THE EVALUATION OF NOVEL METAL-BASED COMPOUNDS AS POTENTIAL BACE1 LIGANDS

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biochemistry, School of Agricultural, Earth and Environmental Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the National Research Foundation (NRF) of South Africa.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Signed: Dr Raymond Hewer

Date: 12 February 2019

DECLARATION: PLAGIARISM

I, Jezelle Karrian, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

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ABSTRACT

The proposed primary cause of Alzheimer's disease (AD) is the cleavage of the Amyloid-β precursor protein by the β- Amyloid cleavage enzyme 1 (BACE1). As such it has been proposed that inhibiting this enzyme would help reduce the formation of Aβ plaques and slow down the progression of the disease. However, there is currently no treatment available that slows down the progression of AD and current drugs on the market only treat the symptoms of the disease. Metal-based compounds have been studied extensively for use as anticancer treatments. Success in the of these metal-based compounds have introduced new avenues in drug development and as such, there have been studies conducted on the introduction into metal-based compounds as potential AD drug candidates. In this study, 13 novel metal-based compounds were evaluated using five simple assessment techniques to determine potential BACE1 ligands. Molecular docking studies were able to predict that compound 12 was able to bind readily with BACE1 as it had a greater docking score (-4.630) which correlated with the thermal shift assay as the ΔT_m of 9.1 ºC was compared to the other compounds. Compounds 7 and 13 were found to be aggregating compounds when results of the chymotrypsin assay were assessed. In addition, the lack of inhibition in the presence and absence of detergent in the chymotrypsin assay was able to determine specificity of these metal-based compounds to the BACE1 protein. Furthermore, the DNA cleavage assay determined that copper-containing compounds 9, 10 and 11 were able to cause scissions in supercoiled plasmid DNA. Theoretical predictions of the physiochemical properties were evaluated to determine probable CNS/oral drug candidates according to Lipinski's rule of 5 and Veber's rules. All results obtained in this study predicted most favourable results with seven compounds producing an RO5 score of 4 thus making them potential BACE1 ligands with probable CNS/oral drug candidate properties and with fewer toxic effects. Furthermore, the chymotrypsin assay revealed that compound 13 was an aggregator and that compound 7 had binding affinities to both BACE1 and chymotrypsin. Overall assessment of these compounds has revealed that the compounds with the most favourable properties and an oral and CNS drug candidate as well as a good BACE1 ligand was compound 6. In addition, the overall positive outcomes of the molecular docking and TSA indicate that metal-based compounds have great potential in the drug design and discovery of new drug candidates for the treatment of AD.

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LIST OF ABBREVIATIONS

^{o}C	Degrees Celsius
µM	Micromolar
µl	Microliter
Aβ	Amyloid β-peptide
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase Inhibitors
AD	Alzheimer's Disease
AICD	APP intracellular domain
APOE	Apolipoprotein E
APP	Amyloid precursor protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT-DNA	Calf thymus DNA
CTF-α/β	α/β-Carboxyterminal fragments
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
EtBr	Ethidium Bromide
FDA	Food and Drug Administration (U.S.)
ΔG_u	Gibbs free energy
LDH	Lactate dehydrogenase
ml	Millilitre
mM	Millimolar
MMSE	Mini-mental scale exam
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMDA	N-methyl d-aspartate
PAINS	Pan-assay interference compounds
RO5	Lipinski's rule of 5
ROS	Reactive oxygen species
TSA	Thermal Shift Assay

PNS	Peripheral Nervous System
DMT	Disease Modifying Agents

CHAPTER ONE: LITERATURE REVIEW

1.1. Background

Alzheimer's Disease (AD) is a neurodegenerative disease which is linked to dementia and is clinically characterized by the progressive decline in cognitive function and impairment in activities associated with daily living (reviewed in Scarpini *et al*., 2003). In addition, degeneration in the brain caused by AD can also lead to behavioural disturbances such as aggression, depression and wandering (reviewed in Francis *et al*., 1999). Furthermore, this disease affects regions of the brain that are associated with higher mental functions, namely, the neocortex and the hippocampus regions (Gong *et al*, 2003). The impairment in cognition is generally thought to be caused by the build-up and aggregation of proteins in the brain which are often referred to as 'plaques'. These 'plaques' are rich in the amyloid β-peptide (Aβ) that are extracellular deposits derived from the amyloid precursor protein (APP) (reviewed in Small *et al*., 1997). In addition, the formation of intracellular neurofibrillary 'tangles' that contain abnormally phosphorylated forms of the protein tau have also been found to contribute to the impairment in cognition (reviewed in Small *et al*., 1997 and Francis *et al*., 1999).

There are a number of risk factors associated with AD including genetic predisposition, old age and life-style factors including diabetes mellitus type II, hypertension, smoking and obesity (Ewers *et al*., 2008; Selkoe and Hardy, 2016). It is estimated that <1% of patients have developed AD through heritable traits, while the majority of AD patients reported no history of the disease in their family (Bachurin *et al*., 2017). The genetic mutations involved are centred on the genes for presenilin 1 and 2 and the gene for APP which has been established to be related to the early onset of AD (reviewed in Selkoe and Hardy, 2016). People with the mutation in APP are at risk of developing AD while those that have mutations for presenilin 1 and 2 incur a 95% chance of early onset (before 60 – 65 years) of the disease (Goldman *et al*., 2011; Alzheimer's Association, 2017). Another genetic factor that may contribute to the late onset of AD is the APOE genotype (Ewers *et al*., 2008). There are three genes associated with the APOE genotype, namely, e2, e3 and e4 (Ewers *et al*., 2008). People with the e4 gene have three times the risk of developing early onset of AD as compared to those with e2 and e3 (Gaugler *et al*., 2017). However, AD associated with

the APOE genotype occurs very rarely in a population and only slightly increases the overall risk for developing AD (Ewers *et al*., 2008; Gaugler *et al*., 2017).

AD affects an estimated 47 million people worldwide (Khoury *et al*., 2018) that are at the age of 65 or older. The prevalence of this disease increases with the aging of the population with nearly a 50% increase in the population of people over the age of 85 (reviewed in Lahiri *et al*., 2002; Cummings *et al*., 2016; Khoury *et al*., 2018). This not only causes a strain on families in terms of care-giving but also contributes to the economic strain due to the increasing need for treatment and care-giving for institutionalised patients (reviewed in Francis *et al*., 1999 and Lahiri *et al*., 2002).

1.2. Therapeutic interventions for Alzheimer's Disease

AD is a multifactorial disease that has many drug targets (Figure 1.1), however, three hypotheses have been investigated extensively to explain AD development and progression namely the cholinergic, glutamatergic and amyloid hypotheses (Figure 1.1) (Danysz *et al*., 2000). Accordingly, drug development efforts have exploited these hypotheses to produce agents that could alter disease progression. The first drugs produced were based on the cholinergic hypothesis. The glutamatergic hypothesis was then interrogated after failures following the release of acetylcholinesterase inhibitors (AChEI). Current efforts to alter progression of AD are predominantly based on the amyloid hypothesis (reviewed in Terry and Buccafusco, 2003).

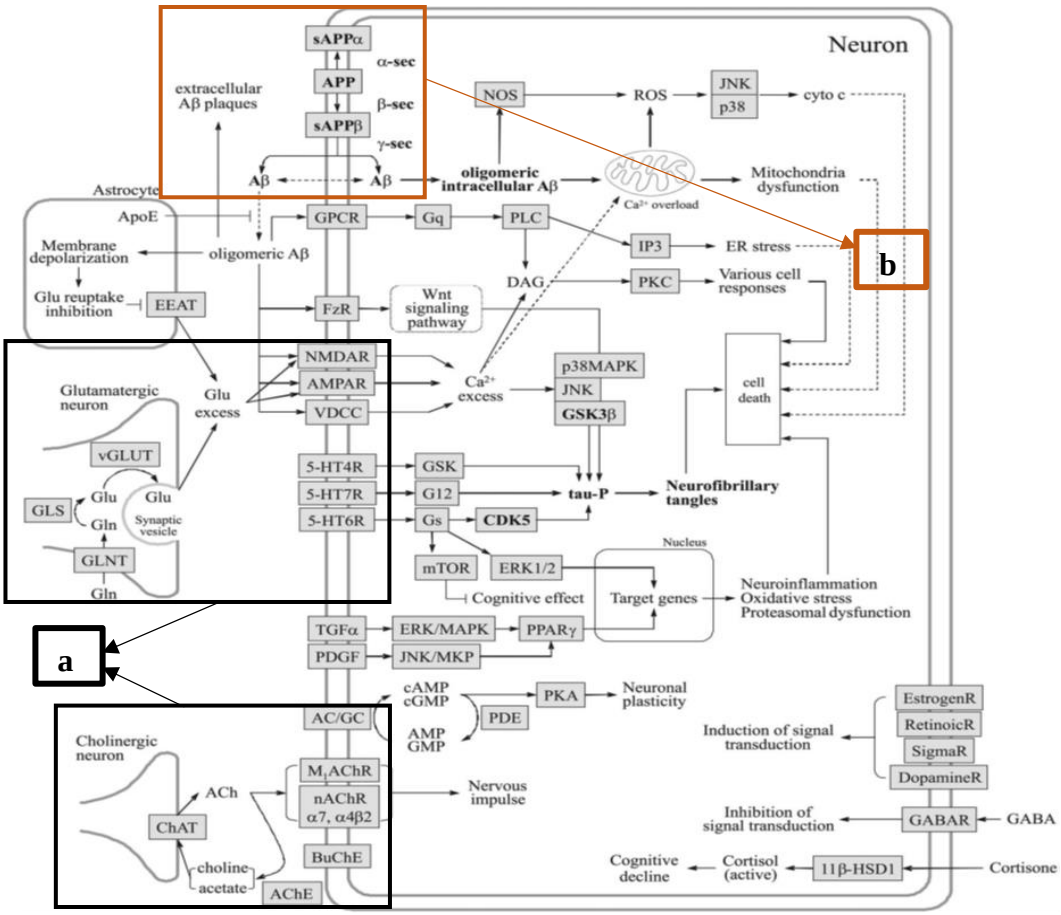


Figure 1.1: Schematic diagram of pathways associated with Alzheimer's disease and possible drug targets for therapy. (a) Alzheimer's disease is a multifactorial disease and because of this there are many targets for drug treatment. Of the pathways, the neurotransmission through the cholinergic system was looked at extensively as well as the NMDA-glutamate system which produced drugs that had positive effects on the symptoms of AD but proved to be ineffective in disease progression. (b) The Aβ pathway has produced many drugs that are still in trials but there are no new drugs available on the market. Bachurin *et al*, 2017.

1.2.1. The cholinergic hypothesis

The cholinergic hypothesis proposes that the decline in cognition in AD patients is due to the impairment of acetylcholine producing neurons in the brain (reviewed in Terry and Buccafusco, 2003). Several studies have demonstrated that the basal forebrain and the rostral forebrain cholinergic pathways, as well as the converging of the projections to the thalamus have important roles in consciousness, attention, memory and mnemonic processes (reviewed in Terry and Buccafusco, 2003). Therefore, damage or abnormalities in these pathways can lead to cognitive decline in aging or AD brains (reviewed in Terry and Buccafusco, 2003).

The formation of Aβ oligomers over the course of AD, as well as the neurotoxicity exhibited by these proteins can have a negative impact on the cholinergic synapse (reviewed in Ferreira-Vieira *et al*., 2016). The loss of these synapses plays an important role in the cognitive function in AD patients (reviewed in Ferreira-Vieira *et al*., 2016). The most affected cholinergic neurons are in the nucleus basalis of Meynert which decrease from approximately 500 000 in healthy adults to < 100 000 in AD affected patients (reviewed in Terry and Buccafusco, 2003 and Ferreira-Vieira *et al*., 2016). Furthermore, choline acetyltransferase (ChAT) transcription in the cholinergic neurons is greatly reduced, thus decreasing its activity which leads to the progression of dementia (reviewed in Terry and Buccafusco, 2003 and Ferreira-Vieira *et al*., 2016). These changes observed in the cholinergic pathway are closely related to impaired attention and memory seen in patients with AD (reviewed in Terry and Buccafusco, 2003; Ferreira-Vieira *et al*., 2016).

AChE in learning and memory and the relation of AChE in the cholinergic hypothesis are primary determinants in the development of treatments for AD (Ferreira-Vieira *et al*., 2016). Chemical agents that target aspects of the cholinergic hypothesis are directed towards increasing the activity of cholinergic neurons (reviewed in Francis *et al*., 1999 and Scarpini *et al*., 2003; Bachurin *et al*., 2017). These drugs include those that increase acetylcholine synthesis as well as cholinesterase inhibitors that reduce synaptic acetylcholine degradation (reviewed in Francis *et al*., 1999 and Scarpini *et al*., 2003; Bachurin *et al*., 2017).

1.2.1.1. Acetylcholinesterase inhibitors

AChE, an enzyme concentrated at the cholinergic synapses in the central nervous system, catalyses the hydrolysis of acetylcholine to choline and co-enzyme A (Downes and Granato, 2004). This recycling of choline makes AChE an important factor in the cholinergic system and for cognitive function (Hasselmo, 2006). In an AD brain the amount of acetylcholine is reduced and therefore, the use of acetylcholinesterase inhibitors (AChEI) would likely lead to increases in the levels of acetylcholine near the synapse (reviewed in Scarpini *et al*., 2003).

1.2.1.2. Types of acetylcholinesterase inhibitors

Development of agents for the treatment of impaired cholinergic system have produced four drugs that have been FDA-approved (reviewed in Lahiri *et al*., 2002). The drugs are tacrine, donepezil, rivastigmine and galantamine, which entered the market in 1993, 1996, 2000 and 2001 respectively (reviewed in Lahiri *et al*., 2002). Tacrine is a centrally active aminoacridine with reversible non-specific cholinesterase inhibitory activity with a half-life elimination of less than seven hours and recommended dosage of four times a day (reviewed in Mangialasche *et al*., 2010). Donepezil can act non-competitively and has a longer half-life as compared to other drugs in its therapeutic class with a recommended dosage of once daily. These properties have led to the use of donepezil as a preferable early treatment of mild-to-moderate cases of AD (reviewed in Scarpini *et al*., 2003). It is a selectively reversible drug that can be metabolised in the liver by the cytochrome P450 isoenzymes CYP2D6 and CYP3A4. Galantamine acts by binding allosterically to nicotinic acetylcholine receptors and actively increases acetylcholine being produced thus increasing neurotransmission in the brain (reviewed in Scarpini *et al*., 2003). As with donepezil this drug is metabolised by the cytochrome P450 isoenzymes CYP2D6 and CYP3A4 and the recommended dosage is twice daily (reviewed in Scarpini *et al*., 2003 and Mangialasche *et al*., 2010). Rivastigmine is a reversible acetylcholinesterase inhibitor that has low binding plasma-protein affinity (reviewed in Scarpini *et al*., 2003 and Mangialasche *et al*., 2010). In addition to inhibiting acetylcholinesterase this drug also inhibits butyrylcholinesterase and thus dual inhibition of both acetylcholinesterase and butyrylcholinesterase can effectively decrease acetylcholine metabolism (Ferreira-Vieira *et al*., 2016). Rivastigmine is hydrolysed by esterases but not cytochrome P450 and has a recommended dosage of twice daily due to a short half-life (reviewed in Scarpini *et al*., 2003).

1.2.1.3. Efficacy of acetylcholinesterase inhibitors

AChE inhibitors have been proven to improve cognition thus contributing to their efficacy (reviewed in Hansen *et al*., 2008). There are many studies that have been conducted to determine the efficacy of AChEI and these studies were directed using randomised, double-blind, parallel groups and improvement in cognition was monitored in comparison to placebo-controlled trials. Meta-analysis studies for mild to moderate cases of AD were conducted to illustrate the extent of

treatment for the improvement of cognition and it was reported that 18% to 48% of patients treated with AChEI drugs had improved cognition (Lanctot *et al*., 2003). These studies were conducted for different periods and based on various cognitive rating scales to determine their efficacy and tolerance, i.e. a drug that can be used for prolonged treatment (reviewed in Mangialasche *et al*., 2010). Some studies presented comparisons to each other in terms of cognition, behaviour and function to determine which drug is better suited for treatment (Mimica and Presecki, 2009; Winblad *et al*., 2001).

There are many clinical reports that have exposed tacrine to have adverse effects (reviewed in Hansen *et al*., 2008; Mimica and Presecki, 2009). In a meta-analysis of these clinical trials, tacrine caused elevated alanine aminotransferase above normal levels in 29% of patients (Mimica and Presecki, 2009). In addition, tacrine caused nausea and vomiting in 28%, diarrhoea in 14%, dyspepsia in 9% and myalgia in 7.5% patients (Mimica and Presecki, 2009; Winblad *et al*, 2001). With dosage requirements being more than once a day and adverse effects as well as the introduction of second-generation therapies has led to the discontinuation of tacrine (Mimica and Presecki, 2009).

There are approximately 1276 clinical trials (reviwed in Hansen *et al*., 2008) that have been reported on donepezil, galantamine and rivastigmine which indicated that these drugs have had significant improvement in cognition when compared to placebo controls (Wilcock *et al*., 2003; Jones *et al*., 2004). Meta-analysis of donepezil, galantamine and rivastigmine had a pooled weighted mean difference between active treatment and placebo of 2.67, 2.76 and -3.01 respectively (Wilcock *et al*., 2003; Jones *et al*., 2004). Two trials presented a comparison of donepezil to galantamine in head-to-head comparative open-label trials (Wilcock *et al*., 2003; Jones *et al*., 2004). One study was conducted over a 52-week period and the other was conducted over a 12-week period (Wilcock *et al*., 2003; Jones *et al*., 2004). Dosages of these drugs were 10 mg/day (donepezil) and 24 mg/day (galantamine) in the 52-week trial (Wilcock *et al*., 2003; Jones *et al*., 2004). The 12-week trial had varying dosages of donepezil (5 – 10 mg/day) and galantamine (2 – 24 mg/day). The 12-week trial presented significant differences in cognition for donepezil and galantamine (Alzheimer's disease assessment scale-cognitive subscale (ADAS-cog) 4.7 vs. 2.3 respectively) but there were no differences indicated for the 52-week trial (Wilcock *et al*., 2003; Jones *et al*., 2004).

Comparison between donepezil and rivastigmine was conducted in 12-week and 2-year double-blinded random trials (Bullock *et al*., 2005). The 12-week trial had no significant difference between donepezil and rivastigmine in terms of cognition (Bullock *et al*., 2005). Donepezil and rivastigmine had flexible dosages at 5 – 10 mg/day and 6 – 12 mg/day respectively (Bullock *et al*., 2005). This trial used the severe impairment battery (SIB) scale to measure changes in cognition. The 2-year trial used flexible dosages of 5 – 10 mg/day and 3 – 12 mg/day for donepezil and rivastigmine respectively (Bullock *et al*., 2005). This trial displayed similar changes in cognition between donepezil and rivastigmine (SIB mean changes 9.9 vs. 9.3 respectively) (Bullock *et al*., 2005).

Although these tests provide valuable information of the efficacy there is still no significant evidence for prolonged treatment (reviewed in Hansen *et al*., 2008). Most studies conducted on these drugs do not have longer exposure times and those studies that do display longer exposure of the drug have reported limitations with their methodology (Winblad *et al*., 2001). Other meta-analysis on the safety and tolerability of these drugs indicate that 76% of patients treated with donepezil, galantamine and rivastigmine had at least one side-affect (reviewed in Hansen *et al*., 2008). In these trials conducted, the most frequently reported adverse effects was nausea (13%), diarrhoea (11%) dizziness (10%) and weight loss (9%) (reviewed in Hansen *et al*., 2008). Donepezil exhibited the least cases of adverse effects (11% nausea, 7% vomiting, 8% dizziness and 7% weight loss) when compared to galantamine and rivastigmine (reviewed in Molino *et al*., 2013). Galantamine displayed 24% adverse effects for nausea, 14% for vomiting, 10% for dizziness and 10% for weight loss (Hansen *et al*., 2008). Rivastigmine had 44% for nausea, 30% for vomiting, 22% for dizziness and 11% for weight loss (reviewed in Hansen *et al*., 2008).

Comparisons between donepezil, galantamine and rivastigmine in some trials suggests that donepezil is more efficacious over other drugs (Hansen *et al*., 2008). Furthermore, donepezil demonstrated lower adverse effects as compared to the other drugs. Although donepezil, rivastigmine and galantamine have all demonstrated positive effects on cognitive function they however, fall short on slowing down the progression of the disease and therefore improved drug therapy is still required (reviewed in Scarpini *et al*., 2003 and Kavirajan and Schneider, 2007 and Mangialasche *et al*., 2010).

1.2.2. The glutamatergic hypothesis

The glutamatergic hypothesis proposes that the decline in cognition in AD patients is due to glutamate-mediated neurotoxicity in which the subtype glutamate receptor, N-methyl d-aspartate (NMDA) is over activated in a tonic way (reviewed in Maragos *et al*., 1987 and Danysz *et al*., 2000). The continued activity of the NDMA receptor causes chronic conditions in the brain that leads to neuronal damage and decrease in cognition (reviewed in Danysz *et al*., 2000). Development of drugs to improve cognition using the glutamatergic hypothesis has produced one major drug that is currently available on the market (reviewed in Danysz *et al*., 2000).

1.2.2.1. The N-methyl d-aspartate antagonist: Memantine

Glutamate is a major excitatory neurotransmitter in the central nervous system and it also provides plasticity (Danysz *et al*., 2000). There are different types of glutamate receptors such as the NMDA, (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) AMPA and kainite subtypes but the NMDA receptor is of greater interest in the treatment of AD (reviewed in Scarpini *et al*., 2003). The NMDA receptor has a complex structure with binding sides for NMDA and glutamate with a central ion channel (reviewed in Scarpini *et al*., 2003). The binding of NMDA and glutamate to the NMDA receptor leads to the activation of the receptor and therefore the activation of the ion channel which increases the flow of Ca^{2+} into the neurons in the central nervous system (Figure 1.1) (reviewed in Scarpini *et al*., 2003). This activation of the Ca^{2+} channel is an important process in learning and memory (reviewed in Scarpini *et al*., 2003). However, the increase in the amount of glutamate in AD patients leads to the over-activation of the NMDA receptor and ultimately to the accumulation of Ca^{2+} which can cause damage to neurons which leads to neuronal death (reviewed in Scarpini *et al*., 2003).

Memantine is a drug that has been used for the treatment of AD and an antagonist of the NDMA receptor (Danysz *et al*., 2000). This drug can bind non-competitively to the NMDA receptor thus protecting the neurons from damage without deactivating the NMDA receptor and ultimately improving cognitive function (reviewed in Scarpini *et al*., 2003 and Mangialasche *et al*., 2010).

