

One of the most researched transporters involved in drug resistance and drug-drug interactions is P-glycoprotein, it is expressed in numerous organs, including the liver, kidney, small intestine, and blood-brain barrier (Glasear, 2011). A p-glycoprotein substrate is a material that makes use of the P-glycoprotein transporter for a number of purposes, such as drug excretion and absorption, as well as other crucial processes that may alter the body's composition or the way that other medications affect it (Lin and Yamazaki, 2003; Glasear, 2011). Compound **1**, **3, 4** and **5** are non-substrate of P-gp (PGP-) which is represented by the red dot, this means that these compounds are not substrate of this transporter. Compound **2** and **6** are predicted to be actively effluxede by P-gp (PGP+) represented by the blue dot. Altogether this demonstrate that compound **1**, **3** and **4** are predicted to passively crossing the BBB (in the york) and not subjected to active efflux, compound **2** is predicted to passively crossing the BBB (in the york) and PGP+ (blue dot), whereas compound **5** and **6** are predicted to be well-absorbed but not accessing the brain (in the white), compound **6** being (PGP+), and compound **5** being (PGP-).

The understanding of the interactions between the small molecules and cytochromes P450 (CYP) is very crucial in drug discovery. Molecules that can interact with cytochromes P450 (CYP) are either called inhibitors or inducers, CYP enzymes are membrane-bound proteins that regulate how quickly drugs are metabolized in our bodies and how long they stay in our bodies. If CYP enzymes are not active enough, drugs can stay in our bodies for longer periods of time, which can result in toxicity. CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 inhibition

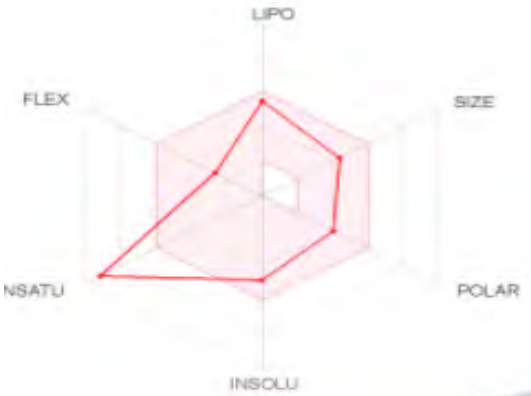
is believed to be one of the main causes of pharmacokinetics-related drug-drug interactions (Hollenberg, 2002; Huang *et al*, 2008). Table 6 demonstrated that compound **1** and **2** inhibit most of the isoforms except for CYP1C9, also compound **6** also inhibits all the isoforms except for CYP1A2. Compound **4** is a non-inhibitor to all the five isoforms, and compound **3** and **5** inhibit only CYP1A2 and induce all rest of the isoforms. When a drug inhibits the CYP isoforms, it decreases the action of the Cytochrome P450 to break it down, resulting in an increase in the pharmacological action of that drug and other drugs. In AD patient inhibition of these isoforms may result in toxicity of un-wanted drugs and accumulation of the drug and other drugs due to lower clearance of these drugs since the patient may be taking other medication.

Table 6; Calculated ADME and pharmacokinetic parameters.

Properties	**Comp1**	**Comp2**	**Comp3**	**Comp4**	**Comp5**	**Comp6**
GI absorption	high	High	High	High	High	High
BBB permeant	Yes	Yes	Yes	Yes	No	No
P-gp substrate	Yes	Yes	No	No	No	Yes
CYP1A2 inhibitor	Yes	Yes	yes	No	Yes	No
CYP2C19 inhibitor	Yes	Yes	No	No	No	Yes
CYP2C9 inhibitor	No	No	No	No	No	Yes
CYP2D6 inhibitor	Yes	Yes	No	No	No	Yes
CYP3A4 inhibitor	Yes	Yes	No	No	No	Yes
Log Kp (skin permeation)	-5.63cm/s	-5.03cm/s	-6.57cm/s	-6.55cm/s	-7.14cm/s	-5.51cm/s

