INVESTIGATING THE *IN-VITRO* ANTI-DIABETIC, ANTI-ALZHEIMER AND ANTIOXIDANT ACTIVITIES OF SCHIFF BASE VANADIUM COMPLEXES

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ABSTRACT

Diabetes mellitus (DM) and Alzheimer‘s disease (AD) characterised by their progressive debilitating effects have secured spots in the list of the leading causes of morbidity and mortality worldwide. Despite intensive research and clinically available drugs, finding a cure to both diseases has remained elusive. Several therapeutic strategies have been developed to manage these diseases but majority of treatment options result in various side effects and are merely management rather than curative therapies. Current oral therapeutic agents used in the treatment of diabetes may be helpful at the initial stages. However, as patients progress towards complete beta cell failure, the efficacy of these oral agents diminishes and dependence on insulin therapy becomes inevitable in other to maintain normoglycemia and prevent diabetic complications. Similarly, the impact of AD therapies on cognitive functions, quality of life and their clinical significance are marginal to non-existent. Common aetiological features of DM and AD have been established and oxidative stress and inflammation are examples of some of common linkages identified. Therefore, this study was conducted to identify possible drug candidates with a potential to target multiple pathways envisaged to bring about the desired therapeutic effect in DM and AD, see the graphical abstract in Appendix 6. This multitarget approach seeks to address drug-related interactions associated with polypharmacy but also seeks to find lead molecules with the potential to provide better pharmacological management compared to conventional therapies. Schiff base vanadium complexes were selected for this study due to their diverse application and reported biological activities. *In-vitro* studies were augmented with physicochemical and pharmacokinetic predictions to assess the druggability and physicochemical properties of the vanadium complexes, which plays a role in their overall activity, bioavailability and their safety profiles. Molecular docking simulation was carried out where indicated to assess the preliminary mode of action of these Schiff base complexes.

The three synthesized Schiff base vanadium complexes at 20, 40, 60, 80 and 100 µg/mL concentrations were evaluated *in-vitro* for inhibitory activity against α-glucosidase, α-amylase, and dipeptidyl peptidase-4 (DPP-4). These complexes were also evaluated for inhibitory activity against acetylcholinesterase, matrix metalloproteinase-1 (MMP-1) enzymes, advanced glycation end products and beta amyloid aggregation. The antioxidant capabilities of these compounds were also investigated. Reference compounds were used to validate the assay protocols and the data obtained by spectrophotometric/fluorometric analysis were converted to percentage inhibitions. Enzyme kinetic studies were conducted using the Lineweaver Burk

plot. One-way ANOVA was performed to test for statistical significance at p value ≤0.05 using GraphPad prism 6.

Statistically separate introduction of vanadium complexes showed significant inhibition as measured with α-glucosidase, DPP-4 and antioxidant assays. On the other hand, minimal inhibitory activity was observed for alpha amylase(α-amylase), acetylcholinesterase, beta amyloid aggregation and advanced glycation end products in comparison with the control. In contrast, enzyme activation was observed with MMP-1. The vanadium complexes were predicted to have drug-like characteristics according to Lipinski parameters; however, their predicted gastrointestinal absorption and blood brain barrier permeability was poor as shown by their locations on the boiled egg model and other generated parameters.

Of the three investigated vanadium complexes, vanadium complex 3 showed the most potential towards diabetes and Alzheimer‘s disease. Based on the results obtained it is evident that these complexes show good anti-hyperglycaemic and antioxidant activity with minimal anti-Alzheimer activity. This study provides evidence to suggest that the Schiff base vanadium complexes may be promising candidates to explore in the control and management of hyperglycaemia, oxidative stress related complications. However, their predicted physicochemical properties may hinder their progress to further phases of drug discovery. This calls for further investigation into alternative means to improve their chemical properties to streamline their inhibitory actions towards multiple targets implicated in AD and DM pathogenesis.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Alpha
Aβ	Amyloid beta
Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer‘s disease
ADME	Absorption, distribution, metabolism and excretion
AGEs	Advanced glycation end products
ANOVA	Analysis of variance
APP	Amyloid precursor proteins
BBB	Blood brain barrier
CNS	Central nervous system
DM	Diabetes mellitus
DPPH	Diphenyl picryl hydrazyl
DPP-4	Dipeptidyl peptidase -4
ECM	Extracellular matrix
FRAP	Ferric reducing antioxidant power
GH	Glucose homeostasis
GDM	Gestational diabetes mellitus
GIP	Glucose insulinotropic hormone
GIT	Gastrointestinal tract
GLP	Glucagon-like peptide.
GLUT	Glucose transporter
IC50	Concentration of drug that reduces enzyme activity by 50%
IU/ml	International unit per milliliter
IRS	Insulin receptor substrate
Km	Michaelis constant
MMP	Matrix metallo proteinases
NMDA	N-Methyl-d-aspartate
PTP	Protein tyrosine phosphatase
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species

RO5	Rule of five
SD	Standard deviation
µ	Micro
Vmax	Maximum velocity

CHAPTER 1: LITERATURE REVIEW

1. Introduction

Diabetes mellitus (DM) and Alzheimer‘s disease (AD) are two progressive chronic diseases characterised by increased prevalence with aging, a genetic predisposition, and comparable pathological features in the pancreatic islet and the brain (1). They are amongst the most challenging leading global health problems in the 21st century. Existing statistical data shows that in 2019 alone diabetes resulted in 4.2 million deaths globally. Approximately 463 million adults (20-79 years) are living with DM with numbers expected to rise to 700 million by 2045 (2). DM is a group of metabolic disorders commonly presenting with chronic hyperglycaemia and glucose intolerance, as a result of lack of insulin, defective insulin action, or both (3). Oral anti-diabetic agents used alone; or sometimes in combination with injectable insulin and supplemented with lifestyle modifications are strategies to treat DM. Without appropriate treatment options, this condition can result in secondary complications such as diabetic retinopathy, diabetic nephropathy and diabetic neuropathy (4). Anti-hyperglycaemic agents currently in use are associated with undesirable effects including episodes of hypoglycaemia, diarrhoea, lactic acidosis and increased risks of cardiovascular and hepatic hazards (5).

Similarly, 50 million people globally have AD or other types of dementia and is projected to reach 82 million in 2030 (6). AD is a slowly progressive disease of the brain that is characterised by impairment of memory, reasoning, planning, language, and perception regarded as cognitive functions (7). Despite the intensive investigation of mechanisms involved in the pathogenesis of AD, little has been achieved in terms of effective treatments or approaches to manage the condition. Two main drug classes available to manage AD include cholinesterase inhibitors and N-Methyl-d-aspartate (NMDA) antagonists. However, these existing drug therapies do not seem to significantly improve the outcomes of the disease; therefore, they are best viewed as palliative rather than disease-modifying treatments (8). Literature evidence exists to support the inter-relationship between AD and DM with several studies reporting that up to 81% of AD patients exhibit impaired fasting glucose and diabetes (1). The dysregulation of several similar cellular, molecular and metabolic processes has been identified as key players in the pathogenesis of both DM and AD and of most significance is glucose regulatory and redox pathways (9). These anomalies are present in the early stages, progressive as well as late stages of the diseases where they result in long-term complications. A few pharmacological drugs have proved to be beneficial, however shortfalls do exist, due to

several undesirable effects associated with these drugs and their ineffectiveness in managing these diseases on the long term. Therefore, the search and discovery of novel pharmacological modalities should be continued to increase the management spectrum which is important considering the shortfalls associated with conventional drugs. Vanadium complexes have gained interest in medicinal chemistry especially in the context of drug development showing promising activity against DM and AD as reported by several scientific papers (10).

This study is an attempt to address the issue of untoward effects and shortfalls in conventional anti-diabetic and anti-Alzheimer treatments by investigating Schiff base oxovanadium complexes as novel management strategies. These complexes were evaluated *in-vitro* for their potential biological activity through enzymatic and peptide assays. The purpose was to determine their potential multiple inhibitory properties in the treatment of DM, AD and their associated complications. This multi-targeted approach could make an excellent strategy to discover drug candidates as it helps address the issues that come with polypharmacy. Although polypharmacy is not necessarily synonymous with inappropriate treatment, it can in some cases lead to significant drug–drug interactions, synergistic toxicities or reduced efficacy (11). The outcomes of this study if successful may ultimately provide treatment options with better efficacy, improve drug adherence, foster better therapeutic outcome and improve patient‘s quality of life.

1.1 Glucose homeostasis in the body

Most body tissues and organs need glucose constantly as an important source of energy. Low blood glucose concentrations or hypoglycaemia can at worst case scenario cause seizures, loss of consciousness, and death, while chronic elevated blood glucose concentration or hyperglycaemia can result in vascular and organ damage (12). Therefore, blood glucose concentrations need to be maintained within narrow limits and this is known as glucose homeostasis(GH) (13). This balance is achieved through insulin-dependent and insulin-independent mechanisms that are triggered when blood glucose deviates from normal (14). Secreted by the pancreas in response to rising glucose levels, insulin promotes glucose uptake into insulin-sensitive tissues (e.g. adipose, muscle) while also inhibiting hepatic glucose production, lipolysis and reduction of glucagon levels (15). Glucagon opposes these effects. The blood glucose level is also sensed by the brain through the action of the autonomic nervous system (ANS). When blood glucose concentrations fall below the euglycemic level, rapid

activation of the autonomic nervous system triggers a counter-regulatory response to restore normoglycemia (16,17). Figure 1.1 below gives a graphical summary of the action of insulin on tissues and organs to maintain glucose homeostasis.

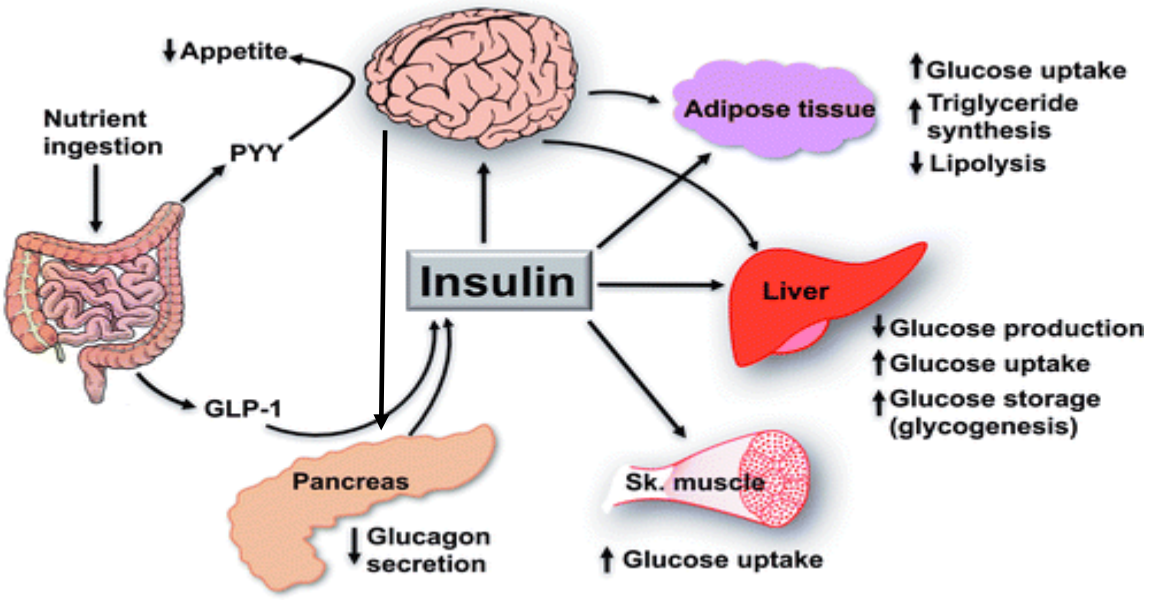

Figure 1.1: A network showing Insulin mediated glucose homeostasis on target cells and tissues of the body. Nutrient ingestion causes a rise in glucose concentration which triggers the glucagon like peptides in the GIT and the pancreas to secrete insulin which acts on the brain, liver and tissues to restore glucose homeostasis. Image adapted with slight modifications (18).

The mechanism of glucose homeostasis is a multicomplex process that involves various enzymes, hormones, tissues or organs which work together to ensure normoglycemia is always maintained . The gut plays its role by ensuring less glucose is produced from carbohydrate breakdown and the pancreas secretes the glucoregulatory hormones that act on tissues and organs to achieve the desired effect, while the brain partly co-ordinates these regulatory processes. Therefore, these target organs are commonly explored in therapeutic management of hyperglycaemia induced pathologies such as DM and AD. The physiological roles of some of these tissues and organs in maintaining GH are detailed below highlighting their contribution to the pathology of DM.