1.2.2.2. Efficacy of memantine treatment

Studies conducted on the efficacy and safety of memantine were based on the randomized, double-blind methodology (reviewed in Scarpini *et al*., 2003; Mangialasche *et al*., 2010). The study was also conducted using placebo-controlled trials with which the efficacy of memantine was compared and the mini-mental scale exam (MMSE with moderate to severe ratings of 3-14) to determine the improvement in cognitive function (Winblad and Poritis, 1999; Reisberg *et al*., 2003). Patients with severe AD also had vascular dementia and a MMSE rating of <10 with a mean MMSE of 6.3 and some studies conducted on severe AD patients with MMSE of 3-14 were treated over a 28-week period (Winblad and Poritis, 1999; Reisberg *et al*., 2003). All tests indicated that memantine led to a significant increase in cognitive function when compared to placebo (Winblad and Poritis, 1999; Reisberg *et al*., 2003). Other studies conducted on the use of memantine revealed that this drug could be used for the treatment of AD, vascular and mixed dementia however, as with the AChE inhibitors this drug does not have any long-term benefits as well as limited therapy attributes as a monotherapy drug (Winblad and Poritis, 1999; Farlow *et al*., 2003)

1.2.3. The amyloid hypothesis

The amyloid hypothesis proposes that the toxic cause of neural or synaptic damage is due to the amyloid clusters in the brain of AD affected patients (Gong *et al*., 2003). Therefore, the development of therapeutic approaches to decrease or remove amyloid oligomers was proposed as a means to yield a positive impact for AD patients. There are many approaches that may achieve such a task however, inhibition of the β-Amyloid cleavage enzyme 1 (BACE1) enzyme has been predominantly explored for this objective.

1.2.3.1. BACE1 as a target for Alzheimer's disease

BACE1 is a 501-amino acid glycosylated type1 transmembrane endo-protease and is part of the aspartyl protease family (Ehlers, 2017). BACE1 is responsible for Aβ processing and is mandatory for forming Aβ in AD affected brains (Figure 1.2) (reviewed in Mirsafian *et al*., 2014). This enzyme is produced at low levels throughout the body and at higher levels in the pancreas (Ewers *et al*., 2008; reviewed in Mirsafian *et al*., 2014; Ehlers, 2017). It is also produced at moderate

levels in the brain however, the activity of the enzyme is higher in the brain than in the pancreas (Ewers *et al*., 2008; reviewed in Mirsafian *et al*., 2014; Ehlers, 2017). The lower levels in the pancreas is attributed to a spliced variant that is missing two-thirds of the exon 3 gene that produces an incomplete protein which is contained in the endoplasmic reticulum (ER) (Ewers *et al*., 2008; Ehlers, 2017).

In the brain, the production of BACE1 is expressed in the neurons predominantly in the presynaptic terminals of the cerebellum, cortex and hippocampus regions (Godyn *et al*., 2016; Ehlers, 2017). The occurrence of BACE1 in AD patients is revealed to be concentrated in the dystrophic presynaptic terminals which are found around the amyloid plaques associated with the disease (Huse *et al*., 2000). BACE1 is synthesized as a zymogen containing a shot pro-domain in the ER (Huse *et al*., 2000). There are many post-translational modifications that occur to form the mature form of BACE1 (Huse *et al*., 2000). Within the ER, BACE1 is glycosylated on the four Asn residues (Huse *et al*., 2000). BACE1 is also subjected to transient acetylation on the seven Arg residues (Huse *et al*., 2000). Furthermore, phosphorylation of BACE1 on the Ser^{498} in addition to the c-terminal dileucine motif, regulate BACE1 recycling between the cell surface and the endosomal compartments (Huse *et al*., 2000). In addition, BACE1 is also s-palmitoylated on four cys residues that is located at the junction of the transmembrane and cytosolic domains. This modification facilitates the partitioning of BACE1 into lipid rafts. The maturation of BACE1 in addition to the low pH levels present in the late Trans-Golgi Network (TGN) as well as intracellular trafficking contributes to increased activity and APP processing (reviewed in Vassar *et al*., 2009).

The amyloidogenic pathway has been frequently investigated for its function in the amyloid plaques that leads to the pathogenicity of AD (reviewed in Ewers *et al*., 2008). Furthermore, aggregation of Aβ peptides in the brain can occur years before the symptoms of the disease is demonstrated. The APP protein which is a type 1 integral membrane protein is cleaved at Asp+1 and Glu +11 (Vassar *et al*., 2009) by BACE1 which releases a soluble form of APP (APP-β) fragment (Figure 1.2). Figure 1.2 depicts BACE1 cleavage of APP at the β-site and the c-terminal fragment in the intermembrane that is proteolyzed by γ-secretase and results in the formation of the amyloidogenic Aβ peptides (Aβ1-40 and Aβ1-42) (reviewed in Bachurin *et al*., 2017). The resulting amyloidogenic Aβ peptides are packaged together to form plaques which accumulate in the extracellular space and causes neurodegeneration (reviewed in Bachurin *et al*., 2017). The

difference in a normal brain and an AD affected brain is the use of the α-secretase and β-secretase respectively to cleave APP which leads to either the amyloidogenic pathway or the non-toxic pathway (reviewed in Bachurin *et al*., 2017).

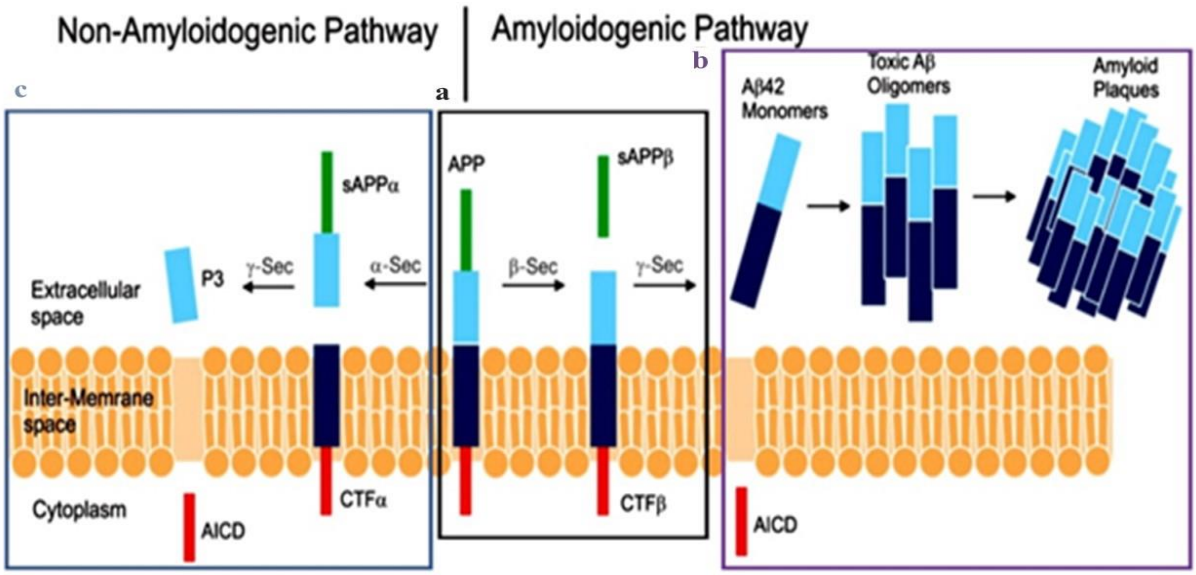


Figure 1.2: A schematic diagram of the amyloid pathway that leads to the formation of amyloid plaques and the pathogenesis of Alzheimer's disease. (a) The pathogenic amyloidogenic pathway is initiated when APP is cleaved by β-secretase thus releasing sAPP-β and then the CTF-β fragment is then cleaved by the γ-secretase which produces AICD and Aβ40-42 monomers. (b) The Aβ40-42 amino acid monomers produced combine to form a toxic Aβ oligomers that eventually lead to packaged toxic amyloid plaques. (c) The non-amyloidogenic pathway consists of the α-secretase that cleaves APP thus releasing sAPPα into the intracellular space. The CTF-α fragment is then cleaved by γ-secretase and the AICD and p3 fragments are then released into the intercellular space. Bachurin *et al*., 2017.

Aβ production in the unaffected brain occurs in the inter-membrane space of the soma and APP is cleaved by the α-secretase to produce sAPP-α that is released into the extracellular space (Alzheimer's Association, 2017; Bachurin *et al*., 2017). γ-Secretase then cleaves the CTF-α thus releasing AICD and p3 fragments into the extracellular space and cytoplasm (Alzheimer's Association, 2017; Bachurin *et al*., 2017). The released fragments and AICD are non-toxic and do not form plaques (Alzheimer's Association, 2017; Bachurin *et al*., 2017). Accumulation of Aβ plaques then leads to synaptic dysfunction and neuronal loss as neurotransmission to signal ion exchange channels in the soma of the brain which are responsible for the function of the neuron is destroyed (Ittner and Jurgen, 2011). This is caused through the release of reactive oxidative species

(ROS) or the binding of Aβ plaques directly to receptors on the neuronal membrane which can alter protein conformations thus leading to abnormal function (reviewed in Huang and Jiang, 2009). The release of ROS and interference with membrane proteins has also displayed mediation in the hyperphosphorylation of Tau in microtubules found in the axon of the neuron (Busciglio *et al*., 1995; reviewed in Huang and Jiang, 2009; Ittner and Jurgen, 2011). When Tau becomes phosphorylated it breaks away from the microtubules and the microtubules loses its integrity and become disassociated (Busciglio *et al*., 1995). This ultimately leads to neuronal tangles and death of neurons in the brain (Bachurin *et al*., 2017).

Therefore, inhibition of the β-secretase enzyme could counteract the pathogenic amyloidogenic pathway and thus could prevent neuronal death (Roberts *et al*., 2001). Several studies have been conducted ensure that BACE1 is the core enzyme responsible for the amyloidogenic pathway of AD (Roberts *et al*., 2001). In one study conducted to reveal the beneficial effects of BACE1 as an AD target, two independent BACE1 gene knockout mice were created (Roberts *et al*., 2001). One gene knockout had a part of exon 1 missing and the other was a deletion of the exons 4-8 which would stop the expression of active BACE1 in these mice (Roberts *et al*., 2001). The production of Aβ was measured in primary cortical cultures which indicated that BACE1 expression was terminated due to the lack of the Aβ peptide in cultures which coincided with reports presented with other BACE1 knockout models (Roberts *et al*., 2001).

1.2.3.2. Possible implications when using BACE1 as a target for Alzheimer's disease

With sufficient evidence which demonstrates that BACE1 inhibitors could be useful in the treatment of AD, many companies have invested in possible drug candidates capable of inhibition (reviewed in Barao *et al*., 2016). However, some reviewers of BACE1 inhibitors have claimed that using these compounds can be detrimental due to the physiological functions that BACE1 has in the brain (reviewed in Barao *et al*., 2016). BACE1 has multiple substrates associated with its activity in the brain which is proposed to be needed for normal brain function (Willem *et al*., 2006; reviewed in Mirsafian *et al*., 2014). There are several BACE1 substrates that have been identified such as L1, a close homolog of L1 (CHL1), contactin-2 and seizure protein-6 (SEZ6) (Willem *et al*., 2006; reviewed in Mirsafian *et al*., 2014). These substrates have been found to be involved in

synapse formation and neurite growth as well as in axon guidance (Willem *et al*., 2006; reviewed in Mirsafian *et al*., 2014). BACE1 is suggested to function in the regulation of myelination in the axon of peripheral nervous systems (PNS) via the neuregulin-1 by proteolytic pathways (Willem *et al*., 2006; reviewed in Mirsafian *et al*., 2014). It has also been suggested that the loss of BACE1 could inhibit proper function and promote hypomyelination in the axon which can lead to segregation of small-diameter axons by Schwann cell processes which ultimately leads to pain sensitivity and impaired motor and sensory functions (Willem *et al*., 2006; reviewed in Mirsafian *et al*., 2014 and Barao *et al*., 2016).

To determine whether the deletion of BACE1 would cause mechanism-toxicity, Savonenko and co-workers developed *BACE1* $^{-/-}$ mice that displayed sensory motor-gating deficiency, behavioural signs of glutamatergic hypofunction and other typical endophenotypes of schizophrenia (Savonenko *et al*., 2008). It was also revealed that the presynaptic density protein 95 (PSDS95)-associated ErbB and spine densities were also reduced (Savonenko *et al*., 2008; reviewed in Vassar *et al*., 2009). In addition, Wong and Co-workers also indicated that in BACE1-null mice there was the absence of mossy fiber long-term potentiation (LTP) thus further revealing the important role BACE1 plays in normal brain function (Wang *et al*., 2008). These BACE1 deficient mice models show that strong inhibition of this enzyme can cause mechanism-based side effects and that further investigators should be aware of these problems (reviewed in Vassar *et al*., 2009). However, investigators have tested whether partial inhibition of the enzyme would be able to reduce the formation of Aβ while keeping mechanism-based side effects to a minimum (reviewed in Vassar *et al*., 2009). Laird and co-workers indicated that *APPswe;PSDIDE9;BACE1* $^{+/-}$ mice displayed significant reduction in Aβ production as compared to *APPswe;PSIDE9,BACE1* $^{+/+}$ mice (Laird *et al*., 2005). In addition, McConlogue and collegues reported that *PDAPP;BACE1* $^{+/-}$ mice also had significantly reduced Aβ production (McConlogue *et al*., 2007). These studies indicated that partial if not gradual reduction of BACE1 in AD brains and titration of potential BACE1 drug candidates thus allowing low level inhibition of the enzyme would reduce levels of Aβ without causing side affects associated with complete inhibition of the enzyme (reviewed in Vassar *et al*., 2009).

1.2.3.3. Known BACE1 inhibitors

BACE1 inhibitors are wide spread in clinical trials and of these drugs there are four agents that have been reported that have reached phase III trials (Table 1.1) (Cummings *et al*., 2018). Overall, there are 6 compounds that have been reported to inhibit BACE1 (Table 1.1) which includes compounds currently in phase II/III clinical trials as well as those no longer in trials for the treatment of AD (Cummings *et al*., 2018). Compounds currently in phase III clinical trials include CNP520 and E2609 (Elenbecestat) (Table 1.1) (Cummings *et al*., 2018). CNP520 (Table 1.1) produced by Novartis Pharmaceuticals, was found to be highly specific to BACE1 and does not inhibit BACE2, thus leading to reduced levels of Aβ and no side affects associated with inhibition of both BACE1 and 2 (Neumann *et al*., 2018). In a randomized, double-blind, placebo-controlled study conducted with CNP520 assessing the safety and tolerability of single and mutliple doses demonstrated that this drug candidate was well tolerated and had dose-dependent reduction in Aβ 1-40 concentratios in CSF by >90% (Neumann *et al*., 2018). E2609 (Table 1.1) which was produced by Eisai Biogen Idec for the treatment in prodromal to mild AD (Yan and Vassar, 2014). This compound is a robust small molecule drug that is orally administered, and studies have revealed that this drug can reduce concentrations of Aβ at dose-dependent dosages in the CSF and plasma (Cebers *et al*., 2016). Furthermore, compounds CNP520 and E2609 are also currently in phase II trials assessing safety and tolerability in prodominal and mild-to-moderate cases of AD in a dose-finding study (Table 1.1) (Cummings *et al*., 2018).

Other BACE1 inhibitors that were in phase III clinical trials but have since been terminated include MK-8931 (Verubecestat), JNJ-54861911, AZD3293 (LY3314814) and LY3202626 (Table 1.1) (Cummings *et al*., 2018). MK-8931 produced by Merck & Co. (Yan and Vassar, 2014; Tumer *et al*., 2015; Li *et al*., 2012; Cheron and Shakhnovich, 2017; Cebers *et al*., 2016) was undergoing assessment on the improvement in cognition as well as its safety and tolerability in prodominal and mild-to-moderate AD and was terminated due to lack of efficacy shown in these trials (Cummings *et al*., 2018). Furthermore, AZD3293 produced by AstraZeneca and Eli Lilly (Yan and Vassar, 2014; Tumer *et al*., 2015; Li *et al*., 2012; Cheron and Shakhnovich, 2017; Cebers *et al*., 2016) is a robust compound that can cross the blood brain barrier. This compound was able to inhibit both BACE1 and BACE2 (Cebers *et al*., 2016) however, they were also terminated due to lack of efficacy in clinical trials for the treatment of mild AD (Cummings *et al*., 2018). The Johnson & Johnson Janssen produced compound JNJ-54861911 (Yan and Vassar, 2014; Tumer *et*

al., 2015; Li *et al*., 2012; Cheron and Shakhnovich, 2017; Cebers *et al*., 2016) demonstrated liver toxicity issues and therefore was unable to be continued in trials for the treatment of late-onset AD (Cummings *et al*., 2018). LY3202626 produced by Eli Lilly & Co. is able to reduce the plasma and CSF biomarkers, Aβ40 and Aβ42 however, this compound was terminated in phase III clinical trials due to the lack of clinical efficacy (Parasrampuria *et al*., 2018).

Table 1.1: Summary of available drugs on the market and drug candidates that are currently in phase III clinical trials for Alzheimer's Disease therapy

Name	Company	Target Type	Function	References
Market Drugs				
Tacrine	Pfizer, Shionogi Pharma	AChEI	Reversible non-specific cholinesterase inhibitory activity	[1][2][3]
Donepezil	Eisai Co., Ltd., Pfizer		Inhibits acetylcholinesterase activity	[4][5][1][7][8][2][3]
Galantamine	Janssen, Ortho-McNeil Pharmaceutical, Sanochemia Pharmazeutika, Shire, Takeda Pharmaceutical Company		Selective against acetylcholinesterase with reversible effects	[9][10][1][7][8][2][3]
Rivastigmine	Novartis Pharmaceuticals Corporation		Inhibits both acetylcholinesterase and butyrylcholinesterase activity	[1][7][8][2][3]
Memantine	Eli Lilly	NMDA antagonist	Binds to the NMDA receptor	[11][1][2]
Currently in Phase III Clinical Trials				
CNP520	Novartis Pharmaceuticals	BACE1 inhibitor	High specificity to BACE1	[12][20][21]
E2609 (Elenbecestat)	Eisai Biogen Idec		Treatment of prodominal to mild AD	[12][20][21]

Aducanumab	Biogen Co., Neuroimmune			[12][19]
Solanezumab	Ely Lily & Co.		Acts on aggregating forms of the Aβ protein	[12]
Crenezumab	AC Immune and Genentech			
Gantenerumab	Hoffmann-La Roche			[12][23]
ALZT-OP1a and ALZT-OP1b	AZ Therapeutics	Anti-Aβ agents	Small molecule inhibitor of Aβ aggregation	[12]
GV-971 (Sodium Oligo-Mannurate)	Shanghai Green Valley		Inhibits aggregation of Aβ	
CAD106	Novartis Pharmaceuticals		Stimulates Aβ antibody responses	[12]
TRx-0237 (LMTX)	Janssen, Pfizer, TauRx Therapeutics	Anti-Tau agents	Inhibits Tau aggregation and other enzymes	[12]
TTP488	Janssen, Pfizer, TauRx Therapeutics		inhibits Tau aggregation	
AVP-786	Avanir	NMDA receptor agonists	Treatment of sleeping disorders	[12][26]
AXS-05	Axsome Therapeutics			[12][26}
Escitalopram	NIA, JHSPH Centre for clinical trials	Serotonin agents	Inhibits reuptake of serotonin norepinephrine	[12][13][16] [17]
ITI-007	Intra-Cellular Therapies, Inc.	5-HT2A antagonist	Dopamine receptor modulator	[12][13][16] [17]
Methylphenidate	Actavis Pharma Company	Dopamine reuptake inhibitor	Improve apathy	[12][13][16] [17]
MK-4305 (Suvorexant)	Merck Sharp & Dohme Ltd.	Dual orexin receptor antagonist	Improve sleeping disorders	[12][13][16] [17]
Nabilone	Sunnybrook Health Sciences Centre	Cannabinoid receptor agent	improve neuropsychiatric symptoms	[12][19][25]

Octohydroaminoacridine succinate	Shanghai Mental Health Centre, Changchun-Huayang High-tech Co. and Jiangsu Sheneryang High-tech Co.	AChEI	Cognitive enhancer	[18][24]
Zolpidem	Brasilia University Hospital	positive allosteric modulator of GABA-A receptors	Improve sleeping disorders	[12][15]
Humulin RU-100	NIH	Food supplement	Food supplement that is used to improve lipid composition and homeostasis	[12]
Terminated Phase III BACE1 Inhibitors				
MK-8931 (Verubecestat)	Merck & Co.		Mild-to-moderate AD	[1][6][12]
JNJ-54861911	Johnson & Johnson Janssen		Late-onset AD	[12][13][14]
AZD3293 (LY3314814)	AstraZeneca and Eli Lilly	BACE1 inhibitor	Inhibits both BACE1 and BACE2	[12][20][21]
LY3202626	Eli Lilly & Co.		Reduces plasma and CSF biomarkers	[12][22]

1 – Scarpini *et al*., 2003 **2 –** reviewed in Mangialasche *et al*., 2010 **3 -** Molino *et al*., 2013 **4 -** Winblad *et al*., 2001 **5 -** Feldman *et al*., 2004 **6 -** Scarpini *et al*., 2003 **7 -** Kavirajan and Schneider, 2007 **8 -** Hansen *et al*., 2008 **9 –** reviewed in Lahiri *et al*., 2002 **10 -** Wilkonson *et al*., 2002 **11 -** Thomas and Grossberg, 2009 **12 -** Bachurin *et al*., 2017 **13 -** Yan and Vassar, 2014 **14 -** Cheron and Shakhnovich, 2017 **15 -** Cebers *et al*., 2016 **16 -** Tumer *et al*., 2015 **17 -** Li *et al*., 2012 **18 –** reviewed in Lahiri *et al*., 2014 **19 -** Sevigny *et al*., 2016 **20 -** Adolfsson *et al*., 2012 **21 -** Blaettler, 2016 **22 -** Ostrowitzki *et al*., 2012 **23 -** Donohue *et al*., 2014 **24 -** Black *et al*., 2010 **25 -** Hori *et al*., 2015 **26 -** Ehrnhoefer *et al*., 2008.