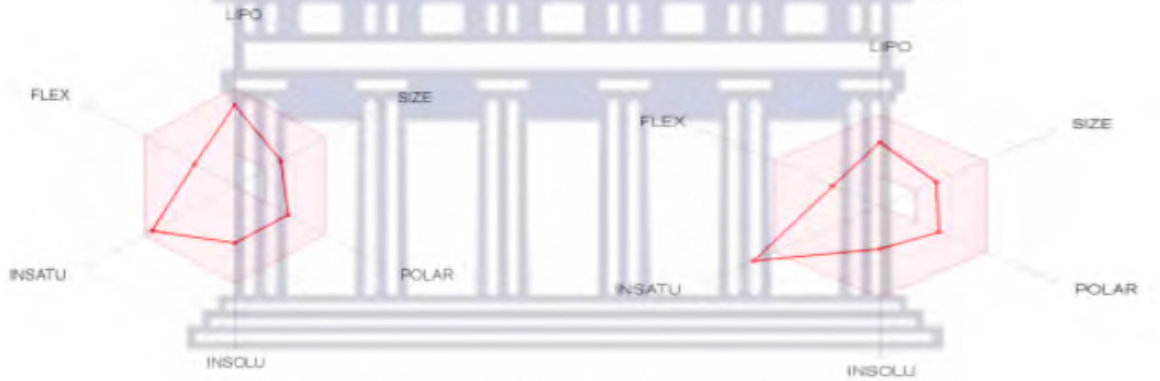
For drug-likeness assessment a bioavailability Radar was used (refer to Figure 25), to be considered as drug-like, a compound has to fall entirely within the pink area (Matlock *et al*, 2018). The bioavailability radar has parameters that have an optimal range in the pink area, those parameters include lipophilicity, size, polarity, solubility, flexibility, and saturation. Lipophilicity (LIPO; 0.7 XLOGP3 +5.0); size (150 MW 500 g/mol); polarity (POLAR; 20 TPSA 130 2); solubility (INSOLU; log S 6); saturation (INSATU; a fraction of sp3 hybridized carbons 0.25); and flexibility (FLEX; 9 rotatable bonds). The bioavailability radar of the six compounds demonstrated that all six compounds fell in the pink area, with exception of

compound **1**, **4** and **5** that have a high INSATU value, and therefore the six ligands fulfilled some properties of drug-likeness.



Compound 1

Compound 2



Compound 3

Compound 4

Compound 5

Compound 6

Figure 25; Bioavailability radars of the six test compounds.

Properties	Comp 1	Comp 2	Comp 3	Comp 4	Comp 5	Comp 6

Log Po/w (iLOGP)	2.63	0.00	2.51	0.00	1.79	0.00
Log Po/w (XLOGP3)	4.02	5.60	3.13	1.83	0.89	5.59
Log Po/w (WLOGP)	3.81	4.56	3.84	1.95	0.36	4.53
Log Po/w (MLOGP)	2.53	2.78	1.58	0.78	1.33	3.62
Log Po/w (SILICOS-IT)	4.42	3.80	4.01	1.45	-0.28	3.97
Consensus Log Po/w	3.48	3.35	3.02	1.20	0.82	3.54

Table 7; Lipophilicity of the tested compounds.

According to the five Lipinski's rules, one of the five factors that determine drug bioavailability is lipophilicity. Lipophillicity is the key property that contributes to the ADMET characteristics, and a compounds that show a log P or D between 1 and 3 seem to be best suited to obtaining the right physiochemical properties (Arnott and Planey, 2012. It is stated that an orally active drug should not violate more than one of the following criteria: 5 H-bond donors, 10 H-bond acceptors, 10 rotatable bonds, molecular mass <500 Da, a lipophilicity parameter (logP) < 5, and a polar molar surface area < 140 (Arnott and Planey, 2012; Lipinski *et al*., 1997; Chagas *et al*., 2018). Based on the predicted physicochemical results all the compounds did not violate the rules expect for compound **6** that had a molecular mass that is greater than 500Da. All six-test compound have predicted a logP values that are less than 5 and are predicted to be lipophilic with consensus values ranging from 0.82–3.54 (Table 6), which is the optimal range for lipophilicity.