1.1.1 Digestion and absorption: The gut

The gut is an important organ that regulates GH by releasing various enzymes and hormones upon nutrient ingestion (19). The gut houses enzymes such as alpha amylase (α-amylase) and alpha glucosidase (α-glucosidase), which digest food to allow for efficient absorption. Pancreatic α-amylase is secreted by the pancreas into the small intestine and is a key enzyme that breaks down dietary carbohydrates and complex polysaccharides such as starch into oligosaccharides and disaccharides (20). The two major starches are amylose, a straight-chain α-1,4-linked glucose polymer, and amylopectin which is a branched starch with a backbone of α-1,4-linked glucose and α-1,6-linked glucose. Amylose and amylopectin account for 20 and 80% of dietary starch, respectively (21). The intestinal epithelium absorbs only monosaccharides, hence for the dietary starch to serve as a nutrient and energy source, it must first be hydrolysed into simple sugars (22). To achieve this, the membrane-bound α-glucosidase enzyme, localized in the brush border of the epithelium of small intestine catalyzes the digestion of these oligosaccharides and disaccharides into simple sugars e.g. glucose for easy absorption into the body (23). These simple sugars on absorption, enter the bloodstream raising blood glucose concentration; this is referred to as post prandial hyperglycaemia (24). In patients with diabetes, postprandial hyperglycaemia is most pronounced following a meal due to the absorption of glucose from the gastrointestinal tract. Therefore, inhibiting glucose uptake in the intestines may be beneficial for these patients to control the blood glucose concentration in the postprandial state (25). The α-glucosidase and α-amylase inhibitors e.g. acarbose and miglitol play a vital role in controlling hyperglycaemia by reducing the intestinal absorption of glucose, suppressing carbohydrate digestion, delaying glucose uptake and consequently, reducing blood glucose concentration (26,27). However, drugs in this class are often reported to cause diarrhoea, flatulence and other intestinal disturbances, which limits their use in clinical practice. Inhibition of these two carbohydrate hydrolysing enzymes is one of the therapeutic approaches for decreasing postprandial hyperglycaemia, hence the inhibitory effect of vanadium complexes on these starch hydrolysing enzymes were investigated.

Similarly, incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are secreted from the intestine on ingestion of glucose or nutrients to stimulate insulin secretion from pancreatic β- cells (28). GLP-1 suppresses endogenous glucose production, suppresses appetite, delays gastric emptying, and improves β-cell insulin sensitivity while the dominant action of GIP is the stimulation of glucose-dependent insulin secretion (29). These incretin hormones bind to their respective receptors on pancreatic β-cells to potentiate insulin secretion. This effect is mediated through elevation of intracellular cyclic adenosine monophosphate (cAMP) concentration and inhibition of

adenosine triphosphate (ATP)-sensitive K^+ channels, which together induces β cell exocytosis (30). Through these mechanisms, these two dipeptidyl dipeptidase (DPP-4) substrates ultimately maintain glucose homeostasis (31). DPP-4 is the enzyme responsible for N-terminal cleavage and inactivation of these incretin hormones (GLP-1 and GIP), and therefore its inhibition has been investigated as a way of ameliorating hyperglycaemia in diabetes (32).

1.1.2 Insulin secretion : Pancreas

The pancreas maintains blood glucose concentration through its various hormones, particularly glucagon and insulin. This is accomplished by the opposing and balanced actions of the hormones‘ glucagon and insulin (14). When blood glucose concentration rises, pancreatic beta cells secrete insulin which binds to its receptor on skeletal muscle and adipose tissue, facilitating insulin-dependent glucose uptake from the blood and storage into these tissues to ultimately lower blood glucose concentration (33). In hypoglycaemic conditions, glucagon is released from α-cells to promote hepatic glycogenolysis. In addition, glucagon drives hepatic and renal gluconeogenesis which results in increased endogenous blood glucose concentration (14).

1.1.3 Insulin signalling

After insulin is secreted, it binds to the insulin receptor at the cell surface and activates its tyrosine kinase activity, leading to autophosphorylation and phosphorylation of several receptor substrates, a process known as insulin signalling (34). Therefore, the molecular basis for insulin signalling involves tyrosine kinase activation (36). Insulin signalling plays a role in GH. Insulin binds to its cell surface transmembrane receptor and stimulates intrinsic protein tyrosine kinase activity, autophosphorylation, and subsequent tyrosyl phosphorylation of insulin receptor substrate (IRS) proteins; and these signalling proteins mediate the biological effects of insulin, which include glucose uptake, glycogen synthesis, lipogenesis, and cell growth (36). Protein tyrosine phosphatase (PTP) is an enzyme that dephosphorylates the insulin receptors and its substrates thereby inactivating the insulin receptors and terminating insulin signalling (37). The negative regulatory effect of PTP1B in insulin signalling pathway is a potential therapeutic target for T2DM. However, the development of efficient highly selective PTP1B inhibitors remains a challenge (38). The main challenges encountered with developing PTP inhibitors have been attributed to cell permeability and target selectivity. The

positive-charged active site prefers negatively charged molecules, which usually lack cell permeability (39). With regard to selectivity, since the active site of PTP enzymes is highly conserved across the family, most therapeutically relevant PTPs have a close homolog, that if inhibited, would have adverse effects (40). Interestingly, vanadium compounds e.g. Sodium orthovanadate have been established as PTP inhibitors. The orthovanadate ion has a tetra-coordinated structure and a negative charge, features that it shares with the phosphate group of PTP substrates. By mimicking phosphate, orthovanadate fits into the PTP active site stabilised by a complex network of hydrogen bonds and in this way, vanadate acts as a broad specific, reversible and competitive PTP inhibitor (41). Having established this mode of inhibitory action by vanadium complexes against PTPs, the inhibitory activity of the evaluated Schiff base vanadium complexes against the PTP enzyme was of interest leading to its investigation using molecular docking studies.

1.1.4 Glucose uptake in insulin targeted tissues

Insulin signalling mechanisms induces the translocation of the glucose transporter 4 (GLUT4) from intracellular membrane compartments to the plasma membrane, where it catalyses the uptake of glucose into adipose and skeletal muscle cells (42). Glucose enters the skeletal muscle cell via facilitated diffusion through the GLUT4 glucose transporter which is translocated from intracellular storage depots to the plasma membrane and T-tubules upon muscle contraction (43). GLUT4 expression is severely disrupted in individuals with type 2 diabetes mellitus (T2DM) and contributes to insulin resistance. Potential causes for resistance to insulin-stimulated glucose transport may be due to defective intracellular signalling of GLUT4 translocation in skeletal muscle from stored intracellular vesicles to active components of the plasma membrane (44). Increasing intracellular concentrations of GLUT4 can improve or even reverse T2DM. The goal is to modulate gene expression to increase total GLUT4 levels, and to promote the translocation of glucose secretory vesicles to the plasma membrane (45). Thiazolidinediones enhance insulin sensitivity and therefore promote glucose uptake by insulin-sensitive tissues (46).

1.1.5 Glucose modulation :The brain

The brain via the sensory action of neurons situated in hypothalamus and brainstem plays a pivotal role in the homeostatic regulation of glucose metabolism (47).The brain senses changes in nutrient and hormone levels and triggers negative feedback responses such as

stimulating pancreatic insulin secretion, hepatic glucose production, and glucose/fatty acid metabolism in adipose tissue and skeletal muscle and cellular glucose uptake to restore glucose homeostasis (48,49). When glucose homeostasis is not tightly regulated, hyperglycaemia may manifest leading to DM and AD which are discussed in detail in the next sections.

1.2 Diabetes mellitus

DM is a group of metabolic disorders commonly characterised by chronic hyperglycaemia resulting from an impairment in insulin secretion, defective insulin action, or both (3). Several distinct types of DM exist and are caused by a complex interaction of genetics, environmental factors and life-style choices (50). This disease condition and its complications give rise to micro and macrovascular diseases which affect eyes, kidneys, heart, blood vessels, nerves and also lungs via multi-complex pathways (51). DM is classified into type 1, type 2 DM and gestational DM. Type 1 DM accounts for 5 – 10% of all diabetic cases and commonly affects children, adolescents and can also occur later in life (52). In type 1 DM, the body‘s immune system destroys part of the pancreatic cells that produces insulin, resulting in insulin deficiency. The factors that initiate this autoimmune response are not known however islet resident macrophages, cluster of differentiation (CD4) and CD8 T-lymphocytes and insulin-specific autoantibodies have been implicated (53,54). Without a normal functioning beta cell, the pancreas is unable to respond to an increase in blood glucose concentration (55). Individuals with type 1 DM show classical symptoms of insulin deficiency such as polyphagia, polyuria, polydipsia, weight loss and diabetic ketoacidosis (56). Pharmacological treatment for type 1 DM is exogenous insulin.

Type 2 diabetes mellitus (Type 2 DM) accounts for approximately 90% of all DM cases and is influenced by genetic predisposition as well as lifestyle (57). The risk of developing this form of diabetes increases with age, unhealthy diet, obesity, and lack of physical activity (58). Type 2 DM can be characterised by both insulin resistance and insulin deficiency. Insulin resistance in type 2 DM patients increases the demand for insulin in target tissues. Sometimes the increased demand for insulin cannot not be met by the pancreatic β-cells due to the gradual destruction of these cells and a decrease in insulin secretion (59). This explains why some of the type 2 DM patients move from being insulin independent to becoming dependent on insulin overtime. This form of diabetes frequently remains undiagnosed for many years since the

hyperglycaemia develops gradually, and at earlier stages is often not severe enough for the patient to notice any of the classic symptoms of diabetes (56).

Gestational diabetes mellitus (GDM) is as a result of hyperglycaemia that develops during pregnancy. Statistically, GDM manifests in approximately 14% of pregnancies worldwide (60). Over the course of gestation, the mother‘s body undergoes a series of physiological and metabolic changes in order to support the demands of the growing foetus. One important metabolic adaptation is with insulin sensitivity. During early gestation, insulin sensitivity increases, promoting the uptake of glucose into adipose stores in preparation for the energy demands of later pregnancy (61). As pregnancy progresses, blood glucose is slightly elevated, and this glucose is readily transported across the placenta to fuel the growth of the foetus (62). The body and other placental hormones such as oestrogen, progesterone, cortisol, leptin and human placental lactogen can block insulin which promotes a state of insulin resistance and decreased insulin sensitivity (63). In cases where the balance between insulin resistance and secretion cannot be achieved, the symptoms of gestational diabetes are manifested. Several risk factors have been implicated in the development of GDM such as increased maternal age, obesity, ethnic background, family history of T2DM and a previous history of GDM (64). The primary intervention recommended for GDM is dietary counselling in combination with physical activity and self-monitoring of blood glucose. When these measures are insufficient in terms of achieving optimal glycaemic control, pharmacological agents such as insulin and metformin are used as first line treatment (65,66).

1.2.1 Aetiology of diabetic complications

The goals of treatment of diabetes are to maintain glycaemic control, prevent or delay the onset of complications, decrease mortality, and maintain a good quality of life (67). Diabetic complications can be classified into microvascular complications and macrovascular complications. The macrovascular complications include coronary artery disease, peripheral arterial disease and stroke while the microvascular complications include diabetic nephropathy, neuropathy and retinopathy (9,68). Long-term uncontrolled hyperglycaemia in diabetes mellitus is the primary instigator of diabetic complications. Several other possible mechanisms, including increased aldose reductase related polyol pathway, increased advanced glycation end products (AGEs) formation, and excessive oxidative stress have been proposed (69,70). Diabetic retinopathy is an eye condition that can cause vision loss and blindness in people who have diabetes. On a molecular level, hyperglycaemia can lead to intramural

pericyte death and thickening of the basement membrane, which contribute to changes in the integrity of blood vessels within the retina, fluid accumulation, altering the blood-retinal barrier and vascular permeability (71). Diabetic peripheral neuropathy involves the presence of symptoms or signs of peripheral nerve dysfunction in people with diabetes. The pathophysiology of diabetic neuropathy is primarily due to the increase in sorbitol and fructose levels, glycated end products, reactive oxygen species (ROS) and activation of protein kinase C in the diabetic state with hyperglycaemia playing a central role (72). Diabetic nephropathy is the result of many changes in the kidney that includes glucose metabolism, renal haemodynamic changes and ischaemia associated with increased oxidative stress, inflammatory effects as well as increase in the circulation of AGEs. These cause functional and structural changes in the nephron ultimately resulting in progressive loss of kidney function and renal fibrosis (73). The pathological role of the non-enzymatic modification of proteins by glucose, a process that is known as glycation, has become increasingly evident in numerous diseases. Early glycation products undergo progressive modification over time *in-vivo* to form irreversible cross-links, after which these molecules are termed AGEs (74). AGEs are a diverse group of highly oxidant compounds with pathogenic significance in aged-chronic disease, including diabetes, cardiovascular disease and neurodegenerative disease. They are produced by non-enzymatic reaction between reducing sugars and free amino groups of proteins, lipids, or nucleic acids also known as the Maillard reaction (75,76). The formation of AGEs is a part of normal metabolism, but if excessively high levels of AGEs are reached in tissues and the circulation, they can become pathogenic (77,78). AGEs play a role in the pathogenesis of diabetic complications such as retinopathy, nephropathy, neuropathy and cardiomyopathy. This occurs due to the chronic hyperglycaemic state in diabetes mellitus allowing reducing sugars e.g. glucose and di-carbonyl compounds to form covalent adducts with the plasma proteins forming AGEs (79). The kidney has an important role in the metabolism of AGEs, because renal proximal tubule cells absorb AGEs from the glomerular filtrate and catabolize them (80). AGEs are involved in the structural changes of progressive nephropathies such as glomerulosclerosis, interstitial fibrosis, and tubular atrophy. These effects are most prominent in diabetic nephropathy (81). AGEs also induce diabetic cardiomyopathy through endothelial cell dysfunction as they directly modify extracellular matrix proteins of endothelial cells – type IV collagen and laminin, this process destroys the normal structure and function of blood vessels, which may result in cardiac fibrosis, atherosclerotic plaques and subsequent cardiac dysfunction (82). AGEs inhibitors such as aminoguanidines, prevent the formation of AGEs

and their cross-links. In this study, the effect of vanadium complexes on AGEs formation was investigated.