1.3. Therapies in phase III clinical trials for the treatment of Alzheimer's disease

There are currently 112 drug candidates that are currently in clinical trials for the development of treatments for AD (Cummings *et al*., 2018). Of these candidates, 26 are in 35 phase III trials, 63 are in 75 phase II trials and 23 are in 25 phase I trials (Cummings *et al*., 2018). Of these compounds

63% are disease modifying agents (DMTs), 22% are symptomatic cognitive enhancers, 12% are symptomatic agents addressing neuropsychiatric and behavioural changes and 3% have undisclosed mechanisms of action (MOA) (Cummings *et al*., 2018). Of the phase III drug candidates, 17 are DMTs, one that is a cognitive-enhancing compound and one that is associated with behavioural symptoms (Cummings *et al*., 2018).

1.3.1. Anti-Aβ Agents and Tau aggregation

Drug candidates that are in their phase III clinical trials for the activity against Aβ protein include Aducanumab, ALZT-OP1a and ALZT-OP1b, Crenezumab, Gantenerumab, GV-971 (Sodium Oligo-Mannurate) and Solanezumab. Of these drug candidates Aducanumab, Crenezumab, Gantenerumab and Solanezumab are anti-Aβ agents that act on aggregating forms of the Aβ protein (Bachurin *et al*., 2017; Cummings *et al*., 2018). ALZT-OP1a and ALZT-OP1b are mast cell stabilizer and anti-inflammatory compounds respectively, that helps to reduce neuronal damage (Cummings *et al*., 2018). CAD106 is an amyloid vaccine that stimulates Aβ antibody responses whilst avoiding T-cell autoimmune responses (Farlow *et al*., 2015). This compound demonstrated favourable outcomes in first-in-human trials (Farlow *et al*., 2015). Due to this, this drug candidate can be used in long-term treatments against Aβ pathology (Farlow *et al*., 2015). The GV-971 drug candidate that inhibits aggregation of the Aβ plaques as well as an inhibitor of neuroinflammation (Cummings *et al*., 2018). TRx-0237 (LMTX) and TTP488 are anti-tau compounds that inhibits Tau aggregation which is related to Aβ aggregation pathogenicity (Bachurin *et al*., 2017; Cummings *et al*., 2018). TRx-0237 also displays other inhibitory effects for other enzymes such as monoamine oxidase as well as inhibitory of nitric oxide production (Bachurin *et al*., 2017).

1.3.2. Antioxidants and inflammatory drugs

Antioxidant drugs that are in clinical trials have multitarget mechanisms that can be beneficial to AD treatment because the CNS degeneration can be caused by oxidative stress which means there is an imbalance of oxidants and antioxidants. The application of Epigallocatechin gallate (EGCG)

which is a multitarget herbal and has strong antioxidant activity. This compound was assessed for its function in early AD to increase cognitive impairment. Another drug that falls into this category is SK-PC-B70M which comes from Korean herbal medicine and is composed of oleanolic-glycoside saponin-enriched fraction (Bachurin *et al*., 2017).

Most potential drug candidates being produced are focused on the causative effects of AD however, some drugs target other factors of the nervous system that may not link directly to the disease but still have positive effects on cognition (Cummings *et al*., 2018). ALZT-OP1b (ibuprofen) is a non-steroidal, anti-inflammatory compound that helps slow down inflammatory in AD affected patients (Cummings *et al*., 2018). This compound used in combination with ALZT-OP1a helps reduce the accumulation of Aβ in AD patients. TTP488 is a RAGE antagonist with anti-inflammatory properties that helps reduce inflammation in glial cells (Cummings *et al*., 2018).

1.3.3. Neurotransmitter, neuroprotective and other phase III drug candidates

There are currently 9 neurotransmitters and one neuroprotective compounds in phase III clinical trials (Cummings *et al*., 2018). The neurotransmitter drug candidates include AVP-786, AXS-05, Escitalopram, ITI-007, Methylphenidate, MK-4305 (Suvorexant), Nabilone, Octohydroaminoacridine succinate and Zolpidem. AVP-786, AXS-05, Escitalopram and ITI-007 is currently being investigated for the improvement of neuropsychiatric symptoms such as agitation and sleep disorders (Cummings *et al*., 2018). AVP-786 and AXS-05 drug candidates are produced by Avanir and Axsome Therapeutics, respectively. These compounds are sigma 1 receptor agonist and a NMDA receptor agonist. In addition, AXS-05 is also involved in the inhibition of the reuptake of serotonin norepinephrine. Escitalopram which is produced by NIA, JHSPH Centre for clinical trials, inhibits serotonin reuptake. ITI-007 is a 5-HT2A antagonist as well as a dopamine receptor modulator and is produced by Intra-Cellular Therapies, Inc. (Cummings *et al*., 2018).

In addition, drug candidates MK-4305, Nabilone, Zolpidem, Methylphenidate and Octohydroaminoacridine succinate were investigated for the improvement in neuropsychiatric symptoms such as sleep disorders and apathy (Cummings *et al*., 2018). Methylphenidate is dopamine reuptake inhibitor that is used to improve apathy in AD patients. The MK-4305

neurotransmitter is a dual orexin receptor antagonist that help improve sleep disorders associated with AD (Cummings *et al*., 2018). Nabilone is a Cannabinoid receptor agent and is produced by Sunnybrook Health Sciences Centre. Zolpidem produced by Brasilia University Hospital and is positive allosteric modulator of GABA-A receptors and in being investigated for the improvement sleep orders in AD patients (Cummings *et al*., 2018). Octohydroaminoacridine succinate is an AChEI that is used as a cognitive enhancer and is produced from three different sources, namely, Shanghai Mental Health Centre, Changchun-Huayang High-tech Co. and Jiangsu Sheneryang High-tech Co. In addition, the omega-3 fatty acid eicosapentaenoic acid (EPA), icosapent ethyl (IPE) protects neurons from pathology associated with AD (Cummings *et al*., 2018).

Other phase III drugs comprise of food supplements that acts on passive processes that have positive effects on cognition in AD patients. Some drugs that have been used for the treatment of insulin homeostasis that are in phase III clinical trials for AD such as Humulin RU-100 which is a short acting recombinant insulin. This drug candidate replaces insulin in the brain thus increasing cell signalling and neurogenesis.

1.4. Discovering new drug compounds

The process for the development of new drug therapies is long, time consuming and filled with obstacles since trying to find the right ligand with specific inhibitory functions, that has no adverse effects can prove to be difficult and can lead to many failed attempts (Cheron and Shakhnovich, 2017). The first step in the early stages of drugs discovery is target identification and validation and this involves the establishment of a valid target which can be proteins, genes or RNA (ribonucleic acid) segments which needs to be 'druggable' (Hughes *et al.*, 2011). The second step includes identifying small molecules that have drug-like properties which need to be evaluated for their ability to produce the desired activity which would be conducted in a compound screen (Hughes *et al*., 2011). Thirdly, these compounds would then be used in preclinical trials and following success of these trials can then proceed to clinical development and ultimately reach the market (Bai, 2014).

Compound libraries that are composed for the discovery of new therapies comprises of small molecules that comply with the Lipinski Rule of 5 (Ro5) (Hughes *et al*., 2011). Compounds

evaluated for their drug-like properties need a balance of solubility and polar or hydrophobic properties that aid in enabling these compounds to be absorbed, permeate membranes and pass other biological barriers (Hopkins and Paolini, 2007). To eliminate compounds that may present problems later in the drug discovery process the Ro5 was developed as a guide to help limit the chemical space in which compounds are designed in (Hopkins and Paolini, 2007). Lipinski and Co-workers demonstrated that compounds within these limits are more likely to be orally absorbed (Lipinski *et al*., 2001). The Ro5 states that molecules should have molecular weights less than 500 Da and with a clogP value, which is the measure of lipophilicity that affects the absorption of the compound into the body, of less than 5 (Hughes *et al*., 2011). In addition, these molecules should also have less than 5 H-bond donors and less than 10 H-bond acceptors (Hughes *et al*., 2011).

1.4.1. Thermal Shift Assay

There are many techniques available that have been used in drug development however, many of these methods require specialized equipment and expertise such as Nuclear Magnetic Resonance (NMR) (Groftehauge *et al*., 2015). A simple method that has been frequently used in drug development recently is the Thermal Shift Assay (TSA) which determines protein stability in response to a temperature gradient (Niesen *et al*., 2007; Groftehauge *et al*., 2015). The stability of a protein is related to the Gibbs free energy (ΔG_u) of unfolding and is temperature dependent. For most proteins ΔG_u decreases as the protein becomes more unstable in increasing temperatures (Niesen *et al*., 2007). The point in which ΔG_u becomes zero is known as the melting temperature, T_m, where the concentration of unfolded and folded protein is equal (Niesen *et al*., 2007; Groftehauge *et al*., 2015). The TSA uses a fluorescent dye that binds to the hydrophobic amino acids of the protein (Niesen *et al*., 2007; Groftehauge *et al*., 2015). As the protein unfolds in increasing temperatures the hydrophobic regions are exposed which allow for the binding of the dye (Niesen *et al*., 2007). When the dye binds it releases a fluorescent signal which is proportional to the unfolding of the protein and thus proportional to protein stability (Groftehauge *et al*., 2015).

There are many factors that contribute to protein stability such as buffers, salts, detergents that interact non-specifically with proteins and ligands which bind to proteins at specific sites (Niesen *et al*., 2007). In addition to stabilizing proteins, protein-ligand interactions are of great interest

when discovering drug candidates as the stability of the protein in the presence of ligand is representative of the ligands binding affinity (Groftehauge *et al*., 2015). When ligands are bound to proteins the ΔG_u increases in increasing temperature which causes an increase in the T_m. The effect of ligands on the stability of proteins is proportional to its concentration and to the ligands binding affinity to the targeted protein (Niesen *et al*., 2007).

In addition to using the TSA for detecting protein-ligand interactions there are many other applications for this assay such as optimizing conditions for crystallization (Groftehauge *et al*., 2015). This assay is suitable for most soluble proteins in most conditions however, some protein samples do not give clear signal owing to denaturation of fibrils, high background caused by the dye binding to the native state of a protein or by the protein lacking sufficient hydrophobic regions (Niesen *et al*., 2007; Lavinder *et al*., 2009; Groftehauge *et al*., 2015). Therefore, to ensure that the TSA is amiable to the targeted protein there needs to be certain criteria that proteins follow in which having sufficient hydrophobic regions is imperative (Lavinder *et al*., 2009). There are other methods that can be used to determine the stability of the protein in the presence of ligands such as, the Differential Scanning Calorimetry (DSC) and Differential Scanning Light Scattering (DSLS) (Groftehauge *et al*., 2015).

1.4.2. Cytotoxicity

Compounds in screening libraries are required to be evaluated for toxicity before they can be considered as lead candidates. Cytotoxicity assays are great tools for the evaluation of compound toxicity (Fotakis and Timbrell, 2006). The use of cell-based assays for the detection of cytotoxicity has increased over the years especially in the case of high-throughput and secondary chemical screening in pharmaceutical companies (Weyermann *et al*., 2005). Most assays used in the screening of compounds are based on either cell death or cell viability in which cell viability is the measure of cells that are still alive and cell death is the measure of the effects of either necrosis, apoptosis or autophagy (Mery *et al*., 2017).

There are various cell-based assays that have been developed to detect cytotoxicity. Determining the correct system for detecting cytotoxicity is important in that results should reflect desired outcomes when tested in these models. Furthermore, it should be noted that most in vitro systems

are based on liver toxicity as the liver is the most effected by compounds that have off-target mechanisms (Vinken and Blaauboer, 2017).

1.4.2.1. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Mitochondria provide cells with energy through a cascade of reactions. These processes however have many potential sites for toxic chemicals to damage which can prevent mitochondrial function. This makes mitochondria a suitable biomarker for cytotoxic activity (Vinken and Blaauboer, 2017). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a commonly used colorimetric assay that uses a tetrazolium salt to produce a coloured product in which viable cells are able to reduce MTT which is yellow in colour to a purple product. This formazan product is insoluble and is retained in the cell. However, with the addition of solvents, i.e. DMSO, formazan is solubilized and released into the cell culture medium. The coloured product released into the cell culture medium can then be subjected to colorimetric measurement where the number of surviving cells is proportional to the amount of solubilized formazan that was released. Proportionality of living cells to formazan product is the underlying theory of the MTT assay in that activity can only occur in living cells. In addition, this can also be a measure of viability or cell number as well as mitochondrial integrity (Vinken and Blaauboer, 2017).

Certain aspects of the assay need to be considered, such as the influence of coloured culture medium that can affect the colour development of formazan. In addition to this some compounds may interact with the tetrazolium salts thus reducing them and rendering the MTT assay inapplicable. In these cases, alternatives such as the bioluminescent measurement of ATP content and the measurement of neutral red dye uptake in the lysosomes of living cells can be considered. (Vinken and Blaauboer, 2017).

1.4.2.2. Lactate dehydrogenase

The lactate dehydrogenase (LDH) assay for detecting cytotoxicity encompasses all plasma membrane damage in which the membrane integrity is lost (Vinken and Blaauboer, 2017). This allows for cytosolic molecules to move freely from inside of the cell into the culture medium. One such molecule is LDH, a stable enzyme which leaks out of the cell at high concentrations. LDH is responsible for the interconversion of pyruvate and lactate with connected interconversion of

reduced and oxidized NADH. This consumption can be monitored spectroscopically and considered proportional to LDH activity. The parameter used in this context is the LDH index which is the ratio of LDH activity in cell culture medium over total LDH activity. LDH index at a cut-off of 20% is typical of normal compounds however higher LDH indexes indicate cytotoxic compounds (Vinken and Blaauboer, 2017). There are alternative methods to detect cytotoxicity based on cell membrane integrity, including the use of reporter dyes that upon addition to cell culture medium move into cells. Such compounds include propidium iodide and trypan blue (Vinken and Blaauboer, 2017).

1.4.3. Challenges in drug discovery

The most prevalent complication in drug discovery is the presence of promiscuous compounds in screening libraries. Promiscuous compounds are the source of many false positive results in screening due to their ability to display drug-like properties and many of these compounds have been published as beneficial drugs. Therefore, it has become important to eliminate these compounds from screening libraries while the need to understand these molecules has become a priority.

The process of drug discovery encompasses an array of assays that determine the effectiveness of a compound in the study (Vinken and Blaauboer, 2017). Compounds that display drug like properties need to inhibit enzymes as a single molecule that blocks the active site of the receptor or can cause a conformational change to the active site (Rishton, 2008). In addition to binding to the active site, compounds need to be specific in their activity so that it binds strongly and exclusively to the targeted protein (Vinken and Blaauboer, 2017). Compounds that display a high selectivity and tractable pharmacology can be considered as potential or lead drug candidates. However, during screening some hits portray peculiar inhibition properties such as non-competitive activity and the lack of structure-activity relationships which are properties of non-drug-like compounds. Furthermore, the properties portrayed by these compounds lead to false positive results (McGovern *et al*., 2002; McGovern *et al*., 2003). Compounds that portray these aspects are termed non-specific promiscuous compounds or PAINS (Pan-assay interference compounds) that have various mechanisms of action such as, non-specific aggregating and micelle formation (Figure 1.4), spectral interference and the disruption of membrane environments that

are required for receptor integrity. In addition, these compounds can also cause chelating of metal ions essential in catalytic redox effects as well as cause covalent modifications to the receptor (Baell and Walters, 2014).

The inhibition of enzyme activity in the presence of these non-specific inhibitors was found to be caused by the formation of aggregates (Figure 1.3) (McGovern *et al*., 2003). At high concentrations, molecules form higher order complexities which sequester enzymes from substrates and provides an impression of inhibitory efficiency (Figure 1.3) (McGovern *et al*., 2003). Although targeted enzymes are inhibited, the fact that compounds of this nature can also act on multiple protein subfamilies and has the potential to bind to gene sequences is disconcerting because it can cause harm to the cells by inhibiting crucial pathways. In addition, the activity of these compounds is not controllable, and it is difficult to pre-determine the amount of compound that is needed to bind to all active sites of the protein (Aldrich et al, 2017). Aggregation is one of the most frequently used explanations for non-drug like properties found in screening (Figure 1.3) (McGovern *et al*., 2003). Aggregation or colloidal aggregation is common in drug discovery and can appear in approximately 2% of most drug discovery programmes (Baell and Nissink, 2018). In addition, these drugs display aggregation at low concentrations and have an impact in screens both empirically or by means of computational methods (Banerjee *et al*., 2013).

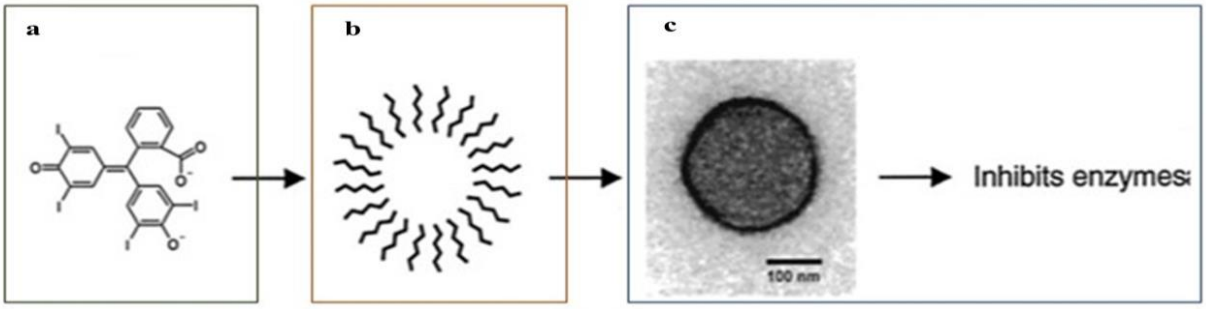


Figure 1.3: The formation of aggregates can provide false inhibitory efficiency of a compound. (a) Compounds that have specific structural and functional properties (b) These compounds then aggregate together to form micelles or aggregates. Compounds that aggregate form micelles around proteins that stop the enzymes activity. (c) A dynamic light scattering image of an aggregate that can sequester enzymes from solution through surface absorption thus, providing false inhibitory efficiency. McGovern *et al*, 2002.

Methods have been developed to detect promiscuous compounds before they pollute screening processes in drug discovery which includes orthogonal assays or computational filters to detect PAINS. In addition, a criterion for the detection of PAINS has been developed based on the

structure of proposed compounds and would allow for implicated compounds to be eliminated before being introduced into screens. According to Baell and Walters, 2014, there are about 400 compound classes that represent PAINS however, 16 out of these compounds classes is enough to eliminate half of PAINS from the screening library. These classes include compounds that produce hydrogen peroxide as a by-product, toxoflavins, covalent modifiers, i.e. isothiazolones and rhodamines and compounds that have by-products which gives artificial signals such as phenol-sulfonamides (Bruns and Watson, 2012; Baell and Walters, 2014). However, the criteria set for detecting PAINS (Bruns and Watson, 2012) is ineffective in detecting aggregating compounds.

1.4.3.1. Assays to detect aggregating compounds

One way to detect the presence of an aggregating compound is with the use of a non-ionic detergent. In the presence of such a detergent, aggregating compounds disassociate and lose their inhibiting ability. It is this decrease in activity that suggests that the compounds are promiscuous (Feng and Schoichet, 2006). The protocol for the detection of aggregating compounds includes the use of 0.01% (v/v) of freshly prepared Triton X-100 or alternatively 0.025% (v/v) of Tween-80 (Feng and Schoichet, 2006). Dynamic light scattering (DLS) is a technique used for determining the size of submicron particles such as proteins, other biomolecules, liposomes, nanoparticles as well as aggregating compounds (Feng and Schoichet, 2006). The formation of 50 – 1000 nm particles characteristic of aggregating compounds in combination with the DLS techniques provides a quick and efficient way of eliminating these compounds and reducing the time and effort in determining lead drug candidates (Feng and Schoichet, 2006). Precipitation via centrifugation is another method that can be used to detect aggregation amongst compounds. For this method the precipitated aggregated particles can be centrifuged and separated before analytical assays are run (Feng and Schoichet, 2006). If the activity of the compound is lower after centrifugation then this suggests aggregation (Feng and Schoichet, 2006). Aggregating compounds can display non-competitive binding and co-operative binding because they do not bind to a specific active site on the protein (Auld *et al*., 2017). In addition, the binding of compounds in this way to proteins causes a rapid increase of apparent inhibition which results in steep Hill slopes. Thus, the use of methods measuring these factors can indicate aggregating compounds (Auld *et al*., 2017). Increasing concentrations of target protein would not decrease the inhibitory activity of

non-aggregating compounds. However, a decrease in inhibition corresponding to an increase in target concentrations would occur for aggregating compounds. A final method that can be used to detect aggregation is a counter screen in which enzymes such as AmpC β-lactamase and malate dehydrogenase as well as trypsin can detect the physiological functions and kinetic abilities of the compound. These enzymes are preferred for the use in counter screening because they are not affected by the addition of detergents but are sensitive to compound aggregation (Aldrich et al, 2017).

1.4.3.2. Detecting DNA binding compounds

Deoxyribonucleic acid (DNA) plays an important role in the biological processes of a cell because it contains information necessary for the synthesis of proteins and enzymes (Zhou *et al*., 2014). This has made DNA an important target in drug discovery (Hurley, 2002). There are many ways in which small molecules used in drug discovery can interact with DNA and this can be either passive or direct (Hurley, 2002). Passive interactions include binding to DNA associated proteins or by interacting with DNA-RNA hybrids. Some small molecules can bind directly to the DNA molecule. Binding of these molecules to DNA can occur by either intercalation or groove binding (minor or major) and in some cases molecules can bind using multiple binding modes (Hurley, 2002; Zhou *et al*., 2014). Furthermore, compounds that exhibit two or more binding modes or bind to both DNA and proteins are considered non-specific and therefore are not good drug candidates (Feng and Schoichet, 2006).

There are two assays that can be used to detect the binding of compounds to DNA and these assays use the fluorescent nature of specific dyes when bound to double stranded DNA. One assay that is commonly used as a diagnostic tool for detecting the binding modes of compounds is the displacement assay (Nonaka *et al*., 1990). This assay was based on the decrease in the fluorescence emitted by DNA bound ethidium bromide (EtBr) in the presence of a competitive ligand (Figure 1.4a) (Nguyen and Anslyn, 2006). Due to the intercalating nature in which EtBr binds to DNA, a decrease in the fluorescence would indicate that EtBr is being displaced by an intercalating compound (Nguyen and Anslyn, 2006). However, dyes such as Hoechst 33258 (Figure 1.4b) are used to determine minor groove binding compounds as the decrease in fluorescence is due to the displacement of this dye from the minor grooves of DNA (Nguyen and Anslyn, 2006). Another

assay that can be used to monitor interactions of compounds to DNA is the DNA cleavage assay by gel electrophoresis (Zhou *et al*., 2014). This method utilizes the three different forms of plasmid DNA on a gel to monitor the extent in which compounds bind to DNA (Zhou *et al*., 2014). Binding of compounds to double stranded supercoiled (form I) plasmid DNA can cause one of the strands to become nicked thus leading to an open circular relaxed form (form II) (Figure 1.4c) (Zhou *et al*., 2014). Extended exposure of DNA to compounds can eventually lead to both strands of the plasmid being nicked and this leads to the appearance of the linear form of DNA (form III) (Figure 1.4c) (Zhou *et al.*, 2014).