Table 8; Water solubility of the test compounds.

Compounds	**LogS (ESOL)**	**LogS (Ali)**	**LogS (SILICOS-IT)**
1	-4.84 5.11e-03mg/ml;1.43mol/l Moderate soluble	-5.31 1.74e-03mg/ml;4,87e-06mol/l Moderate soluble	-7.28 1.88e-05mg/ml;4.87e-06mol/l Poorly soluble
2	-6.32 2.11e-04mg/ml;4.77e-07mol/l	-6.46 1.54e-04mg/ml;3.47e-07mol/l	-7.78 2.90e-05mg/ml;6.55e-08mol/l Poorly soluble

	Poorly soluble	Poorly soluble	
3	-3.51 7.47e-02mg/ml;3,66e-06mol/l Soluble	-4.11 1.88e-02mg/ml;7.68e-05mol/l Moderate soluble	-5.16 1.68e-03mg/ml;6.89e-06mol/l Moderate soluble
4	-6.32 2.11e-04mg/ml;4.77e07mol/l Poorly soluble	-6.46 1.54-04mg/ml;3.47e-07mol/l Poorly soluble	-7.18 2.90e-05mg/ml;6.55e-08mol/l Poorly soluble
5	-2.78 4.20e-01mg/ml;1.66e-03mol/l Soluble	-2.65 5.71e-01mg/ml;2.25e03mol/l Soluble	-3.04 1.17e+00mg/ml;4.84e03mol/l Soluble
6	-6.63 1.22e-04mg/ml;2,35e07mol/l Poorly soluble	-7.30 2.60e-05mg/ml;4.99e-08mol/l Poorly soluble	-7.70 1.04e-05mg/ml;2.00e-08mol/l Poorly soluble

Solubility is one of the most important properties that has an influence on absorption for oral administration. This property is being predicted and carried out in three methods with the output of LogS values in Swiss-ADME. The first one is the application of the ESOL model, and the second one is LogS (Ali) (Yalkowsky and Valvani, 1980). The third predictor was developed by SILICOS-IT, all predicted values are the decimal logarithm of the molar solubility in water (log S) (Lipinski *et al*, 1997; Ertl and Schuffenhauer, 2009). Estimation of the water solubility showed that compound **4** and **5** are soluble in all three predictions, compound **6** and **2** are poorly soluble, whereas compound **1** is moderately soluble to poorly, compound **3** is soluble to moderately soluble (table 7).

Table 9; drug-likeness of the six test compounds.

Compound	**Lipinski**	**Ghose**	**Veber**	**Egan**	**Muegge**	**Synthetic accessibility**
1	Yes	Yes	yes	Yes	Yes	2.93
2	Yes	No	yes	Yes	No	2.85

3	Yes	Yes	yes	Yes	Yes	2.67
4	Yes	Yes	yes	Yes	Yes	1.89
5	Yes	Yes	yes	Yes	Yes	2.49
6	Yes	No	yes	Yes	No	4.32

Table 9 shows the drug-likeness feature. Five of the test compounds complies with all the drug-likeness rules, and none of them violate more than one of them. The exception is compound **6**, which broke both Muegge and Ghose rules. The six compounds were evaluated on a scale of 1 to 10, where 1 is very easy to synthesis, and 10 is very difficult to synthesize. The synthetic accessibility of all of them is almost 3, and this means that these compounds are easy to synthesize. Compound **6** with a value of 4.32 makes it between easy to hard to syntheses.