In addition to AGEs, hyperglycaemia induced oxidative stress causes vascular damage resulting in the progression of DM associated pathologies (83). Oxidative stress results when there is a significant imbalance between the production of free radicals and antioxidant defence, leading to potential tissue damage (84). The free radical species are highly reactive molecules produced from cellular oxidative processes, e.g. mitochondrial processes. The precise mechanisms by which oxidative stress accelerate the development of complications in diabetes are not fully known. Studies suggesting the protective effect of antioxidants have been presented in experimental, clinical, and epidemiological studies, which have demonstrated that antioxidants might be helpful in treating diabetes and its complication (85).

The significant role of oxidative stress in the pathology of DM emphasized the need to investigate the anti-oxidative activity of the vanadium complexes in this study. Managing diabetic complications in the clinical settings can be challenging due to the multifactorial nature of these complications (86). Therefore, it is necessary to identify and validate useful therapeutic drug targets by investigating novel treatment to manage these complications. Pharmacological interventions are crucial in managing DM to prevent the emergence of diabetic complications which will be covered in the next section.

1.2.2 Current pharmacological managements and control of DM

Good glycaemic control remains the main foundation of managing DM. Such approaches play a vital role in preventing or delaying the onset and progression of diabetic complications. It is important that a patient-centred approach is used to guide the choice of pharmacological agents. The factors to be considered include efficacy, cost, potential side effects, comorbidities, hypoglycaemia risk, and patient preferences. Pharmacological treatment of T2DM is initiated when glycaemic control is not achieved or if HbA1C rises to 6.5% after 2 – 3 months of lifestyle intervention.

1.2.2.1 Insulin

Insulin preparations are classified based on their onset of action and duration of action in the body: Rapid-acting insulin; short-acting insulin; intermediate-acting insulins and long-acting insulin. Overall, insulin therapy has proved to be very beneficial in the management of DM.

However, insulin therapy is associated with side effects such as hypoglycaemia, lipodystrophy, dose-related problems, inconsistencies due to multiple dosing (87).

1.2.2.2 Biguanides

This class of drugs are commonly known as the first line treatment for Type 2 diabetes. This drug acts specifically by lowering the production of glucose in the liver and improves the sensitivity of the body to insulin thereby increasing the absorption of glucose by the cells, hence increasing its effectiveness. Some of the key side effects of biguanides are nausea, vomiting, stomach problems which tend to subside once the patient's body gets used to the drug (88). An example of drugs in this class is metformin. Lactic acidosis is a rare but serious side effect of this drug and must be treated as a medical emergency. The increased risk of metformin induced lactic acidosis generally arises in patients with renal, pulmonary or cardiac insufficiency or with a history of liver disease (89).

1.2.2.3 Alpha amylase and alpha glucosidase inhibitors

The inhibition of α-amylase and α-glucosidase enzymes plays a major role in the management of postprandial hyperglycaemia. Their inhibition leads to a reduction in starch hydrolysis and this reduces the intestinal absorption of the sugars leading to a reduction of postprandial glycaemia. Examples of drugs in this class are acarbose and miglitol.

1.2.2.4 DPP-4 inhibitors

Sitagliptin, saxagliptin, linagliptin, and alogliptin are the DPP-4 inhibitors currently on the market (90). DPP-4 inhibitors prevent the breakdown of GLP-1 and GIP , two incretin mimetics which promote glucose-dependent insulin secretion (91). The most common adverse reactions occurring in 5% of patients or more who received DPP-4 inhibitors are upper respiratory tract infection, nasopharyngitis, and headache.

1.2.2.5 Sulphonylureas

Sulphonylurea stimulates insulin secretion from the pancreatic beta cell. The hypoglycaemic effect is brought about by closing the ATP sensitive potassium (K^+) channels in the beta cell plasma membrane, resulting in insulin being released and available for uptake by the cells. Hypoglycaemia is the major side effect of all sulphonylureas, while minor side effects such as headache, dizziness, nausea, hypersensitivity reactions, and weight gain are also common. Sulfonylureas are contraindicated in patients with hepatic and renal diseases and are also

contraindicated in pregnant patients due to the possible prolonged hypoglycaemic effect to infant (92). Examples of drugs in this class include glipizide and glimepiride.

1.2.2.6 Thiazolidinediones

Thiazolidinediones is a class of antidiabetic drug that activates the gamma isoform of the peroxisome proliferator-activated receptor (PPAR-receptor) thereby causing an alteration of several genes involved in glucose absorption, which helps with improving insulin action and lowering the blood glucose concentration. Some of the key side effects include; weight gain, peripheral oedema which can precipitate or worsen congestive heart failure and rarely hepatotoxicity (93). Examples of drugs in this class include rosiglitazone and pioglitazone.

1.2.2.7 Glucagon-like peptide-1 receptor agonist

GLP-1 is an incretin hormone that stimulates insulin secretion to maintain glucose homeostasis. This hormone increases glucose uptake in the skeletal muscles, decreased glucose production in the liver, neuroprotection, and increased satiety due to direct actions on the hypothalamus. GLP-1 receptor agonists stimulate insulin secretion after oral glucose ingestion by delaying gastric emptying and inhibiting the production of glucagon from pancreatic α- cells when blood glucose concentration is elevated. Examples of drugs in this class include exenatide, liraglutide which are given by subcutaneous injection (94). The most common side effects associated with the use of GLP-1 receptor agonists are gastrointestinal symptoms, mainly nausea, injection site reactions, headache, and nasopharyngitis.

1.2.2.8 Sodium-glucose linked transporter (SGLT) inhibitors

The SGLT inhibitors block the reabsorption of glucose in the kidney thereby causing the excess urine to be excreted in the urine. Due to the increase of glucose levels in the urine, urinary tract infections are therefore a common side effect of this drug class (95). Examples of drugs in this class is dapagliflozin and canaglifloxin.

1.2.2.9 Aldose reductase inhibitors

The aldose reductase inhibitors aim to inhibit or reduce secondary complications induced by diabetes, specifically in tissues in which glucose uptake is not insulin-dependent such as the neural tissue, the lens, and glomeruli (96). Examples of drugs in this class include sorbinil, tolrestat and epralrestat. Discovering better treatment options to DM remains a subject of focus.

1.3 Alzheimer's disease

Alzheimer's disease is a degenerative disorder that involves progressive and irreversible loss of neurons in regions of the brain, particularly the prefrontal cortex and hippocampus which are responsible for cognitive functions, including language, judgment, decision making, orientation, learning and memory (32). There is a significant reduction of the volume of the brain in AD patients as compared to healthy patients due to atrophy resulting from the degeneration of synapses and the death of neurons in the hippocampus (33). Age is the highest risk factor for AD with reports that the risk of Alzheimer's doubles every five years after age 65, with the risk reaching nearly one-third after age 85 (39). AD brain is mainly characterised microscopically by the combined presence of two abnormal structures. These structures are extracellular beta amyloid (Aβ) plaques and aggregated tau proteins, both of which comprise highly insoluble densely packed filaments (100). Aβ peptides are proteolytic fragments of the transmembrane amyloid precursor protein, which are markers of AD in the brain and are believed to be the front liners in initiating the pathological cascade of the disease (101). With ageing, the rate of Aβ production increases, whereas the rate of clearance decreases, resulting in Aβ deposition, which activates hyper phosphorylation of tau, resulting in tau aggregation and eventually leading to neurotoxicity and synaptic damage (102). The next paragraph explains four mechanisms involved in the pathogenesis of AD. There are many hypotheses to explain AD pathogenesis, involving the cholinergic hypothesis that includes glutamatergic neurotransmission alterations, the amyloid peptide theory, the involvement of oxidative stress and the role of tau proteins. These hypotheses are further described below.

1.3.1 The cholinergic hypothesis

The cholinergic hypothesis is the first and most studied approach that describes AD pathophysiology. It was defined more than 30 years ago as a primary degenerative process capable of selectively damaging groups of cholinergic neurons that play functional roles in conscious awareness, attention, learning and memory (103). The activity of choline acetyltransferase, the enzyme responsible for the synthesis of acetylcholine (Ach) a reliable marker of cholinergic neurons and synapses, was found to be remarkably decreased, sometimes in a rather severe way, in pathological samples from the cortex and hippocampus of Alzheimer's patients (104). Ach is a neurotransmitter that serves both excitatory and inhibitory functions, which means it can both speed up and slow down nerve signals depending on the postsynaptic neuron receptor. In the central nervous system, its role is primarily excitatory

(105). It plays a role in arousal, memory, learning, and neuroplasticity which is the brain‘s ability to alter the structure of its neural network allowing it to form new neurons, make new neural connections, and rearrange or eliminate pre-existing connections (106). Acetylcholinesterase (AChE) is the primary enzyme responsible for the hydrolytic metabolism of the Ach into choline and acetate which results in termination of signal transmission between nerve cells (107). AChE has long been an attractive target because of the pivotal role in the nervous system. The therapeutic goal is to increase the synaptic ACh by blocking its degradation, such that there is a net increase in cholinergic receptor activation. Increasing the number of acetylcholine molecules within synapses by inhibiting AChE can counteract a deficiency in either the release of neurotransmitter or a reduction in cholinergic receptors/signalling (108). Cholinesterase inhibitors (CIs) e.g. Donepezil, rivastigmine and galantamine act by increasing concentration of acetylcholine at the synapse by binding to and inactivating cholinesterase. However, cholinesterase inhibitors can't reverse Alzheimer's disease or stop the destruction of nerve cells and as the disease progresses, they eventually lose effectiveness due to less ACh produced by the dwindling brain cells. As an attempt to find drug candidates with ability to inhibit acetylcholinesterase activity while also preserving brain cells, the anticholinesterase activity of vanadium complexes was investigated.

1.3.2 The Amyloid cascade hypothesis

Amyloid refers to misfolded peptides or proteins that demonstrate a stable, cross-beta super secondary structure that renders it insoluble, fibrous-like, and resistant to proteolysis (109). It is formed from the breakdown of a larger protein, called amyloid precursor protein (APP). Aβ is a peptide with high resistance to proteolytic degradation. It consists of 37–43 amino acids, in which the isoforms 1–40 and 1–42 are the most common (110). Aβ is thought to be especially toxic and may alter the normal function of tissues and cause serious changes in tissues and organs of the body (111). The amyloid cascade hypothesis is believed to play a significant role in neuropathogenesis of AD (109). This hypothesis suggests that the dysregulation in APP processing occurs early in the disease process resulting in the overproduction of $A\beta_{42}$ which clump together to form plaques between neurons and disrupt cell function (112). As the plaque ―matures", the Aβ peptides reorganise into β-pleated sheets and fibrilise into neuritic plaques (113). The formation of these neuritic plaques result in microglial and astrocytic activation, oxidative injury which drives neuronal degeneration and cognitive decline in AD patients (114). An imbalance between production and clearance of amyloid β is a triggering event responsible for other abnormalities observed in AD (115).

1.3.3 Tau hyperphosphorylation hypothesis

Tau is a microtubule-associated protein of the cytoskeleton best known for their role in stabilizing neuronal microtubules. Structurally, tau is a natively unfolded protein, highly soluble and shows little tendency for aggregation (116). In AD, impaired glucose metabolism can facilitate hyperphosphorylation of tau and their dimerization *in-vivo*, potentially leading to cross-linking and the formation of pairs of helical filaments and subsequently neurofibrillary tangles, cytoskeletal abnormalities and axonal transport disorders, synaptic loss and finally dementia (117).

1.3.4 Oxidative stress and inflammation

The brain is an organ that requires a high oxygen demand to perform its activities properly. However, the brain has limited antioxidant substances and is rich in polyunsaturated fatty acids that are susceptible to peroxidation (118). In addition, Aβ accumulation in the brain attracts immune system cells such as astrocytes, activated microglia, and macrophages, among others which trigger inflammatory response and promote oxidative stress state (119). A critical property of AGEs is their ability to activate receptor for advanced glycation end products (RAGE). Increased RAGE expression and modified proteins can lead to mitochondrial damage, induce reactive oxygen species which promote oxidative stress, the formation of amyloid plaques, neurofibrillary tangles and inflammation which is a common feature in the AD brain (120).