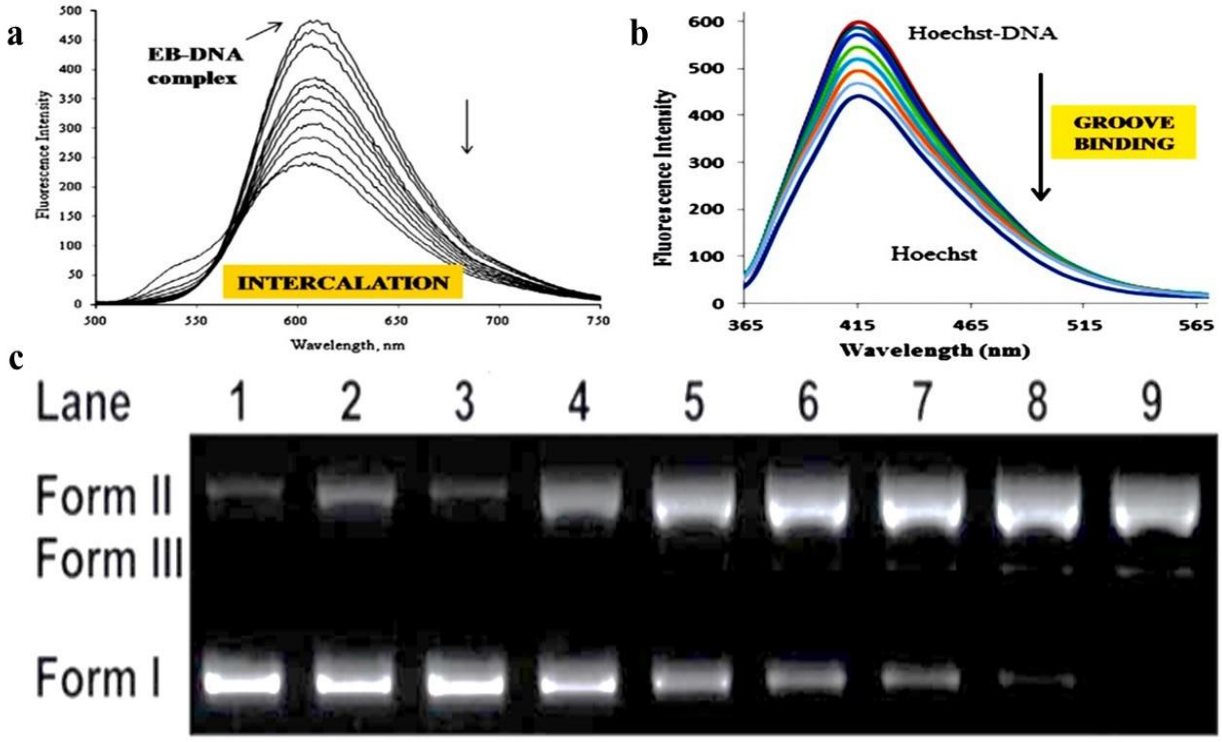


Figure 1.4: Visualization of the outcomes of the two assays for the detection of DNA binding compounds. (a) The decrease in fluorescent intensity due to the displacement of EtBr from DNA thus demonstrating intercalator binding mode. Adapted from Rehman *et al*., 2015. (b) Minor groove binding compounds displacing Hoechst 33258 from DNA (Adapted from Rehman *et al*., 2015) (c) Cleavage of DNA by a copper (II) complex [Cu(Hypy-ttpy)Br_2]Br (CTB). Lanes 1 – 3: DNA, DNA incubated with ascorbic acid and DNA with CTB complex. Lanes 4 – 9: DNA with ascorbic acid and CTB at different incubation times (15, 30, 45, 60, 75 and 90 minutes respectively). Zhou *et al*., 2014.

1.5. Benefits of metallo compounds as potential drugs

There have been many described applications for metal-based compounds in medicine (reviewed in Mjos and Orvig, 2014). Metallo-compounds have been used as antimicrobial and antiparasitic

as well as antiarthritic and antidiabetic treatments (reviewed in Mjos and Orvig, 2014). Other uses of metal-based compounds include the treatment of psychotropics, (i.e. the treatment of bipolar disoders) and as a chelating proligand drug in metal overload disorders (reviewed in Mjos and Orvig, 2014). Furthermore, metal-based compounds have been evaluated as antiviral agents for the treatment of herpes simplex virus type1 (HSV-1) (reviewed in Mjos and Orvig, 2014) and for their potential benefits as polynuclear talates or Polynuclear, transition-metal oxyanions (POMs) for the treatment of HIV and acquired immuno deficiency syndrome (AIDS) (Lebon *et al*., 2002; reviewed in Mjos and Orvig, 2014).

Metal-based compounds as therapeutic avenues gained significant attention in drug discovery after their introduction and success as anticancer treatments (reviewed in Van Rijt and Sadler, 2009). Cisplatin is a FDA-approved chemotherapeutic and had prominent success in the treatment of metastatic testicular, ovarian and bladder cancers (reviewed in Van Rijt and Sadler, 2009 and Ndagi *et al*., 2017). In addition, after decades of being on the market, it is still the best-selling anticancer treatment (reviewed in Mjos and Orvig, 2014). However, cisplatin is only successful in the treatment of certain types of cancers and there are still drawbacks associated with the use of this drug such as severe side effects which include, nausea, bone marrow suppression and kidney toxicity (reviewed in Ndagi *et al*., 2017). Furthermore, some tumours had developed resistance to cisplatin which makes treatment with this drug difficult (reviewed in Ndagi *et al*., 2017). To remedy the limitations involved with cisplatin, many derivatives of the platinum based metallodrug were developed (reviewed in Van Rijt and Sadler, 2009 and Mjos and Orvig, 2014 and Ndagi *et al*., 2017). These included paraplatin (carboplatin) and eloxatin (oxaliplatin) which are FDA approved drugs that are used in the management of ovarian cancer and in the management of colon cancer and lung cancer, respectively (reviewed in Ndagi *et al*., 2017). In addition, aqupla (nedaplatin) and lobaplatin are also platinum-based compounds that were developed as anticancer drugs but were not as effective as the other derivatives (reviewed in Ndagi *et al*., 2017).

Although these derivatives had success in the treatment of cancer they however, were unable to address the limitations associated with cisplatin (reviewed in Ndagi *et al*., 2017). Therefore, the development of other drugs based on different metal centres had been introduced (Lebon *et al*., 2002). These drugs were based on different metals such as titanocences, copper complexes as well as ruthenium, gold and silver (Lebon *et al*., 2002). Gold complexes were considered due to its

effectiveness against cancer cells (Lebon *et al.*, 2002). In addition, gold(III) complexes have activity that was dissimilar to that of cisplatin which would address the limitations associated with platinum-based agents (reviewed in Ndagi *et al*., 2017).

Furthermore, the development of metal-based compounds in the treatment of cancer as well as the success associated with this therapy has encouraged the use of such compounds in other diseases (Dingwall, 2007). Metal-based compounds have been described to have advantageous structural benefits over commonly used organic-based drugs (Dingwall, 2007). These drugs have a wide range of coordination numbers and geometry as well as a wide range of structural diversity (Ismaili *et al*., 2016). Furthermore, they also have adjustable thermodynamics and kinetics of substitution as well as accessible redox states which makes optimization for a drug target faster and easier (Ismaili *et al*., 2016).

1.5.1. Potential benefits of metalized compounds in Alzheimer's disease therapy

The use of metal-based compounds as a means for the treatment of AD has only recently been presented (reviewed in Kenche and Barnham, 2011). Treatment of AD with metal-based compounds are linked to metal homeostasis by preventing the interaction of Aβ with metals thus preventing the pathological features associated with AD (reviewed in Kenche and Barnham, 2011 and Tougo *et al*., 2011). It was found that the interaction of the Aβ aggregates with the biometals, Cu (II) and Zn (II) plays an important role in toxicity associated with AD (reviewed in Tougo *et al*., 2011). Furthermore, the coordination of Cu (II) by Aβ which leads to increased levels of ROS and ultimately oxidative stress and neurotoxicity (reviewed in Tougo *et al*., 2011). Metal homeostasis is needed to maintain neurotransmission at glutamatergic synapses in the cortex and hippocampal regions (reviewed in Tougo *et al*., 2011). The disruption of this homeostasis can lead to synaptic disfunction and ultimately lead to the loss of neurotransmission associated with AD (reviewed in Tougo *et al*., 2011). Additionally, Aβ peptides (Aβ42 and $A\beta_{40}$) have been demonstrated to bind copper, iron and zinc ions under physiological conditions due to the rich portion of histidine residues close to the N-terminus (reviewed in Kenche and Barnham, 2011). The binding of these ions can influence the aggregation and pathogenicity of Aβ plaques by affecting the rate at which aggregates form as well as changing the nature and morphology of aggregates (reviewed in Kenche and Barnham, 2011).

Three histidine residues, namely, His-6, His-13 and His-14 enable ions to bind to the protein thus enabling aggregation and is considered a good target for drug therapy (reviewed in Valensin *et al*., 2012). Therefore, finding compounds that can selectively bind to these sites would prove beneficial in stopping Aβ / metal interactions by blocking sites so that no intracellular metals can bind to Aβ. However, a simple 'lock and key' design can be problematic due to Aβ being unstructured in the absence of metal ions. This can be avoided with the addition of metal-based compounds that are able to change the morphology of Aβ and block its activity thus preventing aggregation and ultimately stopping progression of the disease (reviewed in Valensin *et al*., 2012). Phenanthroline, Pt(II) complexes and L-$PtCl_2$ compounds were used to determine if targeting the aggregation of Aβ would be a beneficial target in the treatment of AD (Dingwall, 2007). It was found that these compounds could interact with Aβ and reduced Aβ-induced synaptotoxicity in mouse hippocampal slices (Dingwall, 2007). Furthermore, L-$PtCl_2$ exhibited reduction in the metal-mediated ROS associated with aggregate formation. The interaction of these compounds with Aβ demonstrated that targeting His 6, 13 and 14 residues could ultimately reduce neurotoxic and synaptotoxic effects caused by aggregation (Dingwall, 2007).

The interaction of metal-based compounds with Aβ to prevent aggregation has led to the identification of other metal mediated targets associated with AD which may be beneficial as future drug targets (Ismaili *et al*., 2016). It was shown that the enzyme responsible for the formation of Aβ, BACE1, was able to bind to intracellularly available copper (Ismaili *et al*., 2016). When intracellular copper is high, the cleavage of APP by BACE1 increases (Dingwall, 2007; Ismaili *et al*., 2016). The binding of copper to BACE1 ultimately leads to the over-production of Aβ and further aggregate formation (Dingwall, 2007; Ismaili *et al*., 2016). Therefore, inhibiting the activity of BACE1 can occur in two ways and includes the standard method of inhibition involving the binding of compounds to the enzyme thus causing a conformational change thus leading to the decrease in Aβ (Dingwall, 2007). Alternatively, the inhibition of BACE1 can occur in the same manner as was seen with Aβ/metal interactions in which metal-based compounds compete for the metal binding site on the enzyme thus preventing the over-production of Aβ (Dingwall, 2007). However, the use of metal-based compounds in the inhibition of BACE1 has not yet been fully explored but may provide breakthrough therapies for the treatment of AD (Ismaili *et al*., 2016).

1.6. Aims and objectives:

The aim of this study was to evaluate 13 novel metal-based compounds as potential BACE1 ligands. To achieve this, the following objectives were derived:

a) Evaluate the compounds through theoretical prediction software programs to determine potential drug-likeness and physiochemical properties

b) Undertake thermal shift analysis to determine in vitro binding to the purified BACE1 protein

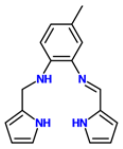
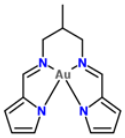
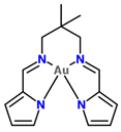
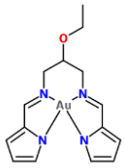
c) Investigate potential non-specific interactions through in vitro aggregation and DNA-binding assays

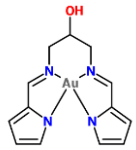
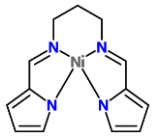
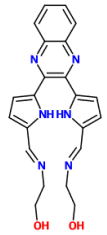
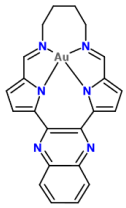
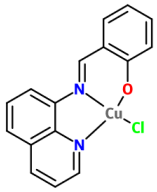
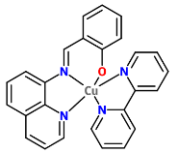
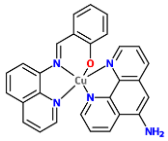
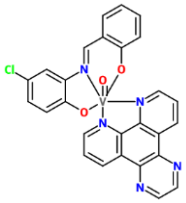
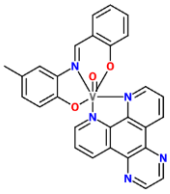
CHAPTER TWO: METHODS

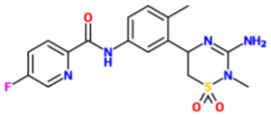
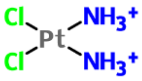
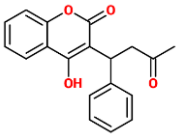
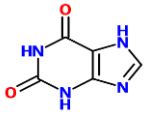
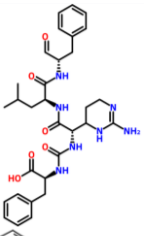
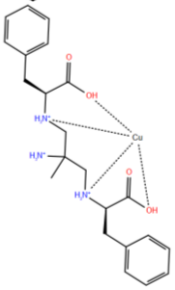
2.1. Compound structures

Complexes 1 – 11 were synthesized and provided by Dr Matthew Akerman and the oxyvanadium (IV) DPQ complexes 12 and 13 were synthesized and provided by Ms. Kristy-Lyn Barry (Table 2.1). Cisplatin, warfarin and caffeine (all from Sigma-Aldrich, USA) were used as control compounds in the prediction of physiochemical properties, DNA cleavage assay and chymotrypsin assay. The prediction of the physiochemical properties of each compound was established using the OSIRIS DataWarrior program which determines the cLogP, cLogS, hydrogen bond donor and acceptors, rotatable bonds, TPSA, drug-likeness and drug-score values based on the structure of the compound. These values were then compared to RO5 and Veber's rules to establish if these compounds would be good drug candidates. Verubecestat (Qi *et al*., 1994; Kennedy *et al*., 2016) was used as a control in the molecular docking studies. All compounds were prepared as 10 mg/ml stock solutions in DMSO (Sigma-Aldrich, USA) unless stated otherwise and stored at – 20 ºC.

Table 2.1: Chemical structures and molecular weights for synthesized novel compounds and control compounds

Compound	Structure	Molecular Weight (g/mol)
1		276.34
2		602.65
3		486.75
4		612.26

5		474.70
6		284.97
7		402.45
8		708.35
9		346.27
10		502.45
11		541.49
12		544.84
13		542.42

Verubecestat		523.43
Cisplatin		300.01
Warfarin		308.33
Caffeine		194.19
Chymostatin		607.71
((2S,8R)-5-amino-2,8-dibenzyl-5-methyl-3,7-diazanonanedioate)copper(II) (Control compound)		607

2.2.Molecular Docking studies

Molecular docking studies were conducted using the Maestro 11.2 software (Schrödinger, USA) which utilizes the Glide app in order to superimpose the structure of compounds to the X-ray model of the BACE1 protein. The PDB file (PDB code: 2ZHT) (Shimizu *et al*., 2008) for BACE1 was obtained from the RCSB PDB website (https://www.rcsb.org/, USA). The docking of compounds to proteins is a step by step procedure and requires ligand and protein preparation as well as the generation of a receptor grid in order to properly predict binding of molecules. Ligand and protein preparation were conducted using the LigPrep and Protein Preparation Wizard applications provided in Maestro. Different states were generated for the compounds at pH range of 7.0 with

the use of the OPLS3 force field. Protein preparation was conducted at default settings however, due to the nature of the metal compounds, no minimization step was conducted after pre-processing and optimization. Receptor grid generation was conducted under default settings and the enclosing box was set to cover the entire protein in order to determine binding on the entire surface of BACE1. Docking of compounds to BACE1 was conducted under default settings using the Ligand Docking application provided by Glide however, no post-docking minimization step were performed on metal-containing molecules. Active site generation was conducted using the SiteMap application in Maestro to identify top-ranked potential receptor binding sites. Interaction diagrams and measuring of the distance between compounds and the active site was generated and evaluated using the interaction software provided in Maestro.

2.3.Displacement Assay:

Extraction of plasmid DNA:

The alkaline lysis plasmid mini-prep previously described (Sambrook and Russell, 2006) was used to isolate the pGEX-4T-1 plasmid from 10 ml Luria Broth (LB) cultures containing *Escherichia coli* cells incubated overnight at 37 ºC. Thereafter, cells were centrifuged at 4 ºC for 5 minutes (16 000 xg) and resuspended in GTE solution (200 µl, 50 mM Glucose, 25 mM Tris-Hydrochloric acid (HCl), 10 mM ethylenediamine tetraacetic acid (EDTA), pH 8). RNase A solution (2 µl, 20 mg/ml stock) was added to the resuspended cells and incubated for 5 minutes at room temperature. The cell suspension was then transferred to a fresh tube (1.5 ml microtube) and sodium hydroxide (NaOH)/ sodium dodecyl sulfate (SDS) (400 µl, 0.2 M NaOH (Sigma Aldrich, USA), 1 % SDS (Sigma Aldrich, USA)) was added. The tube was inverted 3 times and then incubated for 5 minutes at room temperature. Potassium acetate (3 M, 300 µl) was then added, and the tube inverted an additional 3 times and then incubated for 5 minutes at room temperature. Thereafter, the tube was centrifuged for 5 minutes (10 000 xg) at room temperature. 800 µl of the supernatant was then transferred to a fresh tube and centrifuged again if necessary. Isopropanol (600 µl) was then added to the supernatant and incubated at -20 ºC for 30 minutes. The tube was then centrifuged for 5 minutes (10 000 xg) at room temperature and the supernatant was discarded. 500 µl of ice-cold ethanol was added to the pellet and centrifuged for 1 minute (10 000 xg) at room temperature. Thereafter, the supernatant was removed, and the pellet was left to dry at room temperature for 10

minutes. The pellet was then resuspended in 50 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8).

Assay conditions:

Cleavage of DNA by metal complexes was monitored by agarose gel electrophoresis and the assay was adapted from a previously described protocol (Thota *et al.*, 2016). Reactions (25 µl) containing plasmid DNA (1 µg), HCl (50 mM, pH 7.5), NaCl (50 mM), complexes (0.025 mM – 0.500 mM), 0.1% Triton X-100 (Sigma-Aldrich, USA) and H_2O_2 (60 µM) were incubated for 30 minutes at 37 ºC. After incubation, 4 µl of 6X Loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 60% glycerol) (ThermoFisher Scientific, USA) was added to the reaction mixture. Samples were loaded on a 1% (w/v) agarose gel containing 1 µg/ml EtBr and run at 80V for 1 hour in 1x tris/borate/EDTA (TBE) (90 mM tris, 90 mM boric acid, 2 mM EDTA) buffer and viewed under UV light and photographed on a G:Box (Syngene, UK).

2.4. Chymotrypsin Assay

The activity of chymotrypsin (Sigma-Aldrich, USA) in the presence and absence of chymostatin (ThermoFisher Scientific, USA) was monitored by measuring the increase in the by-product of the substrate N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) (Sigma-Aldrich, USA). Chymotrypsin and BTEE was dissolved in 1mM HCl and 50% methanol, respectively. Stock solutions of compounds were dissolved in DMSO and diluted in tris buffer (80 mM tris, pH 7.8). Assays were conducted in a three-part system in which substrate (at a final concentration of 0.53 mM) was added with $CaCl_2$ (53 mM) and Tris-HCl (37.6 mM) buffer which was then incubated for 10 minutes at 25 ºC. In the second part of the assay, enzyme (at a final concentration of 3.3 µg) and compound (20 µM) was mixed together and incubated at 25 ºC for 10 min. The substrate solution was then added to the enzyme for the last part of the assay and the activity of chymotrypsin was measured at an absorbance of 256 nm for every 1 minute interval for 6 minutes at 25 ºC. Activity of chymotrypsin was determined as the change in absorbance per minute for 6 minutes. Aggregation was determined by the increase in activity in the presence of Triton X-100 at a final concentration of 0.1 %.

2.5. Thermal shift Assay:

The TSA previously described (Lo *et al*., 2004) was used to evaluate compounds 1 – 13 for binding to BACE1. The melting temperature of BACE1 was determined by the increase in flourescence in the presence of the SYPRO orange dye. The increase in fluorescence was measured using the high resolution melt (HRM) at excitation 510 nm and emission 460 nm. All assays were performed in the presence of phosphate-buffer saline (PBS), pH 7.4 and 5x SYPRO orange dye. The SYPRO orange dye was supplied at a concentration of 5000x (Sigma-Aldrich, USA) and diluted in PBS at a higher concentration before it was added to the reaction. Compounds were diluted in PBS before being added to the protein to reduce the concentration of DMSO (<5%) in the reaction. The reaction was carried out using final concentrations of 5.5 μM for BACE1 in the presence and absence of 10 μM compound in 25 μl reactions. The temperature range used for the HRM analysis was between 25 ºC – 95 ºC with 0.5 ºC increments and 5 seconds holding time. Reactions were conducted using the Rotor-Gene 6000 real-time PCR machine (Corbett Research, USA) and thermal melt curves were generated and analaysed using the Rotor-Gene software provided.