4.4 Conclusion

Based on the aim of this research project, which is to discover potential HSP90 inhibitors for the benefit of treating or slowing the neurodegenerative process, the test compounds successfully inhibited the target protein based on the HSP90 inhibition assay. Also, the test compounds did not violate one or more of Lipinski rules, which are rules that are used to predict if a drug is suitable for oral administration.

CHAPTER 5

5. Summary and conclusion

5.1 Introduction

The continuous loss of function and structure of neurons is known as neurodegeneration, and it can lead to debilitating neurological diseases such as Alzheimer's disease (AD) and others (Srivastava and Bulte, 2014). AD is a neurodegenerative disorder characterised by a gain of harmful activity of misfolded proteins. It is a major and growing global health concern, affecting 40–50 million people worldwide (Prince *et al*., 2015). Although there is no cure for

Alzheimer's disease, cholinesterase inhibitors (ChEIs) and *N*-methyl-D-aspartate (NMDAR) antagonists are the most widely prescribed symptomatic therapy for mild to severe AD (Cummings, 2000).

HSPs as molecular chaperones play an important role in the quality control of proteins, and. As a molecular chaperon HSP90, has the ability to prevent protein aggregation, dissolve protein clumps, and targeting protein clients for degradation. Recently, it has been proved that HSP90 might become a potential therapeutic target for AD (Ou *et al*., 2014), this is because HSP90 plays an additional role in protecting neuronal proteins with aberrant aggregation tendency from accumulation and forming toxic aggregates. Therefore, HSP90 inhibition may offer therapeutic approach in AD by activation of HSF-1 and the subsequent induction of heat shock proteins, such as HSP70, HSP90 inhibitor may redirect neuronal aggregate formation and protect against protein toxicity (Zhao *et al*., 2012).
Thus, this study aims to discover HSP90 inhibitors, and these compounds may be beneficial in slowing down or halting the neurodegenerative process

5.2 Virtual screening

The top 10 hit compounds were identified using structure based virtual screening, where HSP90 was docked with the Maybridge Screening Collection using FRED. The top compounds where then analysed for desired interactions using VIDA and the Protein Plus Server. The selected 10 compounds were then drawn on the Sigma-Aldrich website to search for structures that show at least 60% similarity, selected one structure on Sigma Aldrich for each MayBrigde structure that showed the highest similarity percentage. Then the Sigma Aldrich compounds were prepared for docking, as they were docked into the active site of the HSP90 protein, and the results were analysed using VIDA and Protein Plus Server to for interactions with HSP90 active site. Sigma Aldrich compounds were successful purchased, because these compounds would be easily imported to South Africa compared to the Maybridge compounds, and the compounds were evaluated using HSP90 enzyme assay, and evaluated for pharmacokinetics and drug-likeness.

5.3 Fluorescence Polarization (FP) Enzymatic Assay

The compounds were evaluated for inhibitory activity against the HSP90 protein using the HSP90α Assay Kit. The summary of results of this enzymatic assay are shown on figure 26. Based on the results of this assay compounds **1**, **3**, **4** and **5** demonstrated the best inhibition.

Compound **5** had a consistent inhibition on HSP90 protein at two different concentration which are 1 and 10 µM, which is in-line with docking results of this compound. However, further dose response studies and more assay repeats is necessary to unequivocally confirm the above observations.