Similarly, matrix metalloproteinases (MMPs) a newer group of proteins and their role in the pathology of Alzheimer‘s disease have been a newer subject of interest. The extracellular matrix (ECM) in the brain has emerged as an important reservoir of signalling molecules, which can influence synaptic plasticity, synaptogenesis, neurite outgrowth and other processes occurring in central nervous system (121). MMPs are among the major modulators of the ECM and are involved in the proteolysis of extracellular matrix components, growth factors, cerebrovascular basement membrane, tight junction proteins, and cell surface proteins receptors (122). Their proteolytic actions are the main drivers of their pathological expression in diseases such as AD. MMP's released from activated glia potentially lead to damage to the blood brain barrier endothelium due to destruction of collagen in the basal lamina surrounding capillaries, and all layers of arteries and arterioles (123). This damage to the brain's vasculature may lead to leakage of plasma from the blood vessel into the parenchyma, further glial activation and a cascade of events leading to further CNS damage (124). MMPs are also

important mediators during the process of inflammation mediated by activating enzymes that act on signalling molecules such as pro-inflammatory cytokines, cell surface receptors, cell–cell adhesion molecules, and inducing oxidative stress (125). Oxidative stress can impact MMP-1 activity and expression indirectly through the modulation of signalling networks that contribute to its transcription or through direct oxidative activation of the enzyme (126). Furthermore, it has been suggested that Aβ, the major constituent of senile plaques, can cause upregulated expression of MMP's by glia (127). This upregulation of MMP-1 is a response to the elevated Aβ found in AD. This may lead to tissue destruction by MMP's, release of cytokines by damaged cells, further activation of glia and a cascade of increasing cellular damage (128). With several studies done on MMPs in cancer, rheumatoid arthritis lung diseases to mention a few, their molecular interactions and role in AD is not yet clearly defined and understood. This might be pinned to the fact that MMPs are multifaceted enzymes that mediate a myriad of physiological (tissue repair, angiogenesis, neurogenesis) and pathological (neuroinflammation) functions, raising the issue of the risk/benefit outcome (129). MMP inhibitors have been unsuccessful in clinical trials due to lack of specificity within the MMP family, poor pharmacokinetics, dose-limiting side effects/toxicity, *in-vivo* instability and low oral availability and efficacy (130,131). Despite these failures, inhibiting MMP activity is still a rational therapeutic approach, particularly for inflammatory mediated disorders such as AD and DM (132). These challenges emphasize the need to design specific drugs and implement innovative drug discovery strategies that interfere with the harmful effects of MMPs while sparing their physiological actions (133). Therefore, the inhibitory activity of vanadium complexes against MMP-1 was investigated.

1.4 Current pharmacological management of Alzheimer's disease

Due to the complexity of the brain, the requirement of CNS drugs to cross the blood-brain barrier (BBB); and liability of CNS side effects, research and development of CNS drug candidates have been associated with significant challenges resulting in numerous failed clinical trials and prolonged drug discovery to market time (134). Two main drug classes have been approved for the treatment of Alzheimer's disease. Cholinesterase inhibitors (CIs); which are approved for dementia of the Alzheimer‘s type in the mild-to-moderate stage. The second class is the NMDA antagonists which oppose glutamate activity by blocking NMDA receptors.

1.4.1 Cholinesterase inhibitors

Cholinesterase inhibitors (CIs) which include donepezil, rivastigmine and galantamine act by increasing concentration of acetylcholine at the synapse by binding to and inactivating these cholinesterases. Several studies have demonstrated that the cholinergic system plays a role in the processing of memory and learning, modulation of acquisition consolidation and retrieval of memory to mention a few (135). CIs also increase acetylcholine activity in the peripheral nervous system which contributes to the side effects commonly experienced. Anorexia, convulsions and insomnia are some of the side effects experienced with the use of these class of drugs.

1.4.2 NMDA Antagonists

N-methyl-d-aspartate receptors (NMDARs) play a crucial role in the synaptic transmission and plasticity responsible for learning and memory. Synaptic NMDARs are neuroprotective, and their dysfunction is directly involved in AD (136). Memantine, a non-competitive NMDA receptor antagonist, is approved for use in moderate to severe AD. This drug opposes the effects of the excitatory neurotransmitter glutamate. The role of glutamate in AD is not well understood, however excessive glutamate activity is believed to interfere with neurotransmission and contribute to gradual loss of synaptic function and ultimate neuronal cell death (135). Memantine is well tolerated, with fewer side effects than cholinesterase inhibitors, although dizziness, headache, somnolence, constipation and hypertension can occur. While these class of anti-Alzheimer drugs represent the best current available pharmacological treatments in AD, they have relatively insignificant overall effect and do not alter the course of the underlying neurodegenerative process. Hence, there is a need to identify newer drug targets for AD treatment.

1.4.3 Beta amyloid modulators

The conventional approaches targeting Aβ focus on three main therapeutic strategies. Firstly, reducing Aβ peptide production via inhibition of β-secretase and γ-secretase. Secondly, amyloid antiaggregant which inhibit Aβ peptide aggregation e.g. a synthetic glucosaminoglycan, 3-amino-1-propanesulfonic acid and colostrinin. Finally, compounds that facilitate the clearance of amyloid aggregates and deposits, e.g. bapineuzumab and solanezumab, the two monoclonal antibodies that have reached the most advanced stages of

clinical development (137,138). Recently, aducanumab, another monoclonal anti-body was approved for its ability to reduce the number of Aβ plaques in the brain (139).

1.4.4 Advanced glycation end product inhibitors

There are some therapeutic options to reduce AGEs and antagonizing RAGE available which include the following: AGE cross-link breakers which break up the cross-links between AGEs and extracellular molecules e.g. alagebrium and pyridinium derivatives. AGE inhibitors which prevent the formation of AGEs and their cross-links e.g. aminoguanidine. Finally, RAGE antagonists which inactivate rage receptors e.g. azeliragon (140,141). Despite the numerous drug candidates identified and intensively studied, the outcomes have not been promising, as all the drug candidates have failed in clinical trials due to issues with their specificity, side effects, safety and efficacy (114). Nearly all recent AGE cross-link breakers, AGE inhibitors and RAGE antagonists have their weaknesses in pharmacokinetics and clinical effectiveness (140). There is still an urgent need to find efficient drugs that can stop or delay the progression of AD and DM. Their common pathological features further described in the next section, encourages a multi-targeted approach towards mitigating their symptoms.

1.5 Diabetes and Alzheimer's - A pathophysiological link

Several targets of the insulin machinery which may potentially catalyse the development of neurodegenerative disease have been intensively studied and identified. Statistically, the risk of developing Alzheimer's disease was 65% higher in persons with diabetes than in non-diabetic controls as reported from a study (142). This relationship is significant such that AD has sometimes been referred to as the "diabetes of the brain" or "type 3 diabetes (143). When glucose homeostasis is not maintained in the body, this can result in dysregulation of some metabolic, molecular, and cellular processes which can lead to diabetic complications. In addition, these metabolic disturbances may lead to progressive structural and functional abnormalities in the brain, impairing neuronal signalling (144). The brain senses peripheral metabolic signals through hormones such as insulin/leptin as well as nutrients to regulate glucose homeostasis (48). As a response to these signals the brain modulates and coordinates various aspects of metabolism, such as food intake, energy expenditure, insulin secretion, hepatic glucose production and glucose/fatty acid metabolism in adipose tissue and skeletal muscle to maintain glycaemic control (48,145). Accumulating evidence suggests that impaired cerebral glucose metabolism is an invariant pathological feature of Alzheimer's disease.

Chronic hyperglycaemia results in accelerated formation and accumulation of AGEs in various tissues (146,147). AGEs although reported to increase during normal aging process plays a critical role in the pathogenesis of diabetic complications and neurodegenerative disorders, including AD (148). The activation of RAGE by AGEs in a variety of settings triggers rapid generation of ROS and the up regulation of inflammatory pathways. This can ultimately result in diabetic complications, amplification of inflammation and a myriad consequences of natural aging (149). Insulin and insulin-like growth factor (IGF-1) have intense effects in the central nervous system (CNS), regulating key processes such as energy homeostasis, neuronal survival, longevity, learning and memory. The binding of insulin or IGF-1 induces a conformational change of the receptor leading to their auto-phosphorylation an activation of various signalling pathways involved in the maintenance of synaptic plasticity and memory consolidation (150,151). Excess accumulation of Aβ plaques in the brain establishes a vicious cycle of impaired brain insulin signalling, inflammation, and oxidative stress processes that promote neurodegeneration in the AD brain (152). Furthermore, Aβ accumulates in peripheral tissues such as the pancreas and can induce insulin resistance in the liver which suggests that not only can insulin resistance promote Aβ accumulation but also the reverse could occur (153). Several studies have reported results supporting the bidirectional relationship existing between AD and DM. One study found a significantly lower cognitive performance among diabetic patients compared to healthy controls after 4 years follow-up period (154). Another cross-sectional study discovered that subjects with T2D performed worse in all cognitive domains than those with normal glucose metabolism (155). Consistent with these findings, a recent meta-analysis of 144 prospective studies identified a 1.25–1.9 fold increase for cognitive impairment and dementia in patients with diabetes (156). Therefore, by understanding and acknowledging the existence of these overlapping pathogenetic features between AD and DM; we can target these two diseases holistically and find a much anticipated effective treatment strategy with the aim to improve therapeutic outcome for the patient and give them the best quality of life. The illustration below is a representation of the link between DM and AD (**Figure 1.2**).

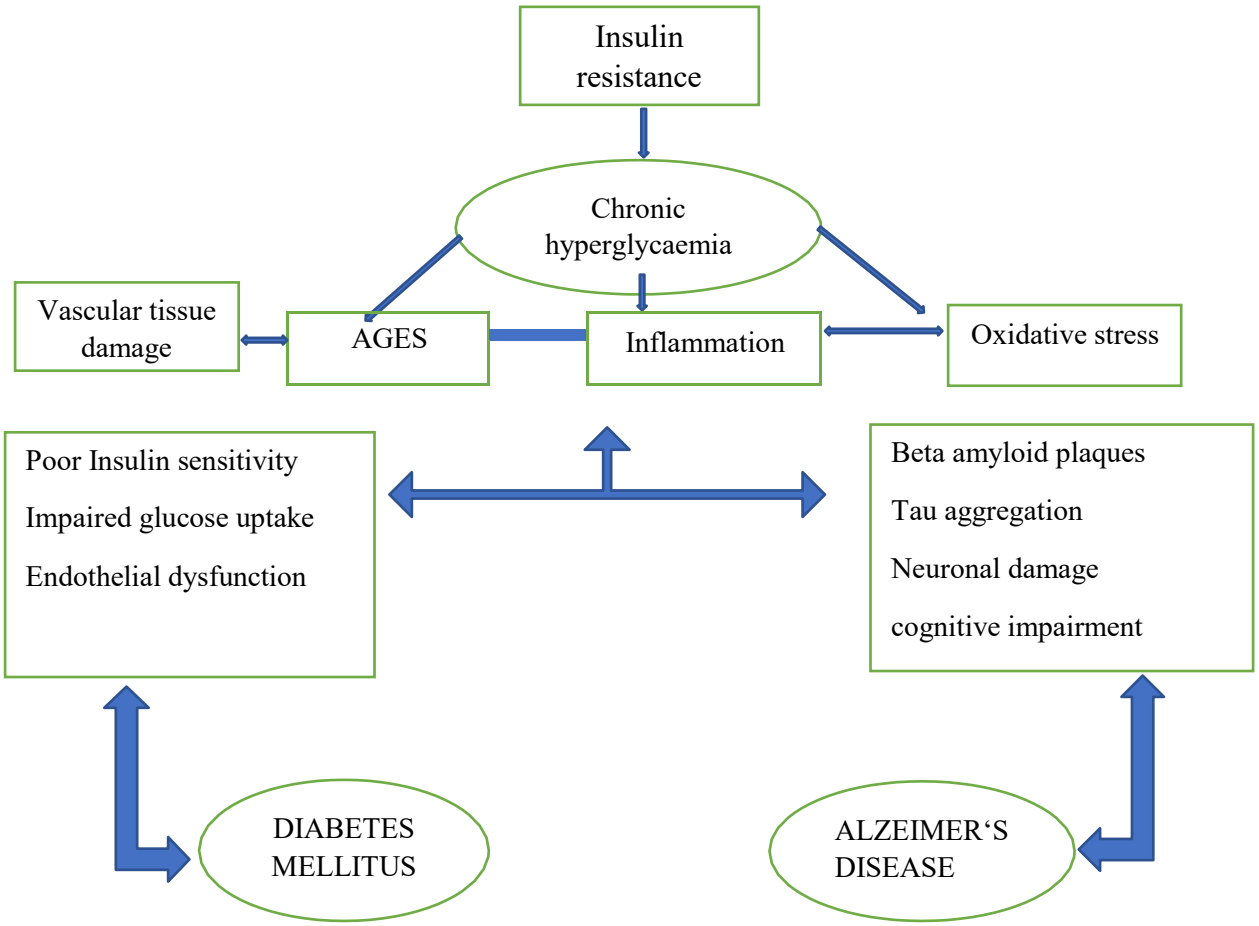

Figure 1.2: An illustration of the mechanisms involved in the pathology of diabetes and Alzheimer's disease. Insulin resistance can result in hyperglycaemic conditions which leads to an array of neurological and vascular inflammatory processes, formation of ROS, neuronal stress, and resulting in neurodegeneration and build-up of abnormal structures in the brain which eventually result in DM and AD symptoms.