CHAPTER THREE: RESULTS

3.1. Prediction of Physiochemical Properties

Compounds 1 -13 were analysed based on their structural properties in accordance to the RO5 and Veber's rules to determine if these compounds had suitable bioavailability profiles (Lipinski *et al*., 2001; Veber *et al*., 2002). The OSIRIS DataWarrior program was used to determine these values based on their structures as well as determine their drug-likeness and their overall drug-score (Table 3.1). Of the 13 compounds evaluated, 3 gold-containing (compounds 2, 4 and 8), 2 copper-containing (compounds 10 and 11) and 2 vanadium-containing compounds (compounds 12 and 13) had molecular weights above 500 g/mol (Table 2.1). All test compounds, excluding compound 12 (clogP of 5.01) produced clogP values below 5 (Table 3.1). The non-metal compounds (1 and 7) had clogP values of 2.00 and 1.41, respectively (Table 3.1). The clogP value of compound 7 and the control compounds cisplatin, caffeine and verubecestat were lower than 2 which suggests that these molecules are more hydrophilic. The metal compounds 2 – 5 and 8 which have gold metal centres as well as compound 6, the nickel-containing molecule, all had clogP values below 5 (Table 3.1). In addition, these compounds also had clogP values below 2, suggesting that these molecules are also more hydrophilic. However, compounds with copper (9, 10 and 11) and vanadium (12 and 13) centres had cLogP values close to 5 (Table 3.1) and are therefore more hydrophobic. All test compounds had less than 10 hydrogen bond acceptors and less than 5 hydrogen bond donors (Table 3.1). The RO5 are rules that have been in place that provides insight on the physiochemical properties for oral drug candidates. In addition to these rules, the Veber's rules provides additional support to the RO5 for further evaluation. As such, the test compounds evaluated according to these rules all had less than 10 rotatable bonds and TPSA values below 140 Å (Table 3.1) which is in accordance to these rules.

In addition to evaluating compounds with the RO5, the OSIRIS DataWarrior program also determines the drug-likeness of a compound based on similarities in structural fragments from commercial drugs and produces a drug-likeness score. The program generates a score between 0 and 5 which ranges from being less similar (< 0) and being more similar (≤ 5) to commercial drugs. The copper-containing (compounds 9 and 10) and vanadium-containing (compounds 12 and 13) compounds as well as the control compounds cisplatin, warfarin and verubecestat (-4.93, -0.77 and

-2.56 respectively) had negative values thus suggesting that they have fragments dissimilar to those found in commercial drugs. However, compounds containing gold (compounds 2 – 5 and 8) and nickel (compound 6) as well as compounds 1 and 7 exhibited positive drug-likeness scores (Table 3.1). Compound 5 presented the highest drug-likeness score (3.32) with compounds 2 and 6 producing the second highest scores (Table 3.1). Furthermore, compound 5 produced a score higher than that of caffeine which produced a score of 2.59 (Table 3.1) suggesting that compound 5 has the highest similarity to commercial drugs compared to all the compounds evaluated.

In evaluating a compounds structure to determine their potential as orally active drugs, there also needs to be reassurance that these drugs would not cause harm to the patient. Therefore analysis of these drugs and their potential to cause mutagenic and tumorigenic problems as well as their ability to be an irratant and have reproductive effects is important. The OSIRIS DataWarrior program determines these potentially harmful effects by comparing the structures of compounds to those found in the Registry of Toxic Effects of Chemical Substances (RTECS). Compounds 2, 7 and 8 was predicted to have high mutagenic properties with compound 13 producing low mutagenic properties. In addition, cisplatin and caffeine displayed high mutagenic properties which has been previously described in literature (Nehlig and Debry, 1994). The control compound caffeine was predicted to have high tumorigenic properties as compared to compounds 6 and 13 which produced low tumorigenic properties. The results obtained for caffeine however do not correlate to the properties of caffeine explained in literature (Misra *et al*., 2016). Of the compounds analysed, compounds 2 and 3 produced high reproductive properties with compounds 4 – 6, 8 and 13 being predicted to have low reproductive effects. Control compounds warfarin and caffeine was also predicted to have reproductive effects which correlates to findings in literature (Hill and Kleinberg, 1984; Temple *et al*., 2017). In addition, warfarin and cisplatin were predicted to be high irritants which was expected (Bochi and Rostagno, 2012). Compound 6 was predicted to have high irratant properties. Furthermore, gold compounds 1 -5 and 8 as well as compound 13 produced low irratant properties. The mutagenic, tumorigenic and reproductive effect properties of compounds was not associated with the metal centres present. In addition, no harmful properties were predicted for compound 1 and the copper-containing compounds 9 and 10 (Table 3.1).

Table 3.1: Molecular property prediction values for test and control compounds

Compound	cLogP	cLogS	H+	H-	Rot. Bonds	TPSA	RO5 Score	Drug-likeness[a]	Drug-score[b]
1	2.00	-2.98	4	3	5	55.97	4	1.58	0.80
2	-0.10	-6.36	4	0	0	34.58	3	2.82	0.13
3	0.22	-6.37	4	0	0	34.58	4	1.35	0.19
4	1.09	-2.00	5	2	8	65.53	3	0.12	0.17
5	0.26	-1.58	5	3	6	76.53	4	3.32	0.34
6	1.28	-1.98	4	2	6	56.30	4	2.53	0.15
7	1.41	-2.80	8	4	8	122.54	4	1.75	0.46
8	3.20	-3.90	6	2	0	82.08	3	1.31	0.10
9	3.00	-3.57	3	1	2	45.48	4	-0.09	0.35
10	4.50	-4.53	5	1	3	71.26	4	-0.36	0.40
11	4.62	-6.14	6	2	2	97.28	3	-0.41	0.40
12	5.01	-5.61	8	2	2	121.45	3	-0.28	0.66
13	4.42	-5.21	7	2	2	104.38	3	-0.46	0.57
Cisplatin	0.73	-0.53	2	2	0	52.04	4	-4.93	0.32
Warfarin	3.28	-3.72	4	1	4	63.60	4	-0.77	0.27
Caffeine	-0.18	-2.98	6	0	0	58.44	4	2.59	0.20
Verubecestat	0.48	-4.07	8	2	3	126.13	4	-2.56	0.40

Values obtained for cLogP, cLogS, Topological Polar Surface Area prediction (TPSA), drug-likeness and drug score were outputs from the OSIRIS DataWarrior program. [a] Positive drug-likeness scores indicate that compounds have fragments frequently present in commercial drugs. [b] The drug score combines all values, i.e. drug-likeness, cLogP, solubility, molecular weight and toxicity risks (determined on program) to determine an overall score.

The overall drug score incorporates the drug-likeness, clogP, clogS, molecular weight and toxicity risks to determine if compounds would qualify as a potential oral drug candidate. The drug score is a value between 0 and 1 with 1 representing a compound with the highest potential of qualifying as an orally-active drug candidate and 0 being the opposite. Compound 1 displayed the highest drug-score from all the tested compounds including the control compounds cisplatin, warfarin, caffeine and verubecestat which had drug-score values of 0.32, 0.27, 0.20 and 0.40 respectively (Table 3.1). Table 3.1 illustrates that the second highest drug-score value was obtained by the vanadium compounds with compound 12 yeilding a value of 0.66 and compound 13 yeilding a

value of 0.57. Gold compounds 2 – 4 and 8 yeilded values below 0.2 with compound 5 yielding a drug-score value of 0.34 (Table 3.1). The copper compounds 9, 10 and 11 as well as compound 7 yeilded drug-score values below 0.5 (0.35, 0.40, 0.40 and 0.46 respectively) which were similar to the values obtained for cisplatin and verubecestat (Table 3.1).

3.2. Molecular Docking

Molecular docking is a widely-used technique that is able to predict the orientation of a molecule when bound to another molecule (Friesner *et al*., 2004). In the field of drug design and discovery, this technique enables multiple compounds to be screened against a target protein for probable binding (Friesner *et al*., 2004). In this study, the Schrodinger software which uses the Maestro program was used to predict binding amongst the 13 metal-based compounds to the BACE1 protein. Docking scores obtained from this program ranks compound conformations to the target protein from compounds that have favourable binding properties to those that are unable to bind to the proposed target (Friesner *et al*., 2004). Increasingly negative docking score indicate a higher binding affinity to the protein (Friesner *et al*., 2004). Analysis of the docking scores of structurally similar compounds revealed that the non-metal containing compound 7 produced a higher score than that of the gold-containing compound 8 (Table 3.2). In addition, compound 7 displayed a docking score that was more favourable than the other 12 compounds tested (Table 3.2). Furthermore, comparison between compound 6 to the structurally similar compounds 1 – 5 revealed that this nickel-based compound produced favourable docking score (Table 3.2) when compared to the gold compounds (Table 3.2). Compounds with vanadium-metal centers (compounds 12 and 13) produced favourable docking scores indicating that these compounds were also predicted to bind to BACE1 however, compound 12 displayed a more negative score (-4.630) compared to compound 13 (-4.184) (Table 3.2).

Compounds 1 – 13 were also evaluated for binding to the active site of the BACE1 protein as potential inhibitors of the enzyme (Figure 3.1 and 3.2). The BACE1 protein active site was determined using the site map analysis included in the Maestro program in which the structure of the enzyme was analysed for potential active binding sites. In addition, the binding site of the ligand from the predicted active site of the protein was measured as the length in Å as the point of contact to the surface area of the active site.

Table 3.2: Docking scores for compounds 1 – 13 representing binding to the BACE1 protein

Compound #	Docking Score [a]
1	-3.604
2	-2.259
3	-2.393
4	-3.638
5	-2.378
6	-4.023
7	-6.022
8	-3.266
9	-3.250
10	-2.630
11	-3.376
12	-4.630
13	-4.184

[a] Docking scores were calculated using the Schrodinger software/Maestro which uses the Glide application that calculates the docking score based on empirical scoring functions. This estimates compound/ligand binding free energy when superimposed to target protein.

The analysis of compounds able to bind to these active sites revealed that compounds 1 and 7 (white/ ball and stick) had binding sites closest to the active site of BACE1 compared to the other 11 compounds tested (Figure 3.1a and 3.2a). Active sites are indicated by the surface areas (red and violet) in Figure 3.1a, with red indicating hydrogen bond acceptors and violet indicating hydrogen bond donors. Comparing the binding site of compound 1 to the structurally similar compounds 2 – 6 revealed that there was no relationship between the docking scores and the binding site of the compounds. The interaction diagram for compound 1 determined from the Maestro program revealed that hydrogen bonds formed between the pyrrole ring structure of the compound to the negatively charged amino acids, Asp 32 and Asp 228 (Figure 3.1b). In addition, evaluation of the predicted binding mode of compound 7 (white/ball and stick) superimposed to the X-ray structure of BACE1 displayed binding sites close to the predicted active site (red and violet surfaces) (Figure 3.2a) as compared to the structurally similar compound 8 (Green/ball and stick) (Appendix 1).

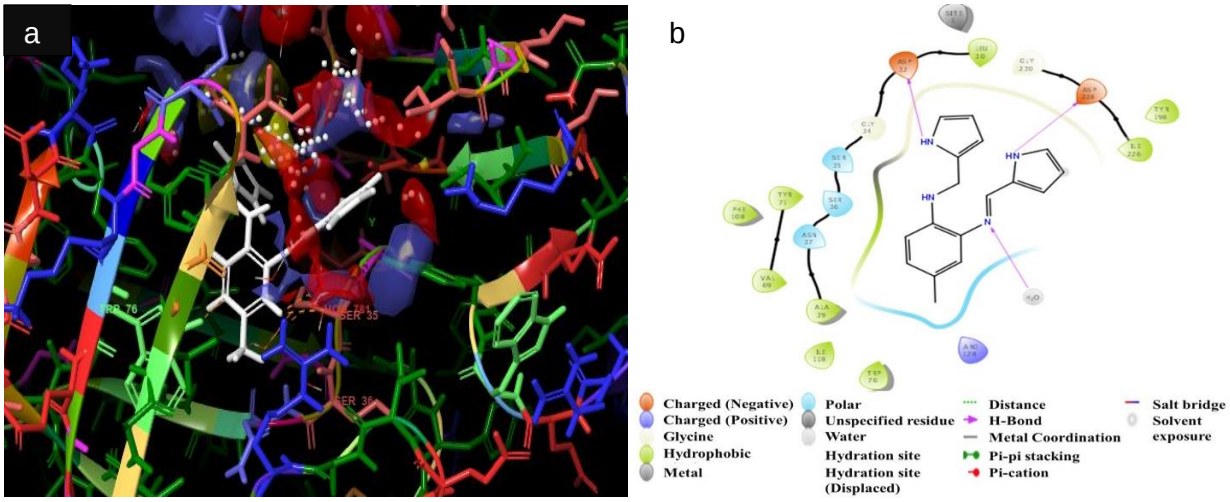


Figure 3.1: Superimposed structure of compound 1 with interaction diagram displaying binding to BACE1. a) Superimposed structure of compound 1 (white/ball and stick) displaying interaction and binding of compound 1 to the BACE1 protein. Surface areas (red and violet) indicate the active site determined by the site map program in Maestro. Red surfaces indicate hydrogen bond acceptor regions with violet surfaces indicating hydrogen bond donor regions. b) Interaction diagram illustrating the bind formations between the compound 1 and BACE1. Compound 1 forms hydrogen bonds to Asp 32 and Asp 228 via the pyrrole groups. The site 1 (grey) indicates the position of the predicted active site of BACE1 to the compound.

Analysis of the interaction diagram revealed that this compound is able to form hydrogen bonds with Glu 364, Asp 318 and Phe 159 (Figure 3.2b) as well as, salt bridges between the amino group and Glu 364 with cation- π interactions with Hip 362 (Figure 3.2b). In addition, one of the amino groups on the compound forms a hydrogen bond with the amino acid, Phe 159 (Figure 3.2b). Measuring the distance of compounds 1 and 7 from the predicted active site revealed that compound 1 (12 Å) was more closely associated to the active site than to compound 7 (31 Å). The molecular docking analysis of compound 1 – 13 was able to determine favourable binding between ligand and protein, illustrating that these metal compounds would likely bind to the BACE1 protein. Predicted binding sites and interaction diagrams illustrated that compounds 1 and 7 are likely to bind close to the predicted active site thus indicating that these compounds are probable inhibitors. In addition, the vanadium compounds (12 and 13) which are structurally similar to compounds 9 – 10 (copper metal centres) had favourable docking to BACE1. Furthermore, the superimposed model of these compounds revealed that although these compounds were able to dock to the protein, their predicted binding site were not in proximity to the BACE1 active site.

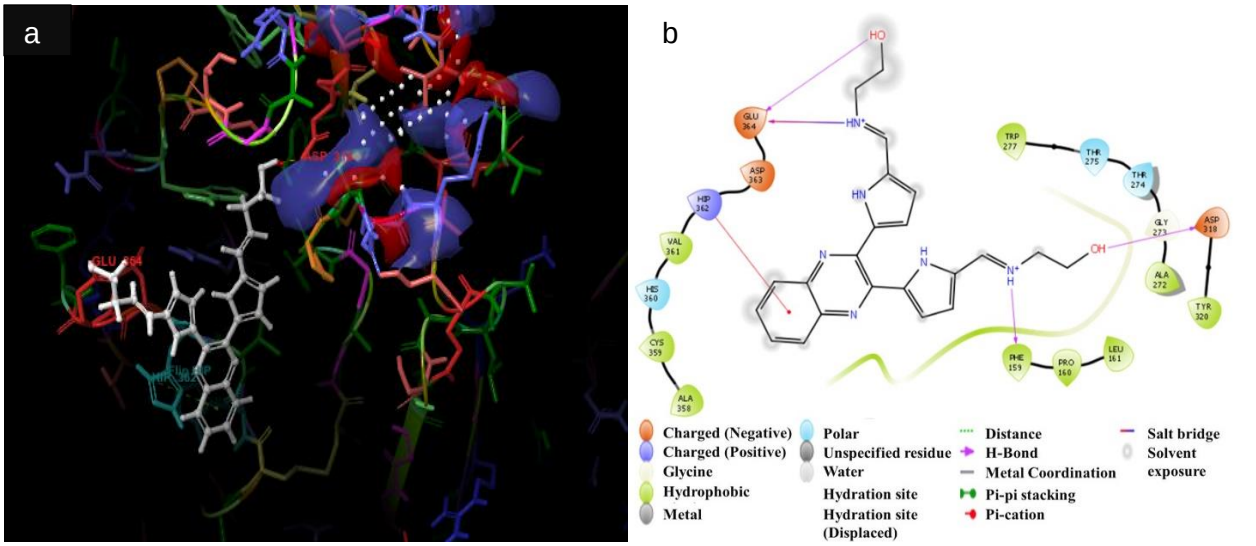


Figure 3.2: Predicted binding model of compound 7 superimposed with the X-ray structure of BACE1 with interaction diagram indicating chemical interactions between protein and ligand. a) Predicted binding model of compound 7 (white/ball and stick) superimposed with BACE1 indicating the binding interactions of compound to protein. Predicted active binding sites are indicated as surface areas denoted red and violet (hydrogen bond acceptors and donors, respectively). Compound 7 binds to the BACE1 protein in close approximation to the predicted active site determined using the site map application in Maestro. b) Interaction diagram between compound 7 and the target protein illustrating the chemical interactions between the structure of ligand and enzyme. Hydrogen bonds are formed between the hydroxyl groups (OH) and the amino acids Glu 364 and Asp 318. A hydrogen bond is also formed between one of the amino groups and Phe 159. The other amino group is able to form a salt bridge to Glu 364. A cation- π interaction formed between the cyclic ring structure of the ligand and the Hip 362 amino acid.

3.3. Thermal Shift Assay

The TSA was approached as a way to verify if the molecular docking studies were able to accurately predict binding of compounds to the BACE1 protein. Optimization of the TSA yielded a T_m of 58.2 ºC for BACE1 at a pH of 7.4. The T_m for the protein reported in literature is 51 ºC at a pH of 4.5 (Hayley *et al*., 2009). Therefore, the deviation from this T_m for the protein calculated in this study was due to the stabilization of BACE1 at the different pH than which has been previously reported (Hayley *el al*., 2009). The first derivative curves produced from the TSA conducted for BACE1 in the presence of compounds 1 – 13, were evaluated to determine possible ligands for the protein. A shift in the peak of the melt curve would indicate possible binding of the compound as well as the stabilization of the protein (Pantoliano *et al*., 2001). Furthermore, the degree of change in the T_m upon binding of compounds has been previously reported to be proportional to the affinity of binding of the ligand (Matulis *et al*., 2005; Vedadi *et al*., 2006; Niesen *et al*., 2007; McMahon *et al*., 2014). Thermal melt curves were also used to determine if

compounds with similar structures displayed similar probable binding and stabilization features. The gold-containing compounds 2 – 5 and the nickel-containing compound 6 were evaluated and compared to the non-metal compound 1 due to these molecules being structurally similar. Figure 3.3 indicates that compounds 1, 2, 4, 5 and 6 were able to cause a shift in the peak (Figure 3.3). However, the degree in which each compound caused a shift in the peak was different despite these compounds being structurally similar to each other. Furthermore, compound 3 was able to cause a shift in the peak but this molecule produced a shift towards the left of the peak for BACE1 alone (Figure 3.3). Comparison between the non-metal compounds 7 and the gold-containing compound 8 revealed that although these compounds are structurally similar, they had different outcomes in the TSA. Compound 8 produced a greater shift in the peak as compared to compound 7 however, they both produced a peak towards the right (Figure 3.3).

Evaluation of the first derivative curves for compounds 9 – 13 revealed that these compounds which are structurally similar were able to cause a shift in the peak when compared to BACE1 alone (Figure 3.4). Compound 11 and 12 shifted the peak towards the right by 3.1 and 9.1 ºC, respectively, which is greater than the shift caused by compound 10 (Table 3.3). Compounds 9 and 13 produced shifts towards the left of the curve for BACE1 alone therefore causing the change in the T_m to be negative (Figure 3.4). Compound 10 yielded a slight shift towards the right with compound 11 producing a 3.1 ºC shift towards the right (Table 3.3). Comparison between the compounds revealed that although compounds are structurally similar they were able to react differently with BACE1 thus producing different shifts in the peak for the first derivative curves. Furthermore, compounds containing vanadium metal centres caused the greatest change in the peak as seen by the positive and negative shifts of compounds 12 and 13 (Figure 3.4).

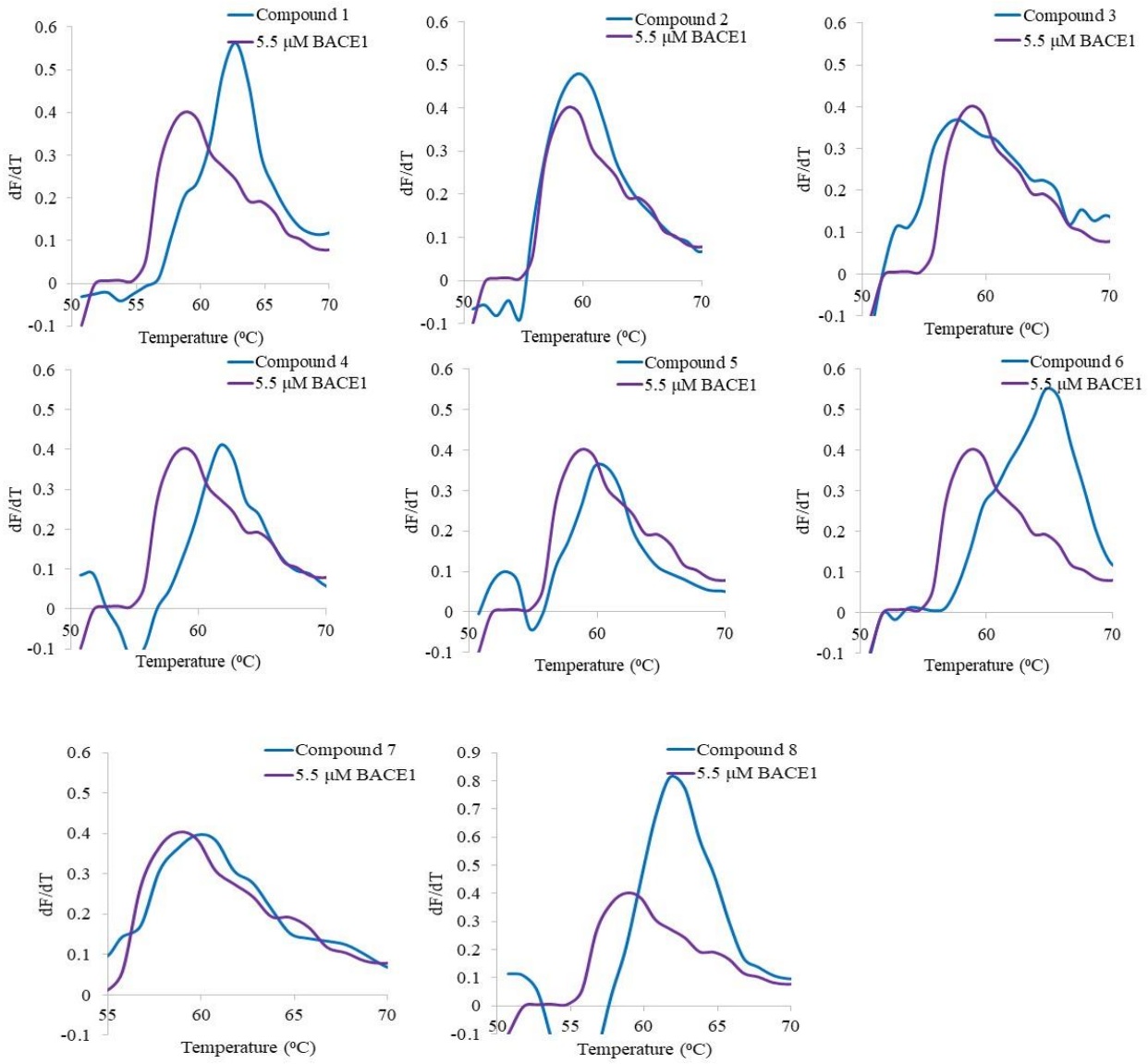


Figure 3.3: The shift in the thermal melt curves for BACE1 in the presence of compounds 1 – 8. Thermal shift assays were conducted from 25 ºC to 90 ºC and the first derivative curves of thermal melt curves were determined. The binding and stabilization of BACE1 was determined as a shift in the peak which indicates a change in the T_m of BACE1. Compounds 1, 2, 4, 5 and 6 were able to shift the peak, indicating that these compounds are able to bind and stabilize BACE1 at 10 µM when incubated for 30 minutes prior to the thermal shift assay. Compound 3 caused a shift to the left which suggests that this compound may be binding to another conformation of the protein. Compounds 7 and 8 were able to bind to BACE1 which is depicted by the shift in the peak.