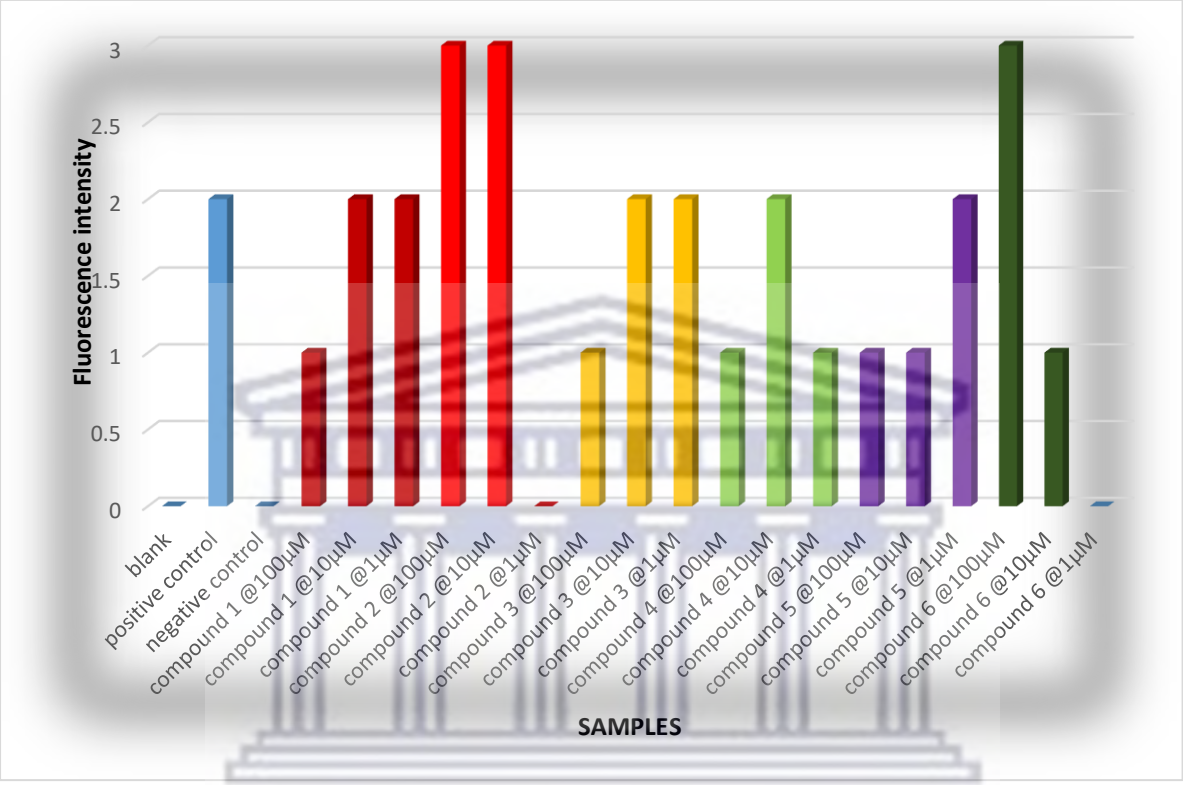


Figure 26: HSP90 inhibition for each test compound at three different concentrations, namely 100 µM, 10 µM and 1 µM.

5.4 Conclusion

AD is the main increasing global health challenge, and the prevalence and effects of AD are expected to rise in the future as the population ages. Therefore, HSP90 is one of the most promising targets for future development of medication regarding AD.

Six novel compounds were successfully purchased from Sigma Aldrich, and were further evaluated for their inhibitory activity against HSP90 enzyme, compound **1**, **3**, **4** and **5** seem to exhibit strong inhibition activity to HSP90 at three different concentrations. These results are in-line with the docking results, and this is because these compounds were all predicted to interact with the crucial amino acids of HSP90 protein. These compounds have Chemguass4 score ranging from -5 to -11. Compound **6** had the weakest Chemguass4 score of -5.43 the *in vitro* results confirmed the weak binding affinity of this compound.

Drug-like and pharmacokinetic studies was done using the Swiss-ADME online tool. All six compounds were predicted not to violate any of the pharmacokinetic and drug-like properties. These compounds therefore have potential to show a good therapeutic index for the treatment of neurodegenerative disease, or have the potential to relieve the symptoms of AD, slow down disease progression and ultimately reduce the burden of the disease.

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