1.6 Multi-target approach in medicine

In the past, drugs with more than one target were deemed as undesirable options due to concerns about unwanted effects arising from mis-targeting other biological targets or off target toxicities (157). Despite the successes of the single-target drugs, the complexity of some diseases such as DM and AD has clearly demonstrated that single-target drugs are inadequate to achieve the desired therapeutic effect (158). This is because the pathogenesis of complex diseases is dependent on a set of biochemical events and several processes operating concomitantly, which makes the pharmacological effect from single targets insufficient (159). This emphasizes the relevance of a multi-targeted approach to come up with effective drugs

to combat such complex diseases. The new paradigm of multi-target approach comes in different forms namely; association of drugs/adjunct therapy, combination of drugs and a single drug with multiple targets; which are all intended for multiple targets (160–163). However, this study is focused on identifying a single drug with multiple targets. This method of drug design is advantageous as it tackles the disease holistically, reduces the incidence of drug-drug interactions, side effect profile and complex dosing associated with polypharmacy. Regardless of the promising potential of a multi-target approach, multi-target drug design can be challenging and requires a good understanding of pathway-target-drug-disease relationships and adverse events profiling (164). The essential requirement is the molecule‘s ability to interact with the desired targets. Therefore the structure–activity relationships that govern these interactions must be taken into consideration when designing the pharmacophore of such molecules (165). The aims of this study are to investigate the multitargeted inhibitory effects of vanadium complexes on enzymes and peptides with pathological role in DM and AD.

1.7 Enzyme targets of interest and their active sites

To successfully develop drug candidates, it is imperative to understand how these enzymes and targets interact at molecular level to bring about these physiological responses that can result in pathological traits. This will allow for better target specificity and drug candidate optimization. This section discusses the general structural compositions of enzymes of interest involved in the aetiology of diabetes and Alzheimer‘s disease as well as proposed strategies to enhance inhibitor interactions with the enzyme to bring about optimum inhibition. This section also briefly highlights some key structural features that may increase the chances of the investigated Schiff base vanadium complexes interacting with the enzymes of interest.

1.7.1 Alpha amylase

The human α-amylase isozymes are composed of 496 amino acids in a single polypeptide chain and bind essential chloride and calcium ions. They have a (β/α) barrel domain which harbours the active site (166). The active site of α-amylase contains a trio of acidic groups and amino acids glutamate 233, aspartate 197, and aspartate 300 which work collaboratively to cleave the connection between two sugars in a starch chain (167). Starch binding in α-amylases, is shown to be influenced by the presence of CH/pi-stacking interaction. This interaction occurs between aromatic residues tyrosine, tryptophan, and sometimes phenylalanine (168). A common tactic

in the design of amylase inhibitors is the incorporation of a nitrogen atom into a substrate-like sugar. The positive charge of the nitrogen is thought to mimic that of the transition state and can develop additional polar interactions within the active site. For example, the inhibitory actions of acarbose and related compounds has been commonly ascribed to the ability to mimic a glucopyranosyl half-chair conformation intermediate of amylase catalysis in combination with interactions between the exocyclic nitrogen atom and the enzyme complexes (169,170). Additionally, the presence of a good leaving group at the anomeric centre of the inhibitor encourages attack by the enzyme‘s catalytic nucleophile. The presence of an electron-withdrawing group, such as fluorine, can destabilizes the formation of the oxocarbenium ion-like transition state and the subsequent hydrolysis step, effectively trapping the enzyme in a covalent complex (171). To improve the likelihood of targeting this enzyme the designed Schiff base vanadium complexes have a nitrogen atom and a good leaving bromine group with one of the complexes also having a powerful electron withdrawing CF_3 group present.

1.7.2 Alpha glucosidase

Human α-glucosidase is of 868 amino acid residues composed of five domains; a trefoil type-P domain (residues 89–135), an N-terminal β-sandwich domain (residues 136–346), a catalytic (β/α)8 barrel domain (residues 347–723) with two inserted loops, which include (residues 444–491). The catalytic triad is located inside the (β/α)8 barrel domain and composed of the amino acid residues Asp443, Asp542, and Glu444 (172,173). The main binding interaction modes between the acarbose and the amino acids at the active site is via a network of hydrogen bonds which maintain complex stability. Twelve hydrogen bonds are formed between the hydroxyl groups of acarbose and the amino acids: Asp587, and Arg571, both originating from the glycoside hydrolase 31 catalytic domain, and by His274, originating from the N-terminal domain loop (residues 271-288) of α-glucosidase (174,175). The first glucose, the N-glycosidic bond and the second glucose of acarbose, all occupy the same site as the substrate α-maltose and have a binding mode identical to α-maltose. This is compatible with a competitive mode of inhibition (176). The Schiff base vanadium complexes have electronegative atoms including oxygen, nitrogen and fluorine which allows for hydrogen bonding interactions.

1.7.3 Dipeptidyl peptidase-4 (DPP-4)

The DPP-4 active site comprises of three pockets for ligand binding, the first of which is the common binding region (CBR), which include S1 (hydrophobic) and S2 (EE-loop/hydrophilic) sites. The second binding pocket is the sitagliptin binding region (SBR)and the third is the

linagliptin or alogliptin binding region(177). The pockets S1 and S2 contain Arg125, Ser209, Phe357, Arg358, Tyr547, Ser631, Val656, Trp659, Tyr62, Tyr666, Asn710, and Val711 considered region of interest for the action of DPP-4 inhibitors (178,179). Site 1 and site 2 of chain B show favourable affinities to polar molecules with hydrogen bond donors and acceptors, as well as positive, hydrophobic and aromatic groups(180). Therefore, it has been speculated that new inhibitors have to contain functional groups that will allow for hydrogen bond interactions, hydrophobic contacts, π-π stacking interactions, π-π T-shaped-like interactions and salt bridges since they are supposed to be coupled in both DPP-4 active sites (chains A and B). The CBR is common to all known DPP-4 inhibitors except covalent inhibitors, which form a covalent bond with S630. The covalent inhibitors include vildagliptin and saxagliptin. To accommodate the CBR region, a ligand should have a positively charged group (mostly a free amino group) to form a salt bridge with E205, E206 and Y662 along with a hydrophobic part to fit into the hydrophobic pocket. The highly hydrophobic S1 pocket of the CBR in DPP-4 is very specific in size and is in nature. Selectivity towards DPP-IV can be achieved by adding electron-withdrawing halogen atoms (especially fluorine) at the 2,4,5 position of the aromatic ring (181). The investigated vanadium complexes possess aromatic groups, hydrogen bond acceptors, aromatic rings for π-π stacking and electron withdrawing substituents which improve the chances of interactions with the DPP-4 enzyme.

1.7.4 Protein tyrosine phosphatase

The key features of the PTP domain is the conserved active site cysteine that serves as a nucleophile to attack the phosphate of the phosphotyrosine residue. The aspartate residue acts as a general acid to provide its proton to the leaving group, resulting in the formation of a cysteinyl-phosphate enzyme intermediate. This aspartate residue then acts as a general base and along with two conserved glutamine residues activates a water molecule to dislodge this intermediate releasing the inorganic phosphate(157). Vanadate ion‘s close resemblance with phosphate allows it to enter the cell via phosphate & sulphate channels (183). The orthovanadate ion has a tetra-coordinated structure and a negative charge, features that it shares with the phosphate group of PTP substrates. By mimicking phosphate, orthovanadate fits into the PTP active site with trigonal pyramid geometry, stabilised by a complex network of hydrogen bonds (41). The similarity in the skeletal backbone of these investigated vanadium complexes(vanadate ion) with the phosphate ion and their ability to form hydrogen bond interactions due to the nitrogen, oxygen and halogen substituents may allow interaction with this enzyme.

1.7.5 Acetylcholinesterase

The acetylcholinesterase (AChE) active site consists of a narrow gorge with two separate ligand binding sites: an acylation site (or A-site) at the bottom of the gorge where substrate hydrolysis occurs and a peripheral site (or P-site) at the gorge mouth (184). Several amino acid residues within the gorge have been identified as important in facilitating efficient catalysis and inhibitor binding. Of particular interest is the catalytic triad, consisting of serine, histidine, and glutamate residues, that mediates hydrolysis and the P-site containing a number of aromatic amino acid residues as well as an aspartate residue that is able to interact with cationic substrates (185). Organophosphates for example inactivate AChE as they pass through the P-site and phosphorylate the catalytic serine in the A-site. AChE inhibitors act by forming a covalent bond with the catalytic serine residue that is either reversible, as for carbamate containing drugs, or irreversible, as for most organophosphates. The carbamate moiety has been, and still is, a key functional group in medicinal chemistry programs to develop drugs targeting AChE, for example the drugs pyridostigmine (186). According to literature, hydrogen bonding, van der Waals, and hydrophobic forces are the main binding interactions for vanadium complexes and AChE (187).

1.7.6 Beta amyloid peptide

Regions that undergo specific interactions and contribute to Aβ aggregation include the N-terminal region , the hinge region and the C-terminal regions (188). The N-terminus region promote oligomerization and fibril propagation and stabilize Aβ aggregates therefore increasing their resistance to proteolysis. Aβ -metal interactions have been well characterized and binding is known to occur at the N-terminal regions of the peptide with the metal binding centre as an ~~–a~~nchor" while surrounding functional groups interact and change the conformation of Aβ peptide (189,190). This may be a potential route for these vanadium complexes. The hinge region joins two hydrophobic segments in space and produces the β-strand structure. In this region, Lys28 forms a salt bridge with Asp23/Glu22, which further stabilizes the structure (191). Finally, the C-terminal region which is considered to play an important role in the structure stability and assembly of Aβ42 oligomers (192). The C-terminal region, which contains hydrophobic segments, interacts with the cell's lipid bilayer therefore, targeting the lipid–Aβ interaction is a viable approach toward blocking Aβ aggregation (188).

These subregions contribute differently to structure formation and Aβ aggregation hence targeting them can lead to the discovery of potential effective inhibitors.

1.7.7 Matrix metalloproteinases

A typical MMP consists of a pro-peptide of about 80 amino acids, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide of variable lengths (also called the ‗hinge region') and a hemopexin (Hpx) domain of about 200 amino acids (193). MMPs are expressed as inactive zymogens which means they are inactive in their natural form. The Zn^{2+} at the active site of the enzyme binds to the conserved cysteine residue in the pro-peptide. When the interaction between cysteine and Zn^{2+} is disrupted, which can be achieved by cleaving the pro-peptide, the proenzyme is activated (194,195). MMPs also contain an S1‘ pocket located near the catalytic Zn^{2+} ion which is important for the recognition of the preferred substrate. The first generation of MMP inhibitors were based on small molecules and relied on targeting the Zn^{2+} ion in the active site of MMPs. This class of compounds contain a hydroxamic acid (RC(O)N(OH)R') motif, which makes a potent interaction with the catalytic zinc ion of MMPs, however, they are unstable and prone to hydrolysis (196). For this reason, MMP inhibition by targeting exosites or allosteric sites have been proposed to represent unique opportunities for the design of selective inhibitors (131). Other novel inhibitor molecules have been designed based on pyrimidine derivatives interacting with catalytic zinc in the S1‘ pocket or with the aromatic residue in the S1 pocket instead of the catalytic site with zinc ion (197). Interestingly, a study on MMP-1 inhibition of synthesized compounds showed that the presence of a trifluoromethyl substituent attached to a benzene ring resulted in a 60-fold higher potency as compared to its analogue and this potency was attributed to a halogen bonding interaction involving the trifluoromethyl group and the ARG214 residue of MMP-1 as well as hydrophobic compatibility with the active site (198). One of the vanadium complexes used in this study has a trifluoromethyl substituent; it will be of interest to see if a similar trend will be observed. In the absence of these key structural moieties proposed to enhance MMP inhibitory activity, the presence of the trifluoromethyl substituent in vanadium complex 3 may enhance its inhibitory effect on MMP-1 over other two vanadium complexes.

With an understanding of the aetiology of DM and AD as well as an understanding of their enzyme and protein targets of interest, Table 1.1 below gives a summary of the enzymes

investigated in this study, their pathological implications and the desired therapeutic outcome from their inhibition.

Table 1.1*:* A summary of the targets of interest implicated in the pathological features of diabetes mellitus and Alzheimer's disease.

Enzyme	**Pathological implications**	**Therapeutic goals**
Alpha amylase	Breaks down complex carbohydrates to simple carbohydrates. Increased glucose intestinal absorption.	Control post-prandial hyperglycaemia. Delay carbohydrate absorption.
Alpha glucosidase	Breaks down simple carbohydrates to sugars.	Control post-prandial hyperglycaemia. Delay carbohydrate absorption.
DPP-IV	Inactivates incretin hormones responsible for glucose-stimulated insulin secretion.	Increase insulin secretion from pancreatic beta cells Decrease glucagon secretion from α- cells.
Protein tyrosine phosphatase	Negative regulator for both insulin and leptin.	Improve glucose homeostasis and insulin signalling.
Acetylcholinesterase	Hydrolyses acetylcholine, a neurotransmitter that promotes signal transmission between nerve cells.	Enhance nerve signal transmission. Improve cognitive function.
Advanced glycation end products	Form cross-links which leads to diminished vasculature in DM. Play a key role in the formation of $A\beta$.	Delaying or preventing the onset of diabetic complications.
Beta amyloid	Protein fragments which aggregate to form neuritic plaques that are harmful to brain tissues.	Halt beta amyloid production and aggregation. Prevent neuronal degradation and improve cognitive function.