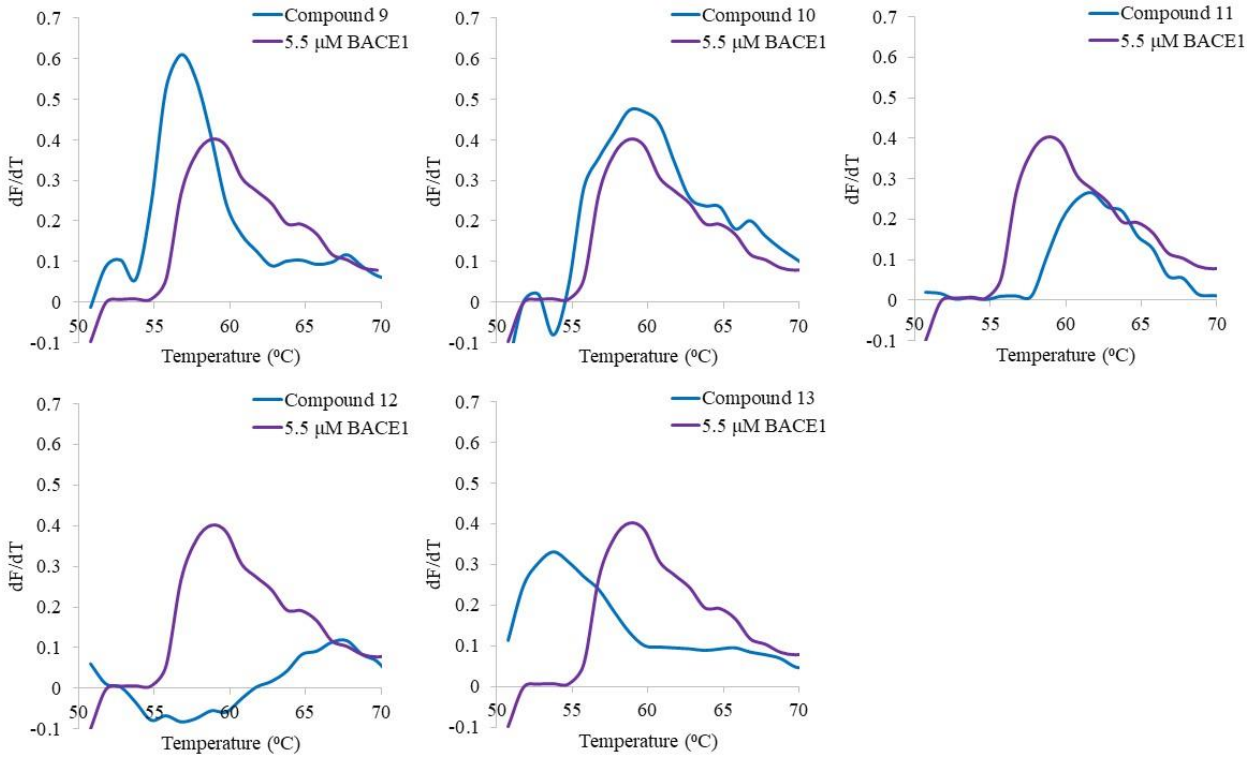


Figure 3.4: First derivative curves of the thermal shift assay for BACE1 in the presence of compounds 9 – 13. Compounds 9 – 10 evaluated in the thermal shift assay to determine probable binding and stabilization of the BACE1 protein. Derivative curves produced revealed that these structurally similar compounds 9, 11, 12 and 13 are able to shift the peak of the curve of BACE1. Comparison of the peak produced by BACE1 to the peak produced in the presence of compounds 9 and 13 revealed a shift towards the left. Compounds 11 and 12 (copper and vanadium metal centre, respectively) caused a greater shift in the peak towards the right as compared to compound 10. Compound 13 produced the greatest negative shift as compared to compound 9.

Comparisons between the T_m values obtained from the first derivative curves revealed that compounds 1, 2, 4, 5, 6, 7, 8, 10, 11 and 12 all caused significant ($p < 0.05$) changes in the melting temperatures as compared to the melting temperature obtained for BACE1 (Table 3.3). Compound 1, 2 and 4 – 6 shifted the peak by 5, 2.1, 3.6, 2.2 and 7.1 ºC, respectively (Table 3.3). Compound 6 which has a nickel metal centre produced the greatest shift in the T_m as compared to the gold-containing compounds similar in structure i.e. compounds 2 – 4 as well as the non-metal compound 1 (Table 3.3). Compounds 3, 9 and 13 produced negative ΔT_m values when the melting temperature of BACE1 alone was compared to the melting temperature in the presence of these molecules (Table 3.3). The negative shifts in the peak all occurred to one compound in the gold-containing, copper-containing and vanadium-containing groups of metal compounds tested in this study suggesting that these compounds are able to bind to different conformations of the protein

(Cimmperman *et al*., 2008; Reinhard *et al*., 2008). These conformations are the native or non-native forms of the protein. Binding of these compounds to either one of these conformations would show either a positive shift (native) or a negative shift (non-native) in the first derivative curves (Cimmperman *et al*., 2008; Reinhard *et al*., 2008). There was however, only one nickel-containing compound available in this study and therefore, a comparison of nickel compounds could not be made. Compounds 12 and 13 which have vanadium metal centres displayed the most interaction with BACE1 as the shifts in the T_m were greater than when compared to the gold and copper compounds but not to the nickel compound (Compound 6) (Figure 3.3 and 3.4). Compounds 6 and 12 in comparison to the other compounds yielded the biggest change in the T_m. Overall comparison of these compounds revealed that compound 12 yielded the greatest shift in the T_m (Table 3.3).

Table 3.3: Comparison of the melting temperatures of BACE1 with compounds 1 – 13.

Compound #	T_m (ºC) [a]	ΔT_m (ºC) [b]	*p*-value [c]
1	63.2 ± 0.64	5.0	0.009
2	60.3 ± 0.42	2.1	0.006
3	57.3 ± 0.63	-0.9	0.844
4	61.8 ± 0.05	3.6	0.001
5	60.4 ± 0.53	2.2	0.030
6	65.3 ± 0.71	7.1	0.005
7	61.2 ± 0.64	3.0	0.023
8	62.3 ± 0.71	4.1	0.016
9	56.3 ± 0.71	-1.9	0.941
10	59.9 ± 0.18	1.7	0.008
11	61.3 ± 0.707	3.1	0.026
12	67.3 ± 0.608	9.1	0.002
13	53.2 ± 0.799	-5	0.988

[a] Standard deviations were determined from the T_m from first derivative curves of the thermal melt curves from duplicate assays for each compound. [b] The ΔT_m was calculated by subtracting the T_m for BACE1 alone from the T_m produced in the presence of compounds. [c] *p*-values were determined by comparing the T_m of BACE1 to the T_m obtained in the presence of compounds 1 – 13.

In order to determine the relationship between the docking scores obtained from molecular docking and the T_m values obtained from the TSA a correlation plot was produced. The Pearson correlation

coefficient (r) was used to examine the strength and direction of the linear relationship between the two variables (Kilambi and Gray, 2017). The correlation coefficient value is within the range of +1 – -1 in which the larger the value is (towards the negative or positive), the stronger the coefficient relationship would be (Kilambi and Gray, 2017). Figure 3.5a reveals a weak positive correlation (r = 0.265) between the two variables. In addition, there was no significant difference ($p < 0.05$) in the r value obtained for the two variables (0.381) when compared to 0 (no linear relationship) in which the r value is too low for there to be a correlation between the docking scores and T_m values (Figure 3.5a). Evaluation of the docking scores and T_m values revealed two outliers from the data set. Eliminating these values from the correlation graph produced an r value of 0.767 which demonstrates a strong positive uphill relationship between the two variables (Figure 3.5b). Furthermore, a comparison of the r value to 0 (no linear relationship) revealed a significant difference ($p < 0.05$) between the r value and 0.

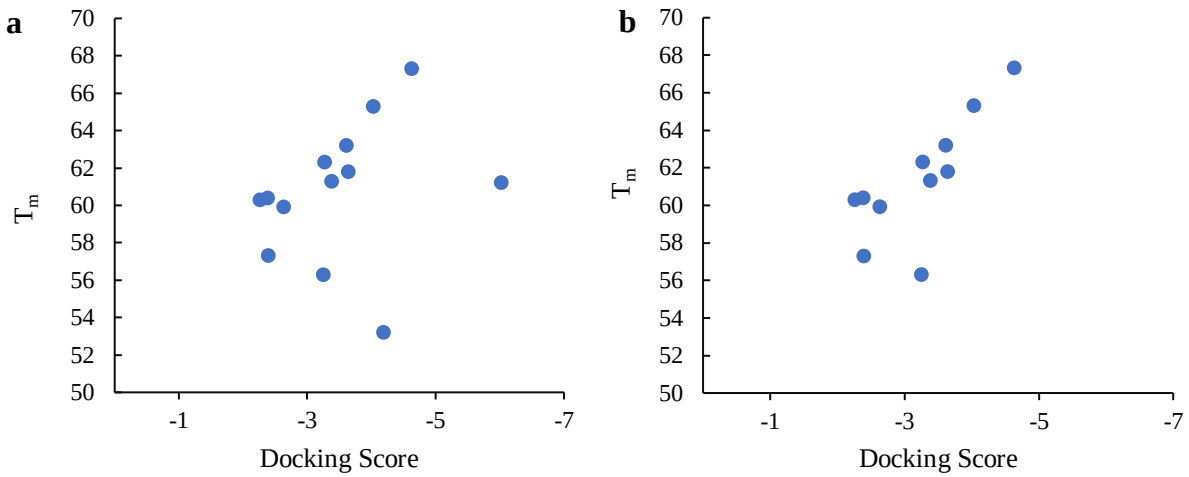


Figure 3.5: Scatterplot used to determine the Pearson correlation coefficient in which the relationship between docking score vs. T_m could be determined. A) Correlation coefficient (r) for the docking score and T_m of all 13 compounds was calculated to be 0.265. A positive r value demonstrates a positive relationship between the two variables. A *p*-value of 0.381 indicates that there is no significant difference from 0, i.e. r = 0 (no linear relationship). B) Removing the outliers from the correlation graph yielded a r value of 0.767 and a *p*-value of 0.0059. Therefore, correlation between the docking scores and T_m of compounds was a strong positive linear relationship which is significantly (0.0059, $p<0.05$) different from 0 (no linear relationship).

Therefore, it can be concluded that there is a relationship between the docking values and T_m obtained for compounds 1 – 13. Furthermore, a positive correlation value (r = 0.767) demonstrates that as the docking scores increases (negative increase) the T_m values increased (Figure 3.5). This

illustrates that the molecular docking of a ligand to a protein with a more negative docking score, i.e. more tightly bound to the protein, is indicative to the compounds having a greater effect on the melting temperature of BACE1.

3.4. DNA Cleavage Assay

The DNA cleavage assay was used to evaluate whether the 13 compounds used in this study would cause scissions to the supercoiled DNA. The cleavage of supercoiled to relaxed DNA by compounds was determined by the appearance of brighter bands for the relaxed form of plasmid (Figure 3.6). Warfarin (lane 2, Figure 3.6) displayed no cleavage activity at 500 μM as was expected. Caffeine (lane 3, Figure 3.6) did not show any cleavage activity at 500 μM as the bands for the relaxed form of the plasmid remained the same as the bands in lane 1 (Figure 3.6) which as compared to previously described results in literature was expected (Baranovsky *et al*., 2009; Banerjee *et al*., 2013). The appearance of a brighter band for relaxed DNA (lane 4, Figure 3.6) correlates with literature in that cisplatin cleaves DNA however, the presence of supercoiled DNA indicates that only partial cleavage was observed at 500 μM. The control compound ((2S,8R)-5-amino-2,8-dibenzyl-5-methyl-3,7-diazanonanedioate)copper(II)) (lane 5, Figure 3.6) at 500 μM yielded a brighter band for the relaxed form of DNA and complete cleavage of supercoiled DNA with complete degradation of the linear form of the plasmid which correlates to the findings in literature (Pamatong *et al*., 1996).

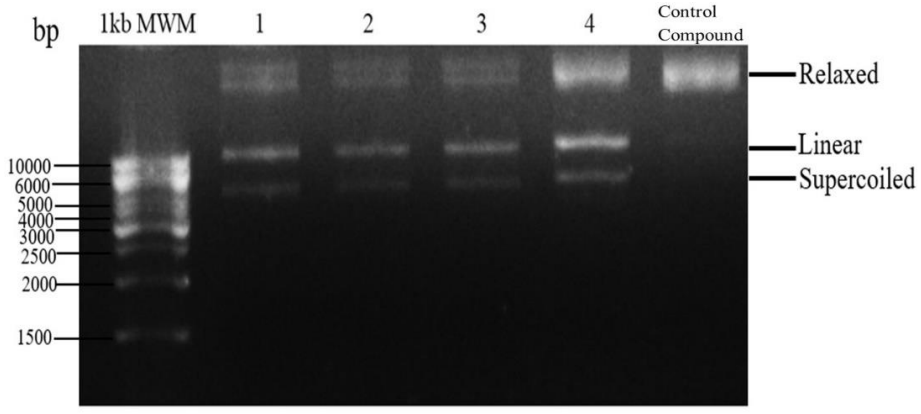


Figure 3.6: The effect of the control compound, warfarin, caffeine and cisplatin on supercoiled DNA. Cleavage conditions: Plasmid DNA (1 ng), H_2O_2 (60 μM), HCl (50 mM), NaCl (50 mM), 0.1% Triton X-100, loading buffer (6x, 4 μl) and compound (500 μM) incubated at 37 ºC for 30 minutes. A 1kb molecular weight marker (MWM) was used to determine the size of the supercoiled plasmid DNA. Lane 1: Plasmid DNA without cleavage buffer incubated at 37 ºC for 30 minutes. Lanes 2 – 5: The cleavage of plasmid DNA under cleavage conditions in the presence of compounds warfarin, caffeine, cisplatin and control at 500 μM, respectively.

Compounds 1 – 13 were subjected to the cleavage assay to determine if these compounds were able to bind and cleave DNA. Compounds 1 – 6, 8, 12 and 13 did not cleave DNA as the intensity of the bands for the relaxed form of DNA remained the same as compared to DNA alone (Figure 3.7a). Compound 7 exhibited partial degradation of the plasmid as indicated by the appearance of additional bands (lane 7, Figure 3.7a) and the decrease in the band intensity for the linear form of DNA (lane 7, Figure 3.7a). Analysis of the percentage band intensity per lane of the relaxed form of the plasmid for compound 7 yielded a *p*-value of 0.00381 (Figure 3.7b) when compared to DNA alone. Furthermore, compounds 9, 10 and 11 as indicated by the white arrows (Figure 3.7a) displayed partial cleavage of DNA by the appearance of brighter bands for the relaxed form and the presence of supercoiled DNA. These compounds produced *p*-values below 0.05 of 0.00084, 0.00136 and 0.00016, respectively, for the percentage of relaxed DNA which as compared to DNA alone indicated that these compounds are able to cleave DNA (Figure 3.7b). As with Figure 3.6, treatment of plasmid DNA with the control compound in Figure 3.7a, yielded complete cleavage of supercoiled DNA and had bright bands (white arrow) for the relaxed form of the plasmid and absence of supercoiled form as indicated by the red arrow (Figure 3.7a). The percentage band intensity of the relaxed form of the plasmid for the control compound yielded a *p*-value of 0.00022 when compared to DNA alone thus indicating significant cleavage of DNA (Figure 3.7b).

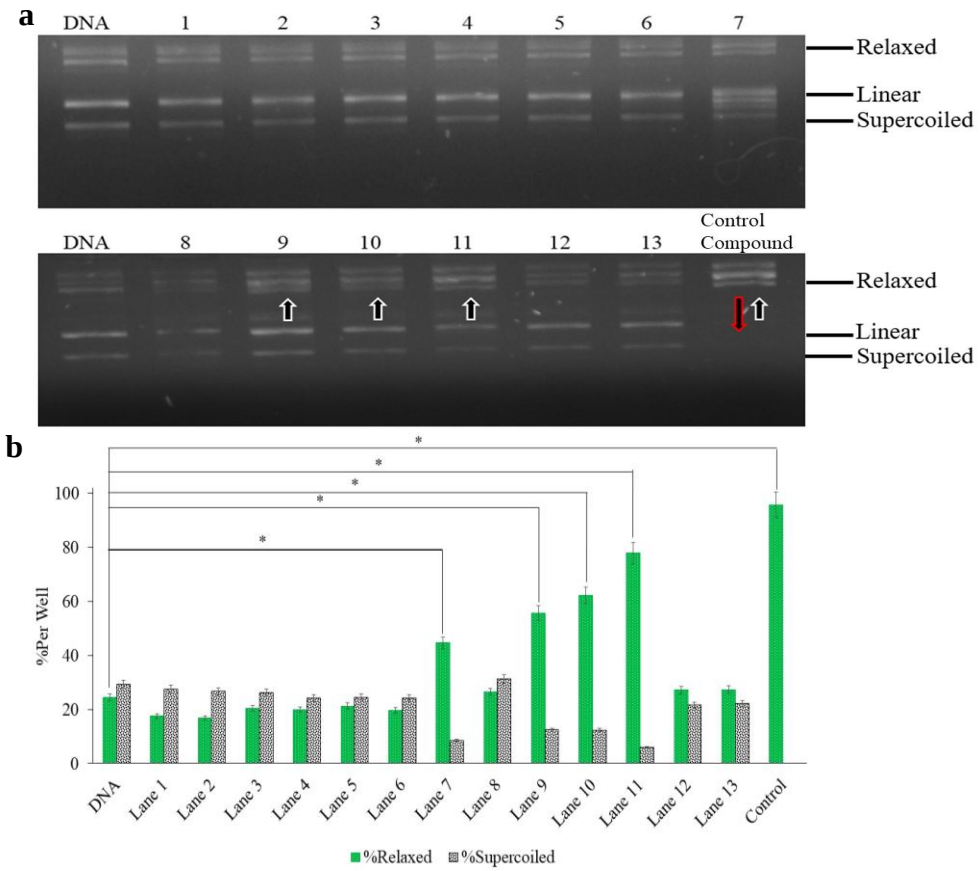


Figure 3.7: The effect of compounds 1 – 13 and control on relaxed, linear and supercoiled forms of plasmid DNA. Cleavage conditions: Plasmid DNA (1 ng), H_2O_2 (60 µM), HCl (50 mM), NaCl (50 mM), 0.1% Triton X-100, loading buffer (6x, 4 µl) and compound (500 µM) incubated at 37 ºC for 30 minutes. a) Lanes 1 – 13: Plasmid DNA in the presence of compounds 1 – 13 at 500 µM. DNA: Plasmid DNA without cleavage buffer incubated at 37 ºC for 30 minutes. White arrows indicate the increase in band intensity for relaxed DNA in the presence of compounds 9, 10, 11 and control at 500 µM. Red arrow indicates the complete cleavage of supercoiled DNA in the presence of 500 µM of control compound. b) Comparison of the percentage of relaxed and supercoiled forms of plasmid DNA per lane for compounds 1 – 13 and the control compound. Statistical analysis of the relaxed form of DNA in the presence of compounds as compared to DNA alone show statistical significance for compounds 7, 9, 10, 11 and control compound ($p < 0.05$).

Comparisons between the structure and cleavage activity of the compounds revealed that compounds with copper and platinum atoms had better cleavage activity than compounds with gold, nickel and vanadium atoms. Compounds 9, 10 and 11 presented cleavage activities against supercoiled DNA and are closely related to each other with a copper atom at the centre. Compounds 12 and 13 are closely related to compounds 9, 10 and 11 but have a vanadium metal centre and showed no cleavage activity to DNA. Compounds 1 – 6 are structurally similar with compounds 2 – 5 having gold metal centres and compound 6 having a nickel atom but all six

compounds yielded no cleavage activity to DNA. Compound 8 has a gold atom centre and is structurally similar to compound 7 and had no cleavage activity to DNA. Compound 7 does not have a metal centre but has 2 hydroxyl groups and yielded partial degradation of the linear form of DNA with slight cleavage of supercoiled DNA and no increase in band intensity for the relaxed form of the plasmid (Figure 3.7a). Comparison between the platinum and copper-based compounds revealed that copper-based compounds had the best cleavage activity which was illustrated by the complete cleavage of supercoiled DNA for the control compound (Figure 3.6 and 3.7) as compared to the cleavage displayed by the platinum-based compound cisplatin (Lane 4, Figure 3.6) which had partial cleavage of the supercoiled DNA.

3.5. Chymotrypsin Assay

The activity of chymotrypsin was determined as the increase in absorbance measured at 256nm over a period of 6 minutes. Control compounds chymostatin, warfarin, caffeine, quercetin and Congo red showed that the assay was effective in determining inhibition and aggregation amongst compounds (Figure 3.8). Chymostatin is a known inhibitor of chymotrypsin and exhibited complete inhibition of the enzyme at 20 μM at 25 ºC (Figure 3.8a) (Fuji *et al.*, 1985). The control compounds warfarin and caffeine displayed no inhibition of the activity of chymotrypsin in the absence of detergent which was expected (Figure 3.8a and b) as these compounds are not serine protease inhibitors. There was no effect on the compounds in the presence of detergent as the activity of chymotrypsin remained the same as in the absence of both inhibitor and detergent (Figure 3.8a and b).

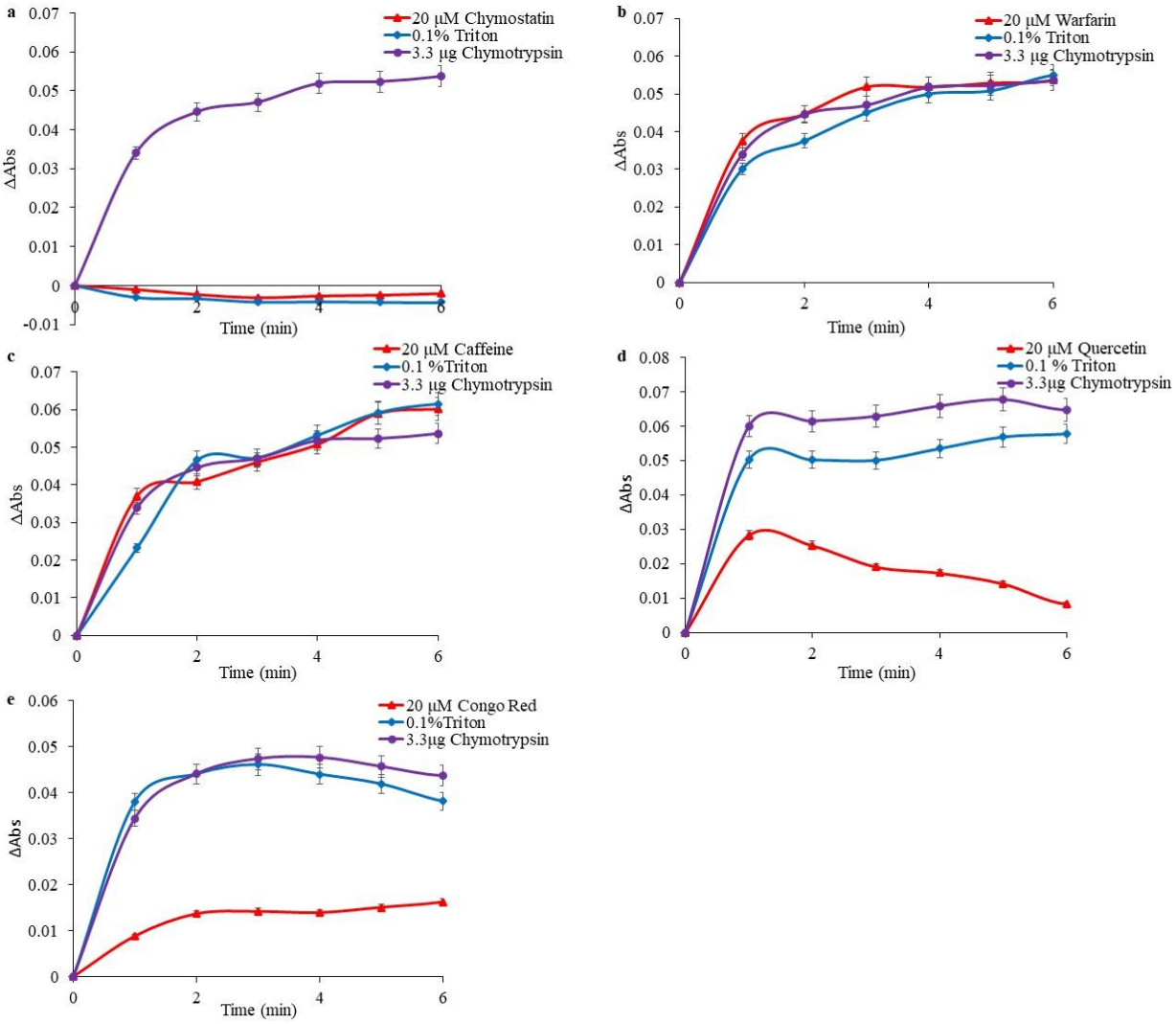


Figure 3.8: The effect of aggregating/non-aggregating inhibitors on the activity of chymotrypsin. Inhibition of chymotrypsin by compounds a) chymostatin, b) warfarin, c) caffeine, d) quercetin and e) Congo red was carried out at 20 µM at 25 ºC for 6 minutes. Absorbance was measured at 256nm and the activity of chymotrypsin was determined as the change in absorbance over time. Known chymotrypsin inhibitor chymostatin was used as a control for this study and yielded complete inhibition of the enzyme at 20 µM. The increase in activity in the presence of detergent (0.1 % Triton X-100) present for compounds quercetin and Congo red was the result of aggregation. Compounds warfarin and caffeine yielded no inhibition on chymotrypsin and therefore, addition of detergent had no effect on the compound.

Compounds 1 – 13 were evaluated as potential inhibitors of the serine protease as well as for the presence of aggregation in the presence and absence of 0.1 % Triton X-100. There was no apparent inhibition displayed by the compounds in both the absence and presence of detergent except for compound 7 and 13 (Figure 3.9). Compound 13 exhibited complete inhibition of chymotrypsin at 20 µM at 25 ºC with an increase in activity of the enzyme with the addition of detergent (Figure

3.9b). Compound 7 yielded a decrease in activity of chymotrypsin however, the addition of detergent to the reaction displayed no change in the inhibitory effect (Figure 3.9a).

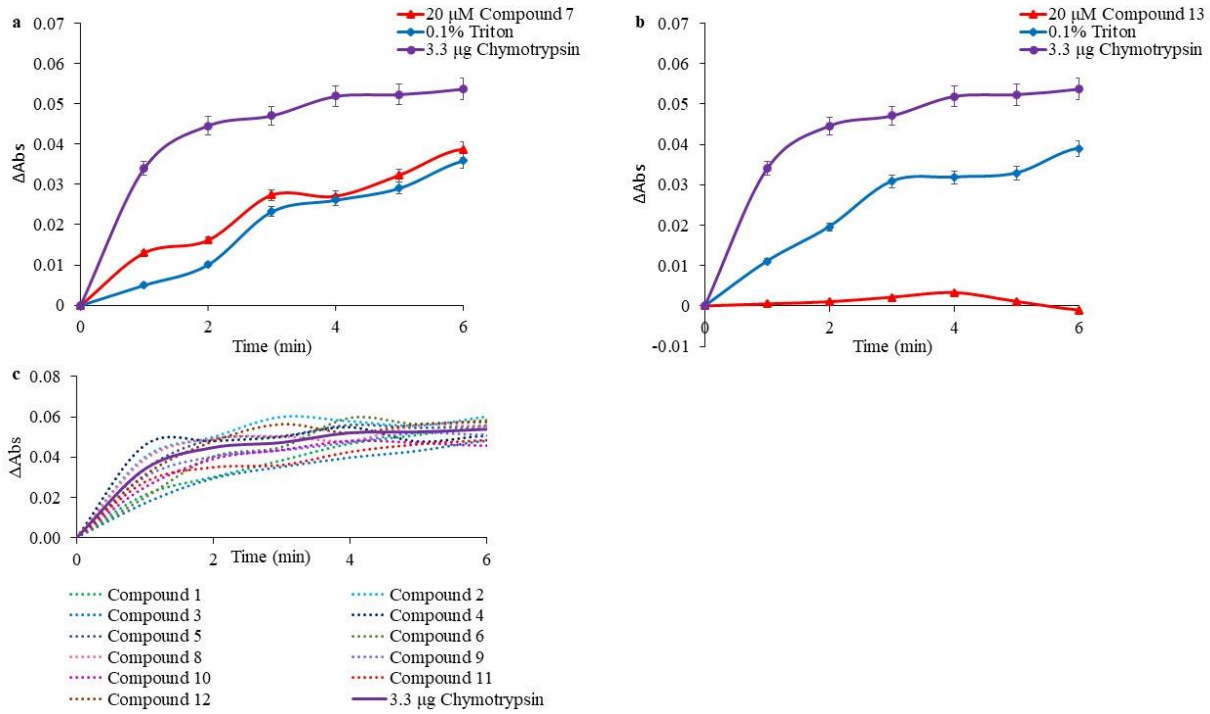


Figure 3.9: Evaluation of compounds 1 – 13 as potential serine protease inhibitors and aggregators. a) Evaluation of all compounds revealed compound 7 to be an inhibitor of chymotrypsin. The addition of detergent to the reaction yielded no increase in the activity of chymotrypsin. b) Compound 13 completely inhibited the activity of chymotrypsin. The compound also displayed aggregation as the inhibitory effect produced by this compound was reduced in the presence of 0.1 % detergent. c) Compounds did not show any inhibitory effect on the activity of chymotrypsin.

The percent activity of chymotrypsin inhibited by compounds 1 – 13 in the presence and absence of detergent presented comparable values in which to assess the degree of inhibition exhibited by these compounds specifically for compounds 7 and 13 (Table 3.4). Analysis of the percent activity of chymotrypsin inhibited by compound 7 revealed that this compound was able to inhibit the serine protease by 32.6 % of total activity of chymotrypsin alone. The percent activity of chymotrypsin in the presence of compound 13 was 4.6 % (Table 3.4). Therefore, this compound was able to inhibit the enzyme by 95.4 %. Activity of the enzyme increase by approximately 50 % with the addition of detergent when chymotrypsin is inhibited by compound 13. However, the

activity of chymotrypsin when inhibited by compound 7 in the presence of detergent did not yield any increase in activity (Table 3.4).

Table 3.4: The percent activity of chymotrypsin when inhibited by compounds 1 - 13 in the presence and absence of 0.1 % Triton X-100

Compound #	% Activity in the absence of detergent [a]	% Activity in the presence of detergent [a]
1	99.3	98.1
2	83.6	84.7
3	86.0	84.8
4	94.0	92.8
5	98.4	97.2
6	93.5	94.7
7	67.4	69.4
8	83.6	84.8
9	98.2	97.1
10	83.8	82.6
11	94.0	92.8
12	90.1	91.3
13	4.6	62.0
Chymostatin	1.7	1.5

[a] Percent activity was determined as the percent increase from the increase observed for chymotrypsin in the absence of detergent and compound.

There was no relationship found between the structure and the inhibitory activity of compounds 1 – 13 when evaluated for structure similarity. Compound 7 is structurally similar to compound 8 however, this compound lacks a metal atom (Table 2.1). Additionally, compound 13 is structurally similar to compound 12 with a vanadium metal centre (Table 2.1) however, compound 12 did not have any significant ($p > 0.05$) inhibition of the enzyme. Similarly, compound 8, which has a gold metal centre, yielded no significant inhibition ($p > 0.05$) of the enzyme when compared to compound 7.

CHAPTER FOUR: DISCUSSION

Establishing the 13 compounds in this study as potential BACE1 ligands was accomplished with the use of molecular docking and further verified by the TSA. In addition to establishing BACE1 ligands, the prediction of the physiochemical properties for the 13 compounds was also able to establish if compounds would be good oral drug candidates. Furthermore, these prediction values also provided insight on permeability and distribution to the CNS as well as through the blood brain barrier. This study was also able to reveal whether compounds would have any properties that would affect its efficacy such as toxicity and off-target binding.

4.1. Theoretical evaluation of compounds as potential oral drug candidates

The overall theoretical drug prediction obtained for the 13 compounds, as well as the control compounds cisplatin, warfarin, caffeine and verubecestat, all yielded results within the values stipulated for good drug candidates (Table 3.1). All tested compounds yielded drug-score values closer to 1 which further indicates that these compounds would be good oral drug candidates (Table 3.1). A better understanding of each parameter however, would help reveal insight into the physiochemical properties of each compound. Furthermore, the analysis would be able to determine if these compounds are able to pass through biological barriers in order to reach the intended target. There is, however, one additional biological barrier that is important to consider for compounds used in this study. This would be the blood brain barrier and as such, there are different requirements that compounds need to adhere to in order to be considered good CNS drug candidates (Gosh and Osswald, 2014).

The RO5 is a guideline in determining if compounds are good oral drug candidates (reviewed in Gosh and Osswald, 2014). Furthermore, compounds that abide by these rules must also ensure a balance of solubility and polar/hydrophobic properties that enable absorption and permeability so that proposed drug candidates would be able to reach the intended target (reviewed in Gosh and Osswald, 2014). However, it is important to note that compounds need to also adhere to metabolism, excretion and toxicity requirements as these also play an important role in the development of new drug candidates (reviewed in Gosh and Osswald, 2014).

4.1.1. Evaluation of solubility and absorption

Solubility and absorption play an important role in the effectiveness of a drug molecule (reviewed in Waring, 2010 and Wan, 2016). Therefore, compounds that have not met two or more of the requirements stipulated by the RO5 should not be considered as good oral drug candidates (reviewed in Waring, 2010 and Wan, 2016). This is because the RO5 assumes that all oral drugs are absorbed and have high oral bioavailability (reviewed in Waring, 2010 and Wan, 2016). As such, compounds that have not met these requirements would have solubility and absorption problems, which would affect the effectiveness of a drug (reviewed in Waring, 2010 and Wan, 2016). Therefore, an overall RO5 score was given to the compounds used in this study in order to estimate their potential as oral drug candidates. Seven compounds from the 13 compounds evaluated in this study yielded scores of 4 out of 4 which suggested that these compounds would be good oral drug candidates (Table 3.1). Compounds 2, 4, 8 and 10 – 13 produced a score of 3 owing to their molecular weights being above 500 g/mol. Compound 8 had the highest molecular weight out of these 6 compounds (Table 2.1). The large molecular weights of these compounds would introduce problems which include passing through biological barriers and reaching the intended target (reviewed in Waring, 2010 and Wan, 2016). In particular the large molecular weights of these compounds would pose problems in traversing the blood brain barrier and as such, compounds 2, 4, 8 and 10 – 13, should not be considered a good CNS drug candidate.

One of the major factors in the evaluation of solubility and absorption, is the logP value (reviewed in Waring, 2010 and Wan, 2016; Arnott *et al*., 2013). This is due to the fact that, lipophilicity of a compound affects many aspects of absorption, distribution, metabolism and excretion and all these properties contribute to the efficacy of a drug molecule (reviewed in Waring, 2010 and Wan, 2016). Poorly soluble compounds require more time and effort in the developmental stages of drug discovery as formulation of these new chemical compounds have low aqueous solubility (reviewed in Waring, 2010 and Wan, 2016). Furthermore, in order for compounds to reach a desirable concentration in the system, the solubility of the compound needs to be increased and as such, a lower logP is more desirable (reviewed in Waring, 2010 and Wan, 2016). Therefore, evaluation of compounds would provide insight on a molecules efficacy as an oral drug candidate (reviewed in Waring, 2010 and Wan, 2016). The clogP value produced by all 13 compounds were <5 which was in accordance to the RO5 (Table 3.1). However, some studies have suggested that to reach optimum solubility without compromising other factors, a logP of <3 would be a better estimation

of solubility to ensure compounds reach therapeutic plasma concentrations after administration (reviewed in Waring, 2010 and Wan, 2016). When compared to the estimated logP value, compounds 1 – 7 all had values below 3 with compound 9 having a clogP value of 3 (Table 3.1). Furthermore, compounds with either a nickel or gold metal centre had logP values <3 with compounds containing either a copper or vanadium metal centre having logP values 3 and above (Table 3.1). There is, however, no association to be found between the molecular weight of a compound and the logP value observed for compounds in this study. This suggests that, compounds with a higher molecular weight are soluble but may possess permeation problems associated with the size of the molecule.

4.1.2. Permeability and distribution

Permeability of a compound is essential for effective delivery to the appropriate tissue or site of action (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). This includes crossing of a lipid membrane or cellular barrier (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). In order for compounds to pass through these biological barriers, they need to have well suited physiochemical properties (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). There are many factors that contribute to a compounds permeability and this includes the number of hydrogen bond donors and acceptors, clogP and TPSA (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). A compound with a high number of hydrogen bond donors (>5) or acceptors (>10) is likely to exhibit lower permeability due to the increased energy requirement needed to break hydrogen bonds when a molecule moves from an aqueous environment to the lipid membrane (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). The 13 compounds tested in this study had <10 hydrogen bond acceptors and <5 hydrogen bond donors as stipulated by the RO5 (Table 3.1). However, compounds intended to reach the CNS as well as cross the blood brain barrier require an optimum of <7 hydrogen bond acceptors and <3 hydrogen bond donors (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). Compounds 7 and 12 were the only two compounds predicted to have hydrogen bond acceptors >7 (Table 3.1). In addition, compound 7 had hydrogen bond donors >3 (Table 3.1). This indicates that these compounds may have permeability problems, especially with oral administration, as the pathway from the intestinal

tract into the CNS would be difficult (Table 3.1) (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016).

The permeability can also be associated with TPSA values as the CNS drugs on the market tend to have lower polar surface areas (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). The evaluation of the TPSA values for oral drug candidates is an addition to the RO5. This rule states that the optimal TPSA values for CNS drug candidates should be <60 – 70 Å (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). Compounds 1 – 4, 6 and 9 all have TPSA values within this range indicating that these compounds have lower polar surface areas (Table 3.1). In addition, control compounds cisplatin, warfarin and caffeine all have TPSA values within the stipulated range (Table 3.1). Interestingly, verubecestat has a TPSA value of 126.13, which is higher than the recommended value. However, this compound still adheres to the rule of <140 Å stipulated by Veber (Veber *et al.*, 2002). In addition, the number of rotatable bonds that allows flexibility of a CNS drug should be <8 (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). All test compounds including control compounds cisplatin, warfarin, caffeine and verubecestat have rotatable bond counts below 8 suggesting that these compounds would, on this analysis alone, be able to pass through the CNS lipid layer and penetrate the blood brain barrier.

Lipophilicity of a compound is another factor that influences permeability and distribution in addition to the number of hydrogen bond donors and acceptors and the total TPSA. It is proposed that the decrease in permeability of a compound is in proportion to its lipophilicity (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). An optimum clogP value was proposed from comparing all marketed CNS drugs (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). This evaluation suggested that clogP values between 1.5 – 2.7 would achieve both good solubility and permeation results without introducing adverse events (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). The 13 compounds in this study, adhered to this criterion and as such, indicated that these molecules would have efficient permeability of the CNS. The well-known CNS drug candidate verubecestat, produced a lower clogP value (0.48) than the stipulated range for CNS drug compounds and this can be associated to its adverse events and removal from clinical trials. However, the revised rules stipulated for CNS drugs by Lipinski in 1999 (Rankovic, 2015; Fernandes *et al.*, 2016) suggests that the clogP value be maintained at <5. Therefore, it can be suggested that compounds with clogP values >1.5 but not exceeding 5 would exhibit optimal CNS

drug properties. Furthermore, compounds with clogP values <1.5 can still be considered as potential CNS drug candidates (Rankovic, 2015; Fernandes *et al*., 2016).

Compounds adhering to the RO5 and Veber's rules are likely to be distributed through the CNS thus enabling it to reach the intended target. Increased lipophilicity would increase a compounds ability to bind to plasma protein and CNS tissue as well as increase permeation of the blood brain barrier (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). Furthermore, improved permeation of the blood brain barrier would also ensure that sufficient concentrations of the compound reaches the brain thus increasing its efficacy (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016).

4.1.3. Toxicity

One of the main factors that contribute to drug compounds being withdrawn or terminated in the drug discovery process is their adverse effects after administration (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). As such, it is important to eliminate compounds with potentially harmful physiochemical properties as to prevent these compounds affecting patients in clinical trials. The OSIRIS DataWarrior program was able to theoretically determine the toxicity effects of each compound based on their structural properties. The program evaluated these compounds according to different types of toxic events such as mutagenic, tumorigenic, reproductive effects and irritation. Compound 2, which is a gold metal compound, yielded high mutagenic and reproductive effects, suggesting that this molecule would not be suitable in drug discovery. Compounds 3 and 6 had high toxicity as reproductive and irritation molecules (Table 3.1). However, toxicity of a compound is dependent on the dosage administered and the concentration in the system. As such, effects of compounds with toxic properties may not exhibit immediate harmful effects upon gradual administration but rather after longer and extensive exposure to increasing concentrations of the molecule (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016).

Toxicity of a compound is also attributed to its lipophilicity as well as their TPSA values (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). This is due to the fact that lipophilicity is also associated with protein binding (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag,

2016). More lipophilic compounds tend to exhibit greater non-specific binding which leads to off-target toxicity (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). In addition, TPSA also plays an important role in the toxicity of a compound as a more polar compound can bind strongly with off target proteins (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). Therefore, the recommended TPSA value for compounds should be <80 Å to ensure compounds produced do not have any off-target binding (reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). The gold compounds, 1 – 6, which have gold metal centres and are structurally similar were predicted to have TPSA values <80 Å (Table 3.1). It is also important to note that, compounds with a high number of rotatable bonds would require TPSA value <140 Å, which is seen for compound 7 (Table 3.1).

4.2. Evaluation of metal compounds as potential BACE1 ligands

The predictive model for these compounds was based on the superimposed structure of compounds to the BACE1 X-ray model. These results indicated that compounds 1 and 7 had significant binding to the BACE1 protein suggesting that these compounds may be good ligands (Table 3.2). In addition, the molecular docking program was able to predict the distance between the compound and the active site. This prediction would provide an estimation on whether these compounds are capable of inhibiting BACE1 through binding to the active site. The active site or in the case of BACE1, the catalytic domain is marked by the centrally located Asp residues, Asp 32 and Asp 228 (Lo *et al*., 2004). This catalytic domain is responsible for the cleavage of APP (Lo *et al*., 2004). In addition, BACE1 also contains a flap-open conformation which is energetically stable due to the presence of multiple hydrogen bonds (Lo *et al*., 2004; reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). The binding of APP to the catalytic domain causes the flap to close thus reducing access to the hydrogen bonds (Lo *et al*., 2004; reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). The flap-closed conformation of BACE1 is stabilised by the formation of hydrogen bonds between Tyr71 and Trp 76 (Lo *et al*., 2004; reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). Selectivity of the enzyme is produced via the cleft which causes a bottleneck effect and favours flexible substrates (Lo *et al*., 2004; reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). The mechanism by which BACE1 cleaves its substrate has been used as the template for the development of drug candidates (Lo *et al*., 2004; reviewed in Waring, 2010;

Wan, 2016; Oo and Kalbag, 2016). BACE1 has been reported to exhibit the same catalytic process as other aspartic proteases (Lo *et al*., 2004). Therefore, current drug candidates for BACE1 were designed based on the transition-state mimetic concept (Lo *et al*., 2004). Ideally, a compound would need to bind to this site as to produce the recommended biological effect, in addition to having good adsorption, distribution, metabolism and excretion properties (Lo *et al*., 2004). Molecular docking studies of the 13 compounds revealed that the predicted binding location of compound 1 was closer to the active site as compared to the other compounds. This suggests that compound 1 has potential inhibitory function (Figure 3.1). In addition, Compound 1 was able to form hydrogen bonds to Asp residues, Asp 32 and Asp 228. It is known that the Asp residues, Asp 32 and Asp 228 are able to cleave a substrate due to the presence of a water molecule in this region (Lo *et al*., 2004; reviewed in Waring, 2010; Wan, 2016; Oo and Kalbag, 2016). It has also been proposed that, BACE1 catalyses peptide bond hydrolysis through an acid-base catalysis mechanism facilitated by the water molecule (Lo *et al*., 2004). The pyrrole groups ability to bind to these residues further suggests inhibitory probability of compound 1. However, metal compounds tested in the molecular docking studies did not bind close to the predicted active site or have any interaction with the catalytic domain residues. Furthermore, these compounds showed binding to other sites on the BACE1 molecule, suggesting that the binding of these molecules may cause other conformation changes to the protein to induce an inhibitory effect.