MMP-1	Destruction of the extracellular matrix in the CNS leading to neuronal dysfunction and death. Mediate the production of beta amyloid and tau aggregates resulting in neuro inflammation.	Prevent neuroinflammation. Prevent Aβ formation Neuroprotection.

1.8 Vanadium as potential candidate for diabetes mellitus and Alzheimer's management

Metal complexes have been identified to play an essential role in pharmaceutical industry as well as applied in the field of medicine to treat several ailments. For example, the use of cisplatin in the treatment of cancer (199). Vanadium is one of such metal also considered to be an essential micronutrient within many organisms. One of its most relevant properties identified is its ability to act as an insulin-enhancing agent, either in the form of its inorganic salts or complexes with organic ligands (200). The insulin-like effect of vanadium salts on cells have been known since the 1980s. However, these compounds suffer from poor absorption and required high doses which resulted in undesirable side effects. This prompted the move to complex vanadium with organic ligands with the aim of reducing the required dose and improve absorption (201). In humans, the vanadium content in blood plasma is around 200 nM; it is also found in bones, liver, and kidneys(202). Vanadium-based complexes have been proposed to exhibit a broad range of pharmacological activities viz; antioxidant, anti-diabetic, anticarcinogenic effect, anti-cholesterol, neuroprotective, osteogenic and cardioprotective effects (203). Once in the bloodstream, vanadium is distributed and stored in different tissues such as the heart, liver, kidney, spleen, brain, muscle, adipose tissue and bones (204). Vanadium regulates the blood glucose concentration through enhancing glucose transport to cells, stimulation of glucose oxidation and glycogen synthesis. Vanadium compounds also stimulate glycolysis, inhibit gluconeogenesis and increase sensitivity to insulin (205). Furthermore, vanadium regulates lipid metabolism through, inhibition of lipolysis and stimulation of lipogenesis, and decreasing plasma triglyceride and cholesterol level (204). In a variety of animal models of diabetes, vanadium salts normalized blood glucose and lipid concentrations, improved insulin sensitivity and impaired antioxidant status (206). Despite the activity shown by vanadium complexes in mitigating insufficient insulin response in diabetes

mellitus, they cannot entirely substitute for lack of insulin (as in type 1 diabetes) but can reduce reliance on exogenous insulin and substitutes for other oral hypoglycaemic agents currently used in treatment of type 2 diabetes.

Although much attention has been focused on antidiabetic activities for vanadium-based compounds, a few vanadium complexes have shown promise in AD. Metal complexes have been developed as chemical modulators toward Aβ aggregation with three proposed mechanisms of action including hydrolysis, oxidation of Aβ peptides and co-ordination to Aβ peptide (207). Vanadyl complexes conjugated with *N*,*N*-dimethylphenylenediamine (DMPD), which is an antioxidant capable of capturing a variety of free radicals was shown to mitigate metal-Aβ induced cytotoxicity *in-vitro* and reduced cerebral Aβ plaques, as well as to significantly improve cognitive defects in mice with Alzheimer's disease (208). Vanadyl (IV) acetylacetonate (VAC) in a mouse model with increased Aβ burden was shown to substantially preserve cognitive function without altering amyloid deposition via inhibition of formation of toxic Aβ species and reduced the level of soluble Aβ oligomers (209). Peroxovanadium complexes have been shown to inhibit amyloid fibril formation via site-specific oxidation of the methionine residues (210).
Table 1.2 below gives a summary of some vanadium compounds investigated on enzymes and peptides of interests from current literature and their proposed pharmacological activities.

Table 1.2: Summary of vanadium compounds investigated for their antidiabetic and anti-Alzheimer activity.

Vanadium compound	**Pharmacological activity/Effects**
Vanadate	Inhibition of protein tyrosine phosphatase (211)
VO(acetylacetonate) VO_2(dipicolinate)	Insulin mimetic effects (212)
Vanadium binding proteins (sea squirt)	Inhibited α-glucosidase activity *in vitro* (213)
Bis(maltolato) oxovanadium(IV) BEOV	Insulin mimetic Decreased Ab level and tau phosphorylation, increased neuron viability (214)
Vanadyl (IV) acetylacetonate	i) Insulin-mimetic

	ii) Glucose-uptake and inhibition of free fatty acids (FFA)-Release in isolated rat adipocytes (215) iii) Upregulated PPARγ expression and activating PPARγ-AMPKα signal transduction in adipocytes and muscle cells (216)
Bis(allixinato)oxovanadium(IV)	Enhanced insulin signalling and induced the GLUT4 gene expression in skeletal muscles (217)
Vanadyl (IV) acetylacetonate	Suppressed neuronal apoptosis under Aβ-related stresses and decreased toxic soluble Aβ peptides (209)

The antidiabetic activity of various vanadium complexes has been studied and a few are based on Schiff base ligands. In AD therapy, the investigation of vanadium compounds as drug candidates is still a growing field. Transition metal complexes containing Schiff bases have been of much interest over the last years, largely because of their various applications in biological processes and potential applications in designing new therapeutic agents (218). The vanadium compound of interest used in this study were synthesised from a Schiff base. Schiff bases may be formed by the condensation reaction of an amine with an aldehyde with release of water and are characterized by imine or azomethine groups (>C=N–) and usually coordinate to a metal through the imine nitrogen centre (219). Schiff bases are a class of organic ligands noted for their multiple medicinal benefits such as antibacterial properties, stability under a range of oxidative and reductive conditions, and their structural and excellent coordination capacity and flexibility is associated with their diverse applications in drug design, development and enhancement of drug performances (220). It is also well known that the formation of complexes with transition metals leads to an enhancement of their biological activities and decrease in the cytotoxicity of the Schiff bases (221). Although preclinical studies using vanadium compounds have and continue to provide promising data, an approved vanadium based drug has yet to reach the market (222). One of the main concerns is their non-selectivity, off-target toxicities and long-term use of vanadium compounds *in-vivo* in humans, hindering their progress through clinical trials (204,223). Despite these current misgivings, interest in these complexes have continued and many believe they could still have desirable therapeutic value. By improving the mode of delivery and tissue targeting of vanadium

complexes we can minimize off-target toxicities and maximize their full potential (222). In the field of drug discovery, metal-based complexes can present novel, unique activities which organic compounds may be unable to duplicate therefore highlighting the need for more research studies of this nature.

1.9 Drug likeness and pharmacokinetic properties of compounds

Drug-likeness defines acceptable ranges of fundamental properties that allows the identification of most likely druggable candidates and this definition may differ depending on mode of administration (224). The Lipinski rule of 5 (RO5), proposed by Christopher Lipinski is relevant in this study because the intention is to design orally active vanadium compounds for treatment of DM and AD. This RO5 curbed from the trend common among orally active drugs that achieved phase II clinical status was formulated as a guide to medicinal chemist due to many synthesized compounds with very poor physicochemical properties, hence the goal was to improve the chemical and physical properties of drug candidates in order to improve oral bioavailability (225).The tenets of Lipinski RO5 (molecular weight must be below ≤ 500, Log P (partition coefficient) must be ≤ 5 and other parameters like hydrogen bond donors (HBD) as well as acceptors (HBA) must be ≤ 5 and ≤ 10 respectively) are particularly important for the following reasons:

- ✓ Firstly, smaller molecular weight compounds are more easily absorbed and distributed into the cell which directly has an impact on the amount of drug available in the body (bioavailability). However, higher molecular weight does not always equate to poor absorption and significantly larger molecules have been efficiently transported into cells (226). Other important parameters such as molecular flexibility (NRB, i.e. number of rotatable bond), polar surface area (PSA) have been identified to be better predictors of bioavailability independent of molecular weight (227).
- ✓ Lipophilicity(Log P) is an important physicochemical parameter that has an effect on transport processes, including intestinal absorption, membrane permeability, protein binding, and distribution to different tissues and organs, including the brain (228). Highly lipophilic compounds may result in high metabolic turnover, low aqueous solubility, poor oral absorption and undesired affinity towards hydrophobic targets other than the desired target, which increases chances of ~~–o~~ff target" toxicity. However, low lipophilicity can also negatively impact permeability and potency and thus results in low bioavailability and efficacy (229). Therefore, a suitable drug

candidate must reflect a good aqueous solubility as well as an acceptable degree of lipophilicity in order to yield the best oral absorption.

- ✓ The presence of functional groups in a drug that are capable of forming hydrogen bonds (hydrogen bond donors (HBD) and acceptors (HBA)) can increase its solubility and the ability to establish important interactions with its biomolecular targets binding and selectivity as well as passive diffusion across cell membranes, a fundamental event during drug absorption (230,231). However, too many HBD/HBA can have a deleterious effect on the drug's membrane partition and permeability.

All these parameters described above are critical factors that determine gastrointestinal absorption of orally administered drugs as well as BBB permeability of drugs targeting the CNS which is relevant in identifying drug candidates for the treatment of DM and AD.

1.10 Specific aims and objectives of the study

A holistic approach to drug discovery recognises the multicomplex yet interconnected roles between body systems to cause disease. Diabetes mellitus and Alzheimer‘s were the diseases of interest in this study due to their increased prevalence in recent times, their pathological link and associated complications, devastating effects on the patients and the dire need for effective pharmacological therapies. Available pharmacological therapies for AD only temporarily improve symptoms but not delay disease progression and are therefore best viewed as palliative rather than curative treatments. Similarly, antidiabetic drugs although with significant effect in type 2 diabetes management, eventually lose effectiveness over time resulting in patients dependent on exogenous insulin which requires a lot of commitment and precision. In addition, these drug therapies are associated with side effects that can sometimes affect adherence and desired therapeutic outcomes. Vanadium and its complexes have been reported by several studies to target regulatory pathways involved in both DM and AD which makes it a potential candidate for investigation. This research pursued in this thesis centred on *in vitro* assays aimed at investigating the inhibitory activities of vanadium complexes against multiple targets involved in the pathogenesis of DM and AD and associated complications. It also incorporates in silico approaches and looks at the physicochemical and pharmacokinetic predictions of the vanadium complexes. The specific objectives were the following:

1. To investigate the inhibitory activity of vanadium complexes against α- amylase, α-glucosidase, dipeptidyl peptidase 4 (DPP-4), protein tyrosine phosphatase 1B, and advanced glycation end products (AGEs) .

2. To investigate the inhibitory activity of vanadium complexes against acetylcholinesterase, matrix metalloproteinases, beta amyloid aggregation.
3. To investigate antioxidant capacity of vanadium complexes using diphenyl-1-picrylhydrazyl (DPPH) and iron-reducing power (FRAP) assays.
4. To investigate the physicochemical and pharmacokinetic properties of the investigated complexes.

CHAPTER 2: METHODOLOGY

2.1 Materials

Sodium chloride, hydrochloric acid, sodium hydroxide, dimethyl sulfoxide were sourced from Merck South Africa. Acarbose , porcine pancreatic α -amylase, dinitro- salicylic acid, starch, sodium potassium tartrate, α-glucosidase from saccharomyces cerevisiae, p-nitrophenyl-α-glucopyranoside, sodium phosphate monobasic, sodium phosphate dibasic, DPP-4 inhibitor screening kit, protein tyrosine phosphatase powder, HEPES buffer, sodium hydroxide, para-nitrophenyl phosphate, sodium-orthovanadate, Tris HCl, EDTA, β-mecarptoethanol, donepezil, acetylcholinesterase enzyme, donepezil, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide, para-nitrophenyl phosphate, aminoguanidine, trichloroacetic acid, ascorbic acid (positive control), methanol, DPPH, potassium ferric cyanide, trichloroacetic acid, tripyridyl triazine, iron(III) chloride, sodium acetate, amyloid beta$_{1-42}$, thioflavin-T, MMP-1 inhibitor screening kit were all purchased from Sigma Aldrich South Africa. Spectramax M3 multi-mode microplate reader (100 – 240V 3.5A 50 – 60Hz; manufactured in China), Incubator (220/240V 50Hz, 0.5kW; Labcon pty ltd), BV1000 vortex mixer (230VAC 50Hz, 0.75amps; Benchmark Scientific Inc. USA), Hotplate stirrer H3760-HSE 230V, 60Hz; (Lasec Laboratories USA), PH-meter (Lasec laboratories USA)

2.2 Sourcing of Schiff base oxovanadium (IV) complexes

Three Schiff base vanadium complexes were provided by the Medicinal Chemistry group led by Prof SD. Khanye from Rhodes University. The vanadium complexes are referred to as vanadium complex 1, vanadium complex 2 and vanadium complex 3, respectively. Each vanadium complex came as dry powder which was further dissolved prior to the biological assays being conducted. Their molecular structures were drawn using the Chemsketch free software.