Compound 7 does not contain a metal centre but was also predicted to bind close to, but not directly to the active site. This was determined from the estimation of the distance between the surface area of the active site and compound calculation by the program (Figure 3.2). This molecule was able to form hydrogen bonds to Glu 364 and Asp 318 (Figure 3.2). Furthermore, this molecule was also able to form salt bridges with Glu 364 and cation- π interactions with Hip 362 residue (Figure 3.2). The different types of binding of the compound to the protein suggests that this compound may be an excellent ligand. However, the distance of the compound from the predicted active site suggests that this compound may not have inhibitory function associated to binding to the active site.

In addition to the interaction diagrams for the superimposition of compounds to BACE1, the docking scores calculated for these compounds also indicated promising protein ligands amongst the 13 compounds. It is important to note that docking scores for these compounds were determined from docking compounds to the whole protein and not particularly to the active site.

The compound with the highest docking score was compound 7 (Table 3.2), further suggesting that this compound is a good ligand for BACE1. However, this compound does not have a metal centre and as such does not reveal the nature of ligand potential of a metal compound. This is further implied as the structurally similar compound 8, which has a gold metal centre, had a lower predicted docking score in comparison to compound 7 (Table 3.2). In addition, comparing the docking scores between compound 1 to the structurally similar metal containing compounds, 2 – 6, also indicates that the predicted scores obtained for these molecules vary (Table 3.2). As such, comparisons of compounds in terms of their metal centres revealed that compounds with a vanadium metal centre had the highest docking scores (Table 3.2). However, the gold metal compound 4, produced a docking score higher than the vanadium metal compound 11 (Table 3.2). It is also apparent that compound 12 had the highest docking score from all 13 compounds evaluated. This suggests that this compound has the most potential to be a ligand despite not binding to the active site of the BACE1 protein.

Docking compounds to the BACE1 molecule revealed that these compounds have good potential as drug candidates based on their predicted docking scores alone. In addition, TSA results revealed that these test compounds were able to bind to the intended target at a pH of 7.4 (Figure 3.3 and 3.4). The TSA was used as a way of determining the binding of the test compounds as well as provide *in vitro* evidence for binding to BACE1. In addition, the TSA helped determine the effectiveness of the molecular docking studies conducted on these metal compounds. Analysis of the first derivative curves produced from the TSA revealed that all compounds tested in this study was able to interact with the BACE1 which was indicated by the shift in the peak of the curve thus, representing a change in the thermal unfolding of the protein (Cimmperman *et al*., 2008). A change in the T_m obtained from the first derivative curves of BACE1 in the presence of a compound is indicative of binding and stabilization of the protein by the ligand (Cimmperman *et al*., 2008). Even a narrow change in the T_m from that of BACE1 alone (58.2 ºC) is indicative to the effect of the compound to the conformation of BACE1. Analysis of the curves for compounds 1 – 8 displayed changes in the peak (Figure 3.3). In addition, the copper compound, 9, also displayed a distinctive shift in the peak but no discernible shift could be determined from the curves for compound 10 (Figure 3.4). Furthermore, the first derivative curve analysis also revealed that some compounds had negative shifts in the peak, i.e. the melting temperature for compounds 3, 9 and 13 had T_m values lower than the T_m obtained for BACE1 alone (Table 3.2). These shifts relate to

the preferential binding of compounds to different conformations of the BACE1 protein (Cimmperman *et al*., 2008; Reinhard *et al*., 2013). Binding of a ligand to a protein always exhibits a stabilizing effect (Cimmperman *et al*., 2008; Reinhard *et al*., 2013). As such, a ligand may bind to either the native or non-native form of the protein (Cimmperman *et al*., 2008; Reinhard *et al*., 2013). In addition, ligands may also bind to a partially unfolded form of the protein (Cimmperman *et al*., 2008; Reinhard *et al*., 2013). This may be due to a non-homogenous solution which contains different conformations of a protein (Cimmperman *et al*., 2008; Reinhard *et al*., 2013). As such, if a compound binds to the native form of the protein then the T_m produced by this compound would be higher than that of the non-native protein as this conformational state has lower energy and would be the most abundant in a mixture (Cimmperman *et al*., 2008; Reinhard *et al*., 2013). Alternatively, if a compound prefers to bind to the non-native or partially unfolded conformation of a protein, which is less populated in the mixture, then this would cause a decrease in the T_m (Cimmperman *et al*., 2008; Reinhard *et al*., 2013).

Comparison of the gold compounds revealed that compound 6 yielded the largest difference in the T_m and as such, this compound would be a good ligand for the BACE1 protein. Evaluation of the copper compounds showed that compound 10 produced the largest difference (Table 3.2). Overall comparison of the T_m differences from the T_m for BACE1 revealed that compound 12 produced the largest difference in T_m when compared to BACE1 alone, suggesting that this compound has good ligand potential.

Evaluation of the T_m in comparison of the docking scores obtained showed that compounds with a greater shift in the peak had a higher molecular docking score apart from two compounds. The correlation graphs showing the relationship between the docking scores and the T_m yielded a positive r value when the outlying compounds were removed (Figure 3.5). This means that a greater shift in the peak for the first derivative curves of the TSA, representing a binding affinity of the compound to the protein, is directly proportional to a more negative docking score (Figure 3.5). Therefore, when looking at this correlation between the two values, compound 12 appears to be the most effective ligand to the protein.

A comparative analysis of the molecular docking, TSA and physiochemical properties of the test compounds in this study would provide an overview as to whether these metal-based compounds are suitable candidates as ligands for the BACE1 protein. As such, evaluation of the

physiochemical properties of the compounds revealed that compound 12 had the highest docking score correlated to the TSA value, however compounds 7 and 13 which also had high molecular docking scores did not correlate with the T_m values. In addition, comparison of these compounds to the predicted values ascertaining to their physiochemical properties allowing for blood brain barrier penetration revealed that compound 7 had hydrogen bond donor/acceptor count and TPSA value count above the stipulated value. In addition, both compounds 12 and 13 had molecular weights and TPSA values above 400 g/mol and 80 Å, respectively, with compound 12 having hydrogen bond acceptors above 7 (Table 3.1). Therefore, these compounds would not be suitable CNS drug candidates as their ADME properties would not be beneficial. Excluding these compounds would mean that the molecule with the highest change in T_m as well as the highest negative molecular docking score would therefore be compound 6. In addition, this compound has all the physiochemical properties associated with a CNS drug candidate and therefore based on this evaluation alone would be the best suited ligand. Based on the molecular docking and TSA results, compounds 12 would have been the preferred compound to be used as a BACE1 ligand. However, association of its physiochemical properties to its efficacy as a CNS drug candidate suggests that, although this compound binds to BACE1, there would have been permeability and distribution problems which would prevent it from reaching its intended target. Therefore, analysis of compounds with the molecular docking and TSA is beneficial in determining potential ligands to an intended target. In addition, these assays support each other in that molecular docking can validate results from the TSA and vice versa, however, these assays would require further information in order to determine good drug candidates especially compounds considered for treatment in the CNS.

4.3. Determining potential DNA cleavage or aggregation in metal compounds

Metal based compounds have been extensively studied as anticancer drugs (reviewed in Ndagi *et al*., 2017). The platinum-based compound cisplatin was one of the first metal-based compound used in anticancer therapy (Maheswari *et al*., 2007; da Silveira *et al*., 2008). One of the mechanisms of action of cisplatin was its ability to induce DNA unwinding and DNA-protein binding (Maheswari *et al*., 2007; da Silveira *et al*., 2008). However, due to the adverse effects outlined for this drug, new drugs based on different transition metals have been extensively studied

(Maheswari *et al*., 2007; da Silveira *et al*., 2008). The use of other transition metals in development has produced many beneficial compounds as potential anticancer candidates with cytotoxicity associated with interactions with DNA (Maheswari *et al*., 2007; da Silveira *et al*., 2008). Metal-based compounds being developed include transition metals such as copper, gold, nickel and vanadium. The DNA cleavage assay is a simple method that enables the evaluation of compounds as potential cleavage molecules that could prove potentially harmful when used as a drug (Maheswari *et al*., 2007; da Silveira *et al*., 2008). The use of biological assays to detect the binding of compounds to DNA have produced many methods and all these methods have been able to identify different modes of action as well as passive or direct binding to DNA (Maheswari *et al*., 2007; da Silveira *et al*., 2008). The alteration of DNA by these compounds have different mechanisms of action such as, causing strand breaks, alkylation or oxidation or by the formation of crosslinks (Maheswari *et al*., 2007; da Silveira *et al*., 2008). In addition, interaction of these compounds with DNA can ultimately lead to cell death thus making these compounds good anticancer candidates (Maheswari *et al*., 2007; da Silveira *et al*., 2008). This is also true for compounds that are aggregators in that sequestering of proteins could have detrimental adverse effects. Therefore, for this study compounds were analysed as potential DNA cleavage and aggregating compounds which would relate to the toxicity of this compound. As such, the test compounds evaluated using the DNA cleavage assay revealed that compounds 7, 9, 10 and 11 were able to cleave supercoiled plasmid DNA (Figure 3.7). This was represented by the increase in the brightness in the band for relaxed DNA. The cleavage of plasmid DNA by these compounds in comparison to the other test compounds as well as the control compound indicated that only metal compounds containing copper had cleavage activity (Figure 3.7). In addition, only compound 13 showed to have increased activity in the presence of detergent (Table 3.4) thus indicating that this is an aggregating compound.

4.3.1. DNA cleavage in copper compounds

The activity of the copper compounds used in this study correlates well with studies that have been conducted on other copper compounds (Maheswari *et al*., 2007; da Silveira *et al*., 2008). The studies conducted on these copper compounds have found that there is significant cleavage of double-stranded DNA by these molecules, specifically, those containing copper (II) atoms as well

as imine structures (Maheswari *et al*., 2007; da Silveira *et al*., 2008). Compounds that contain both imines and a copper atom have the ability to intercalate between the bases of DNA (Maheswari *et al*., 2007; da Silveira *et al*., 2008). It was also found that these compounds were able to participate in catalytic cycles by reacting with reducing and oxidizing agents present in solution. In addition, interaction of these compounds with the reducing and oxidizing agents are able to release ROS that also cause DNA damage (Maheswari *et al*., 2007; da Silveira *et al*., 2008). It was also reported that these compounds were able to interact with membranes and organelles as delocalized lipophilic cations (Maheswari *et al*., 2007; da Silveira *et al*., 2008). Therefore, copper compounds have shown to have very good interactions with not only DNA but have ligand-protein interactions as well (Maheswari *et al*., 2007; da Silveira *et al*., 2008). This makes copper compounds multi-faceted in that they have a wide range of targets. Furthermore, it was also found that the degree of cleavage observed for copper compounds varied even with compounds that were structurally similar (Maheswari *et al*., 2007; da Silveira *et al*., 2008). The possibility of structural differences between compounds containing the same metal centre may play a role in the functioning of metal-based compounds and cleavage activity (Maheswari *et al*., 2007; da Silveira *et al*., 2008). The presence of the metal centre may not always provide good DNA cleavage activity but may influence the binding of the compound to proteins (Maheswari *et al*., 2007; da Silveira *et al*., 2008). In this study, copper compounds had significant binding to DNA and the BACE1 protein but also displayed slight specificity to BACE1 when compared to the chymotrypsin assay as there was no inhibition of activity in the presence of these compounds (Figure 3.10). In addition, these compounds had good absorption and permeation capabilities determined from the calculated values of clogP (Table 3.1). Therefore, these compounds would have good anticancer capabilities in addition to being good BACE1 ligands. However, these compounds would not be suited as BACE1 drug candidates as their ability to bind to both DNA and BACE1 introduces non-specificity and can be associated with increased toxicity not suitable when intended to be used to pass the brain blood brain barrier.

Further analysis of the banding pattern of the compounds reveals that the appearance of brighter bands for the relaxed form of the plasmid is indicative of the molecules ability to bind to one strand of DNA causing a scission (Maheswari *et al*., 2007; da Silveira *et al*., 2008). There was no apparent increase in the band intensity for the linear form of the plasmid in the presence of the copper compounds, which suggests that the plasmid was not subjected to double-strand cleavage as

compared to the banding pattern of compound 7 which indicates multiple binding sites represented by addition banding patterns (Figure 3.7). However, in the published study conducted on the control compound, it was suggested that the rate at which the compound nicked the supercoiled DNA to scission of both strands of the plasmid was faster (Pamatong *et al*., 1996). This would therefore explain the appearance of brighter bands for relaxed rather than linear DNA (Pamatong *et al*., 1996). In addition, cleavage of DNA is supposedly done in a non-random event due to the appearance of a single band as random scission would cause the appearance of multiple fragments (Pamatong *et al*., 1996). Copper compounds appear to have no additional fragments present when compared to DNA alone (Figure 3.7a).

Furthermore, the lack of linear DNA in the presence of the control compound can be attributed to either the disintegration of DNA due to incubation periods or to scission of linear DNA by the molecule to smaller fragments that are able to move further along the gel (Figure 3.7a). In addition, the disappearance of the supercoiled band indicates that scission of DNA at 500 μM is effective. Time dependent and concentration studies reported in literature for this molecule have suggested that this molecule is effective at 123 μM and at pH 7.4, however, reaction conditions also play an important role in the activity of the compound which would explain the differences in the results produced in this study (Pamatong *et al*., 1996).

4.3.2. DNA cleavage and aggregation in gold, nickel and vanadium metal compounds

Studies have described the activity of compounds containing transition metals such as gold, nickel and vanadium (Pamatong *et al*., 1996; Stemmler and Burrows, 2001; Marcon *et al*., 2002; Matkar *et al*., 2006; Maheswari *et al*., 2007; Kong *et al*., 2008; da Silveira *et al*., 2008). These studies have all reported that compounds with these transition metals were able to bind to and cleave DNA (Pamatong *et al*., 1996; Stemmler and Burrows, 2001; Marcon *et al*., 2002; Matkar *et al*., 2006; Maheswari *et al*., 2007; Kong *et al*., 2008; da Silveira *et al*., 2008). The mechanisms of action for these compounds varied according to their structural properties as was seen with the copper compounds (Pamatong *et al*., 1996; Stemmler and Burrows, 2001; Marcon *et al*., 2002; Matkar *et al*., 2006; Maheswari *et al*., 2007; Kong *et al*., 2008; da Silveira *et al*., 2008).

Gold based compounds have reported weak interactions with DNA which was attributed to the interactions that have to occur for these molecules to produce a biological result (Marcon *et al*., 2002). This is a result of gold occurring in either the +1 or +3 oxidation states with the gold (I) state being more stable than gold (III) in aqueous environments. The cellular processes may be different for gold (I) compounds and therefore the cytotoxicity of these molecules may be compromised (Marcon *et al*., 2002). This is indicative of gold (I) compounds being pro-drugs and as such, requiring of a series of chemical transformations in order to generate a pharmacologically active state (Marcon *et al*., 2002). One of the most widely used gold compounds available on the market is auranofin, which is used in the treatment of rheumatoid arthritis (Marcon *et al*., 2002; Roder and Thomson, 2018). The mechanism of DNA cleavage is dependent on the release of ROS rather than actual binding to the DNA strand and causing scissions (Marcon *et al*., 2002). However, whilst some articles have reported binding of some gold compounds to DNA, auranofin has not shown any direct binding (Marcon *et al*., 2002; Roder and Thomson, 2018). The gold compounds in this study may not have been able to cleave the plasmid DNA under these physiological conditions. Therefore, the absence of a brighter band for the relaxed form of the plasmid may not necessarily mean that this compound has no interaction with DNA. Interaction with DNA would therefore be passive rather than direct binding to the double-stranded plasmid. Furthermore, the *in vitro* assay does not consider the interactions that would occur in a cell which may be required by these compounds as suggested by the results from literature for other gold compounds (Marcon *et al*., 2002). Furthermore, reports for DNA cleavage in the presence of gold, nickel, copper and vanadium have all shown that the effect of pH, structure and mechanism of action of a compound all play an important role in the functioning of a compound in order to exhibit the intended biological reaction (Pamatong *et al*., 1996; Stemmler and Burrows, 2001; Marcon *et al*., 2002; Matkar *et al*., 2006; Maheswari *et al*., 2007; Kong *et al*., 2008; da Silveira *et al*., 2008).

There was no cleavage activity or aggregation present for compound 6 which contains a nickel metal atom. As with the gold-based compounds, studies conducted on molecules containing a nickel metal atom have also reported DNA cleavage activity (Matkar *et* al., 2006; Kong *et al*., 2008). Furthermore, these compounds were affected by the presence of hydrogen peroxide present in the reaction (Stemmler and Burrows, 2001; Matkar *et* al., 2006; Kong *et al*., 2008)). Nickel is known to be a potent carcinogen in both mammalian and animal cells (Matkar *et* al., 2006; Kong *et al*., 2008). It also causes chromosomal aberrations via, genotoxicity which includes induction

of sister chromatid exchanges (Matkar *et* al., 2006; Kong *et al*., 2008). However, nickel is an accumulative carcinogen as it is relatively non-toxic when present in common alloys but high levels of exposure to pollution can lead to nasal and lung cancer (Matkar *et* al., 2006; Kong *et al*., 2008). In addition, certain nickel (VII) compounds have various carcinogenic properties and have thus been studied as possible chemotherapeutic agents (Matkar *et* al., 2006; Kong *et al*., 2008). Although nickel is in the same class as platinum there is no significant evidence that suggests that this compound has good anticancer properties because it is considered a weak carcinogen (Matkar *et* al., 2006; Kong *et al*., 2008). Therefore, in order to achieve a biological reaction from nickel complexes there needs to be favourable interactions that allow either water-soluble or -insoluble molecules to accumulate thus producing a biological effect (Matkar *et* al., 2006; Kong *et al*., 2008). Therefore, compound 6, as with the gold compounds, would require modifications in order to be an active DNA cleavage agent.

Of the metal compounds investigated for DNA cleavage, vanadium compounds were reported to bind to and cleave DNA (Stemmler and Burrows, 2001). Vanadium compounds are able to affect metabolic control in cells (Stemmler and Burrows, 2001). In addition, vanadium compounds also influence the activity of the insulin receptor kinase (Stemmler and Burrows, 2001). As such, these metal compounds have been studied for use as anticancer agents and for the treatment of diabetes (Stemmler and Burrows, 2001). Although literature has stated that compounds containing this transition metal cleaves DNA, the results of the DNA cleavage assay conducted in this study has yielded contradictory results (Figure 3.7). Reports on vanadium (IV) compounds conducted in the presence of hydrogen peroxide has revealed that compounds may not always bind to the DNA strand but may cause DNA damage via the production of hydroxyl radicals, superoxide radicals and hydrolysis (Stemmler and Burrows, 2001). One of the main contributors to the activity of metal compounds described in this study is the presence of oxidation or reducing agents (Stemmler and Burrows, 2001). As such, the study of these vanadium compounds has reported that alteration of DNA in the presence of these compounds occur when hydrogen peroxide is used (Stemmler and Burrows, 2001). In addition, the correct pH for these compounds is also important for the appearance of hydroxyl radicals that would enable DNA cleavage (Stemmler and Burrows, 2001). Therefore, the absence of DNA cleavage in the presence of compound 12 and 13 may be attributed to limitations in the assay. However, the absence of DNA cleavage present for compounds suggests that these molecules may only have ligand-protein interactions. Furthermore, compound 12 shows

to have specificity to BACE1 as there was no inhibition of chymotrypsin in the presence and absence of detergent (Figure 3.10). However, compound 13 shows to have inhibited the activity of chymotrypsin in the absence of detergent. Furthermore, the activity of chymotrypsin increased in the presence of compound 13 and detergent suggesting that this compound is an aggregator (Figure 3.10). As such, compound 12 would be considered a good BACE1 ligand and potential drug candidate as indicated by the molecular docking, TPSA and DNA cleavage assays. However, as with the previous evaluation, the permeability and solubility problems associated with this compound suggests that this compound may not be a good CNS drug candidate but may provide benefits as a good drug candidate. Furthermore, the non-specificity of compound 13 suggests that this compound would contribute to adverse events if considered as a potential drug candidate. Off-target binding may cause toxicity problems in other organs of the body especially with compounds that have permeation problems. However, compounds that aggregate but have good permeation and distribution may also induce toxicity as they have equal opportunity to sequester off-target proteins vital for normal cell functions.

4.4. Future studies

The metal compounds evaluated in this study with the use of simple assays have indicated that they are good BACE1 ligands. In addition, these compounds had good physiological properties that can enable them to be considered as good CNS drug candidates. The overall assessment of these compounds allowed for a selection of the best compound that could be used as an oral drug candidate in addition to determining the best ligand for BACE1. Based on the results obtained from the individual assays, compound 6 had the best performance when compared to the other compounds. Evaluation of the metal-based compounds using the molecular docking software presented some limitations. However, paired analysis with the TSA was able to eliminate these limitations to provide a correct assessment of these compounds. The simplicity of these assessment tools enables fast but accurate evaluations of compound properties. However, further assessment of compounds in terms of their mechanism of action for both binding to BACE1 and DNA would have to be determined to prevent any unwanted effects in a cell. The chymotrypsin assay in addition to determining non-specificity amongst the compounds was also able to determine if

compounds had specificity to aspartic or serine proteases which was determined from the inability of these compounds to inhibit chymotrypsin. Therefore, further assessment into the different proteases would give additional information into the mechanism of action and would help in improving the assessment of non-specificity and determine true inhibitors of BACE1.

CHAPTER FIVE: REFERENCES

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