Preparation of vanadium compounds for *in vitro* testing

0.01g of each vanadium compound was weighed out using the Precisa analytical balance, and dissolved in DMSO (2mL) to make the stock solutions (concentration: 5000µg/mL). These stock solutions were diluted further with the appropriate buffer for the specific assay to the desired concentrations (20, 40, 60 80 and 100µg/mL) using C1V1 = C2V2 formula. The desired concentrations were freshly prepared before each assay was conducted.

2.3 Biological assays

2.3.1 Diphenyl 2- picryl hydrazyl (DPPH) scavenging assay

The antioxidant activity of vanadium complexes was determined using the DPPH free radical scavenging assay (232). In this assay, DPPH free radical, which is purple in colour abstracts a

hydrogen atom in a one-electron reaction to form 2,2-diphenyl-1-picrylhydrazine (DPPH-H), which is pale yellow in colour and this colour change is associated with a decrease in absorbance at 517 nm. Therefore, the decrease in absorbance is proportional to the antioxidant capacity of the inhibitor sample.

The scavenging reaction can be written as: $\underset{\text{Purple}}{\text{DPPH} + (\text{H-D})} \longrightarrow \underset{\text{Yellow}}{\text{DPPH-H} + \text{D}}$

The DPPH scavenging activity was determined according to Kwon et al with a slight modification (233). In a 96 well plate, freshly prepared DPPH (0.01 mM) in methanol (100 µL) was added to 200 µL of vanadium complexes at (20, 40, 60, 80 and 100 µg/mL) concentrations, after which it was allowed to stand at room temperature in the dark for 30 mins. Then, the absorbance was measured at 517 nm using the spectramax M3- multimode microplate reader (Molecular devices, China). Ascorbic acid (20, 40, 60, 80 and 100 µg/mL) was used as a reference antioxidant compound. A solution containing buffer instead of vanadium complex was used as the negative control. The percentage of DPPH radical scavenging activity was expressed as:

$$\text{DPPH activity (\%)} = \frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control} \times 100$$

2.3.2 Ferric reducing antioxidant power assay (FRAP)

The FRAP assay is based on the reduction of Fe^{3+}–tripyridyl triazine (TPTZ) complex (colourless complex) to Fe^{2+}–tripyridyltriazine (blue coloured complex) formed by the action of electron donating antioxidants at low pH. Therefore, an increase in the antioxidant activity of the inhibitor compound will cause an increase in the absorbance detected at 593 nm. The FRAP assay was carried out as described by Benzie and Strain with minor modifications for assay on a 96-well microplate (234). The FRAP reagent was freshly prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM TPTZ solution and 20.0 Mm of $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 in volume. Subsequently, 20 µL of vanadium complexes (20, 40, 60, 80 and 100 µg/mL) were then added to the FRAP reagent (200 µL) and the reaction mixture was incubated at 37 °C for 15 mins in the dark. The increase in absorbance at 593 nm was measured using the spectramax M3-multimode microplate reader (Molecular devices, China). The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the Fe(II) standard curve (0-1000 µM), (see Appendix 1) and expressed in µM (Fe(II)/g)

dry mass (mmol $FeSO_4$ equivalents per gram of sample). Ascorbic acid (20, 40, 60, 80, and 100 µg/mL) was used as the reference antioxidant compound.

2.3.3 Alpha amylase inhibition assay

This assay was performed using the 3,5-dinitrosalicylic acid (DNSA) method. The α-amylase activity is measured using a colorimetric method with DNSA reagent. In this method, the substrate (starch) is converted into maltose by α-amylase. Maltose released from starch is measured by the reduction of 3,5-dinitrosalicylic acid, where the absorbance is measured at wavelength of 540 nm. The presence of an α- amylase inhibitor, therefore, decreases the liberation of maltose, hence leading to a decrease in the reduction DNS and absorbance detected. The α-amylase inhibition assay was performed using the 3,5-dinitrosalicylic acid (DNSA) method with slight modifications (233). In a test tube, 200 µL of α-amylase (1 U/mL)) in phosphate buffer solution (0.02 M at pH 6.9) was mixed with vanadium complex (200 µL) and incubated at 37 °C for 10 min. Thereafter, the starch solution (200 µL at 1%w/v) was added to each tube and incubated for 10 min. The reaction was terminated by the addition of 500 µL DNSA reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM of 3,5-dinitrosalicylic acid solution) and boiled for 5 minutes in a water bath at 85 °C. The mixture was cooled to ambient temperature and was diluted with distilled water (5 mL) after which the absorbance was measured at 540 nm using a spectramax M3-multimode microplate reader (Molecular devices, China). The absolute control with 100% enzyme activity was prepared by replacing vanadium complexes with buffer (200 µL). Assay was performed in triplicates. Acarbose was used as the reference compound (20, 40, 60, 80 and 100 µg/mL). The α-amylase inhibitory activity was expressed as percent inhibition and was calculated using the equation given below: The percentage α-amylase inhibition was plotted against the concentration and the IC_{50} values were obtained from the graph.

$$\text{Inhibition of α- amylase activity (\%)} = \frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control} \times 100$$

2.3.4 Alpha glucosidase inhibition assay

α-Glucosidase acts on the substrate p-nitrophenyl-α-glucopyranoside to form p-nitrophenyl and simple sugars. The absorbance detected at 405 nm is a measure of the liberated p-nitrophenol. A potent inhibitor inhibits α-glucosidase activity on the substrate and therefore a reduction in the absorbance which is a measure of the p-nitrophenol formed. The ability of

vanadium complexes to inhibit α-glucosidase activity was assessed according to the standard method with minor modification (235). Briefly, in a 96-well plate, reaction mixture containing 100 µL of phosphate buffer (100 mM, pH = 6.8), 30 µL of α-glucosidase (1 U/mL), and 80 µL vanadium compounds (20, 40, 60, 80, and 100 µg/mL) were pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of 30 µL of para-nitrophenyl α-D-glucopyranoside (5 mM) and the mixture was then incubated for 10 min at 37 °C. The reaction was stopped by adding 100 µL of Na_2CO_3 (0.1 M). The extent of α-glucosidase inhibition was calculated by monitoring the increase in absorbance promoted by the formation of p-nitrophenol at 405 nm using a multiplate reader and compared to the respective control. Acarbose (20, 40, 60, 80 and 100 µg/mL) was used as the reference compound. Wells with phosphate buffer instead of vanadium complex was set up in parallel as a control. Each experiment was performed in triplicates and results were expressed as percentage inhibition, which was calculated using the formula.

$$\text{Inhibition of α- glucosidase (\%)} = \frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control} \times 100$$

Enzyme kinetics and mode of α-glucosidase enzyme inhibition

The mode of inhibition of α-glucosidase by vanadium complex 2 and vanadium complex 3 were determined. The two compounds were selected because they showed the best inhibition profile. To determine kinetics, phosphate buffer (100 µL of 0.02 M at pH 6.9) and 30 µL of enzyme (fixed concentration of (0.5 IU/mL) of α-glucosidase were placed in a 96 well plate followed by the addition of vanadium complex (200 µg/mL). This mixture was incubated at 37 °C for 10 mins. After incubation, increasing concentrations of PNPG substrate in the range (0.125 – 5mM) were added to the enzyme mixture and absorbance was measured at 405 nm. The absorbance was then converted into products formed using the paranitrophenol standard curve (0-1000 µM), (see Appendix 2). The line-weaver Burk plot was constructed and maximum velocity (V_{max}) and the Michaelis-Menten constant (Km) were determined using in graphPad prism 6. By comparing the kinetic parameters of the enzyme (Km and V_{max}) in the presence and absence of inhibitor, the type of inhibition was determined.

2.3.5 Dipeptidyl peptidase -4 inhibition

This assay was performed using a DPP-4 inhibitor screening kit purchased from Sigma Aldrich by fluorometric method. The assay uses the fluorogenic substrate, Gly-Pro-aminomethylcoumarin to measure DPP-4 activity. Cleavage of the peptide bond by DPP-4

releases the aminomethylcoumarin group, resulting in fluorescence that can be measured at an excitation wavelength of 360 nm and λ emission wavelength of 460 nm (236). The introduction of a potent inhibitor reduces the formation of the aminomethylcoumarin fluorogenic substrate and therefore a reduced fluorescence detection. The assay was conducted as per manufacturer's instruction. Briefly, vanadium complexes (12.5 µL) at 20, 40, 60, 80 and 100 µg/mL concentrations were added into the separate wells followed by the addition of DPP-4 enzyme mix (25 µL) in DPP-4 buffer solution (ratio of 1:49 enzymes to buffer). The plate was incubated in the dark for 10 minutes at 37 °C. After incubation, substrate (12 µL) was added and the fluorescence was measured using the Spectramax M3 multi-mode microplate reader at an excitation and emission wavelengths of 360 and 460 nm, respectively. Fluorescent readings were obtained in kinetic mode every minute for 15 minutes at 37 °C. Sitagliptin (12.5 µL) at IC_{50} defined by the manufacturer was used as the reference compound. Wells with DPP-4 buffer (12.5 µL) instead of vanadium complex were set up in parallel as a control. The Percentage inhibition was calculated using the formula:

$$\text{Inhibition of DPP-4 activity (\%)} = \frac{\Delta Fluorescence\ of\ control - \Delta Fluorescence\ of\ sample}{\Delta Fluorescencee\ of\ control} \times 100$$

2.3.6 Molecular docking of protein tyrosine phosphatases with vanadium complexes

In silico protein tyrosine phosphatase inhibition studies of vanadium complexes as a replacement to *in-vitro* studies

Protein and ligand preparation

To investigate the binding mode of vanadium complexes 1, 2 and 3 to the PTP1B, the docking program auto dock 1.5.6 was used to dock automatically the vanadium complexes to the catalytic domain of the PTP1B enzyme. The crystal structure of PTP1B (ID 1NNY; 2.40 Å) was recovered from the protein databank. All heteroatoms and water molecules were removed. Polar hydrogen atoms, Kollman charges and solvation parameters were added by utilising Auto dock tool. The ligand structures where drawn in Chemsketch software and converted to 3D molecular structures before being converted to pdbqt format using open babel software to get them ready for docking. All ligands were retained with polar hydrogen atoms. Gasteiger charges and torsional angles were added by utilizing auto dock tools.

Molecular Docking Simulation

The grid box was centred at the protein binding site (60 × 60 × 60 points with 0.450 Å grid spacing). AutoDock 4.2.6 was used to perform 50 docking runs for each ligand and reference compound with molecular formular - $C_{40}H_{37}N_3O_{10}$ (237). Together with Lamarckian genetic algorithm 2,500,000 energy evaluations and maximum 27,000 generation were employed and the population size was set at 150 with 0.02 and 0.8 mutation rate and crossover rate, respectively. The ligand conformation in the most populated cluster with the least binding free energy was selected for further interaction analysis. The results obtained after the docking run were analysed using the protein-Ligand Interaction Profiler online tool to generate the 3D docking simulations and the bond interactions between the enzyme and ligand (238).

2.3.7 *In-vitro* glycation of proteins

The reaction was set up as described in this study done by Singh et al with slight modifications (239). BSA glycation reaction was carried out in a test tube by incubating 0.5 mL of 10 $mg.mL^{-1}$ BSA in 0.1 M phosphate buffer (pH 7.4) with 0.5 mL of the vanadium compounds at (20, 40, 60, 80 and 100 µg/mL concentrations) and 0.5 mL of 0.5 M glucose solution containing sodium azide (0.1%) as bacteriostatic agent. A blank was prepared with of phosphate buffer (0.5 mL), BSA solution (0.5 mL) and glucose solution (0.5 mL). The reference compound was aminoguanidine (20, 40, 60, 80 and 100 µg/mL). The mixture was incubated at 37 °C for 96 hours. After incubation, BSA glycation was measured at a fluorescence of 370/440 nm using the spectramax M3-multimode microplate reader (Molecular devices China). Percent inhibition of glycation was calculated using the formulae:

$$\text{Inhibition of glycation (\%)} = \frac{Fluorescence\ of\ control - Fluorescence\ of\ sample}{Fluorescence\ of\ control} \times 100$$

2.3.8 Cholinesterase inhibition assay

AChE activity was measured using spectrophotometer based on Ellman‘s method (240). AChE hydrolyses the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman‘s reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 412 nm (241). The absorption intensity of thiocholine is proportional to the AChE activity. Therefore, a potent acetylcholinesterase inhibitor will result in much lower absorbance detected. AChE activity was determined according to Ellman‘s colorimetric method as performed by Ingkaninan et al with slight modifications (242). Briefly, into a 96-well plate 20 µL of DTNB (3 mM), 40 µL of tris-HCL buffer (50 mM) pH 8.5, 40 µL of vanadium compounds (20, 40, 60, 80 and 100 µg/mL) and 40 µL of cholinesterase

enzyme (0.5 IU/mL) were mixed and then incubated at 37 °C for 10 minutes. Absorbance readings were measured as blank to eliminate background. The enzymatic reaction was initiated by the addition of 40 µL of mM acetylthiocholine iodide (ATCI) and the hydrolysis of acetylthiocholine was monitored by reading the absorbance every min for 15 min interval to verify that the reaction occurred linearly. Donepezil was used as the reference compound (20, 40, 60, 80 and 100 µg/mL). For the absolute control, distilled water (40 µL) was used in place of vanadium complexes. Absorbance was measured at 405 nm. The enzyme percentage inhibition was calculated with absorbance at 10 mins (an area when the curve was linear) using the formula:

$$\text{Inhibition of acetylcholinesterase (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Acetylcholinesterase kinetics

The mode of inhibition of acetylcholinesterase by the efficient two inhibitor compounds vanadium complexes 2 and 3 were determined. To determine kinetics, briefly, into a 96-well plate 20 µL of DTNB (3 mM), 40 µL of tris-HCL buffer (50 mM) at pH 8.5, 40 µL of vanadium compounds (200 µg/mL) and 40 µL of cholinesterase enzyme(1 IU/mL) were mixed and then incubated at 37 °C for 10 minutes. After incubation, increasing concentrations of thiocholine substrate in the range (0.5 – 15 mM) were added to the enzyme mixture and absorbance was measured at 405 nm. The absorbance was then converted into products concentration using the thiocholine standard curve (0 – 2 mM), (see Appendix 3.) The line-weaver Burk plot was constructed and maximum velocity (V_{max}) and the Michaelis-Menten constant (Km) were determined using in graphpad prism 6. The type of inhibition was determined by comparing the kinetic parameters of the enzyme (Km and V_{max}) in the presence and absence of inhibitor.

2.3.9 Matrix metaloproteinase-1 inhibition

The MMP-1 activity is measured by hydrolysing a fluorescence resonance energy transfer (FRET) peptide substrate to yield a fluorescent product which is proportional to the enzyme activity present. Therefore, MMP-1 inhibitors act by preventing the formation of this fluorescent product and a subsequent reduction in the fluorescence. This assay was performed as per manufacture‘s instruction. Briefly, in a 96-well fluorescence black plate, 12.5 µL of vanadium complexes (20, 40, 60, 80 and 100 µg/mL) were added, then 25 µL of the enzyme in MMP-1 buffer solution (ratio of 1:49 enzyme to buffer) was added into the wells already containing the vanadium complexes and the mix was incubated at 37 °C for 5 minutes in the

dark. After incubation, 12.5 µL of the fret-tagged substrate was added to the wells and the fluorescence was measured at an excitation wavelength of 490 nm and emission wavelength of 520 nm using the spectramax microplate reader. This was measured at 37 °C in kinetic mode every minute for 30 minutes.

$$\text{MMP-1 inhibition}(\%) = \frac{\Delta Fluorescence\ of\ control - \Delta Fluorescence\ of\ sample}{\Delta Fluorescencee\ of\ control} \times 100$$

2.3.10 Beta-amyloid aggregation inhibition assay

Thioflavin T is a benzothiazole dye that exhibits enhanced fluorescence upon binding to beta-sheet rich structures such as amyloid fibrils and aggregates to give a strong fluorescence signal at an excitation wavelength of 450 nm and emission wavelength of 482 nm (243). Therefore, in the presence of anti-amyloid aggregating compounds, the fluorescence is reduced.

Preparation of peptides

To prepare the peptide for the assay, 1 mg of commercially available β-amyloid protein fragment Aβ1–42 was dissolved in 2 mL of 50 mM sodium hydroxide. The solution was left for 3 min after which deionized water (700 µL) was added to the same tube. The solution was made to 1 mL with phosphate-buffered saline (PBS) to give a final concentration of 25 µg/mL. The peptide solution was sonicated for 3 min. Afterwards, 15 µL of the peptide solution (25 µg/mL) was added to the well, then 200 µL of vanadium compounds and rifampicin (reference compound) was mixed with 85 µL of 10 mM phosphate buffer (final concentration, 5 µM) and incubated at 37 °C. Aliquots were taken at different time intervals (0 and 96 hours) for thioflavin-T (ThT) assay (244).

Thioflavin-T assay

The ability to inhibit ($A\beta_{1-42}$) aggregation for the vanadium complexes compounds was determined using the thioflavin-T method according to Ozadari-Sali et al with slight modifications (158). Briefly, 200 µL of 10 µM Thioflavin T (ThT) solution (prepared in 50 mM glycine-NaOH, pH 8.5) was added to the 20 µL aliquots taken at two intervals of (t = 0 and t = 96 hrs). Increase or decrease in thioflavin fluorescence intensity was measured using a microtiter plate reader (Molecular devices, China) at excitation (450 nm) and emission (480 nm) wavelengths. The control experiment was without the vanadium complexes. ThT solution and phosphate-buffered saline, pH 7.4 was used as a blank. Rifampicin (100 µg/mL) was

assayed as positive controls. A graph of fluorescence intensity versus time was plotted for the inhibitor compounds and compared with the control.

2.4 Calculation of IC_{50}

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function (183). This quantitative measure indicates how much of a drug or inhibitor is needed to inhibit a given process by half. To calculate IC_{50}, the percentage inhibitions obtained for each enzyme inhibitory studies was analysed by non-linear regression analysis using GraphPad prism 6. IC_{50} values were calculated for α-glucosidase, α-amylase, DPP-4, acetylcholinesterase and antioxidant assays, which showed positive inhibition but was disregarded for assays such as aldose reductase and MMP-1 inhibition assays which showed enzyme activation.

2.5 Statistical analysis

The data were expressed as the mean ± SD of three replicates. Analysis was performed using GraphPad prism 6 Software and Excel office 365. One-way analysis of variance (ANOVA) trailed by Tukey post-test were used to test for statistical significance difference at p-values ≤0.05. ANOVA was done on the absorbance readings obtained from spectrophotometric analysis before any normalization. Using the raw data before normalization allows comparison of the investigational compounds against the control which has no active compounds present.

2.6 Predicting the drug-likeness and ADME profiles of the vanadium complexes

The predicted drug likeness, pharmacokinetics properties and bioavailability of the vanadium complexes was done using SwissADME online tool (www.swissadme.ch) of Swiss Institute of Bioinformatics developed by Daina et al (245). The canonical SMILES for each chemical was incorporated in this tool for the computational simulation after which the Lipinski parameters, pharmacokinetic properties and bioavailability radar was generated.

CHAPTER 3: RESULTS

3.1 General structure and characteristics of vanadium complexes

The three synthesised vanadium complexes were synthesised from Schiff bases which are characterised by the presence of azomethine (-CH=N−) group. The azomethine group present in each molecular structure is circled in red. These coordination compounds show a peculiar structure where the Schiff base ligands are linked to the vanadyl cation coordination sphere. While vanadium complexes 1 and 2 have the same molecular weight of 707 g/mol, they differ in the position of the bromine (Br) and nitro (NO_2) groups making them stereoisomers due to the different arrangement of atoms in space. Vanadium complex 3 has a molecular weight of 685 g/mol with the presence of a trifluoro methyl (CF_3) group which differentiates it from the other two vanadium complexes.

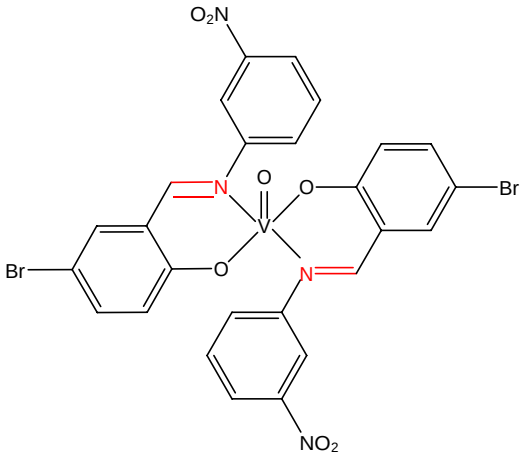

Figure 3.1: The molecular structure of vanadium complex 1 with the presence of azothemine bonds (red). Molecular formula: $C_{26}H_{16}Br_2N_4O_7V$.

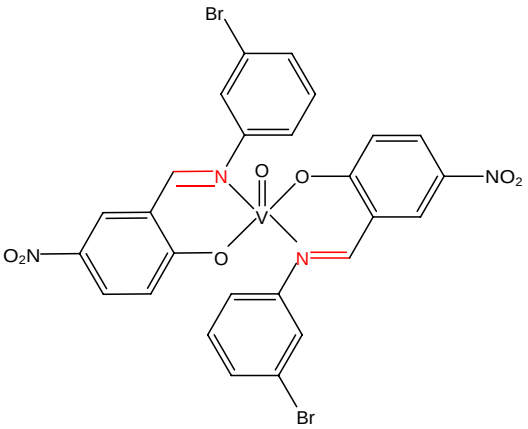

Figure 3.2: The molecular structure of vanadium complex 2 with the presence of azothemine bonds (red). Molecular formula: $C_{26}H_{16}Br_2N_4O_7V$.

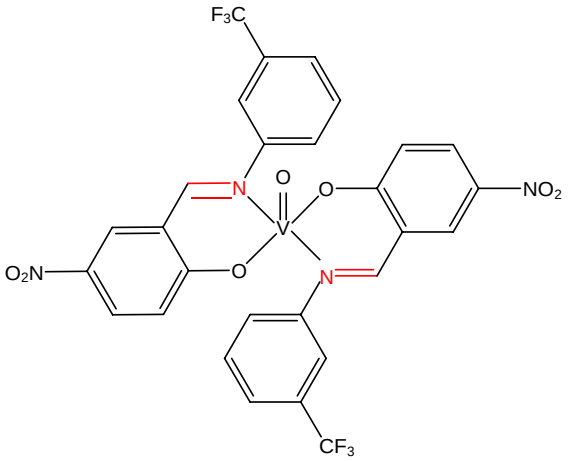

Figure 3.3: The molecular structure of vanadium complex 3 with the presence of azothemine bonds (red). Molecular formula:$C_{28}H_{16}F_6N_4O_7V$.

3.2 Biological assay results

All *in-vitro* results for all the assay are represented in a column chart and data are presented as mean ± standard deviation values represented by the error bars. The asterisks (*) indicate significant statistical difference relative to the control using one-way ANOVA followed by Tukeys multiple comparison test to find differences between the inhibitors and control at p value ≤ 0.05. The arrow from right pointing at the baseline at 0.0 represents the negative control, which is defined to have zero percent inhibition for the purpose of data normalization.

3.2.1 Antioxidant (DPPH) assays

Figure 3.4 shows the DPPH radical scavenging activity for vanadium complexes and reference drug, ascorbic acid at 20, 40, 60, 80 and 100 µg/mL concentrations.

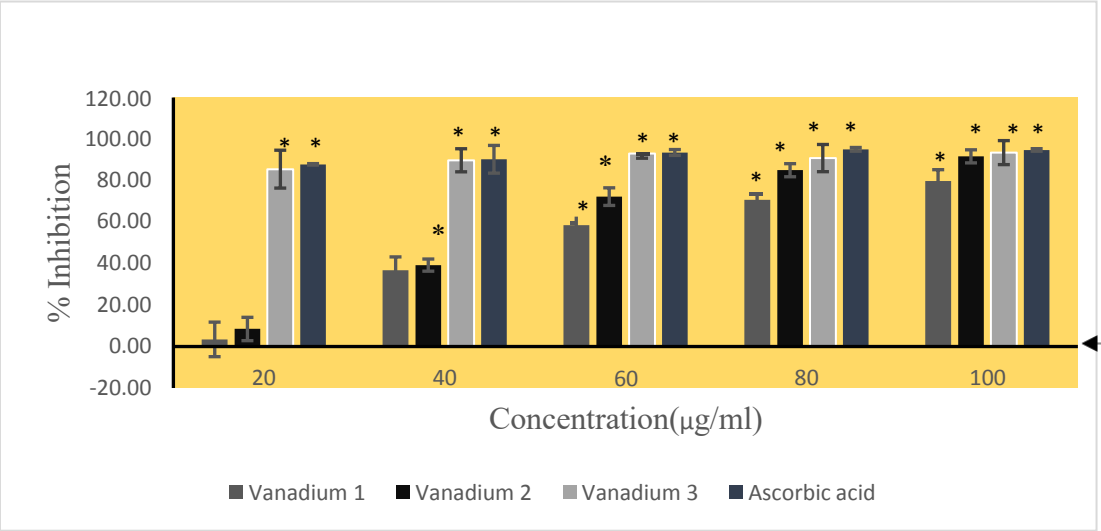

Figure 3.4: DPPH free scavenging activity of vanadium complexes and ascorbic acid at different concentrations (20, 40, 60, 80 and 100 µg/mL). Asterisks show statistical significance.

The vanadium complex 1 showed a free radical scavenging activity, with 60 µg/mL and above showing statistical significance by comparison to the control while vanadium complex 2 showed significance from 40 µg/mL and above ($p < 0.05$). Vanadium complex 3 showed a free radical scavenging activity, demonstrating significance at all concentrations ($p < 0.05$) by comparison to the control. As anticipated, a significant ($p < 0.05$) free radical scavenging activity was observed with ascorbic acid in all concentrations. Of the three vanadium complexes investigated, vanadium complex 3 was more effective in free radical scavenging activity in DPPH assay as evidenced by a smaller IC_{50} (Table 3.1)

Table 3.1: Table of IC_{50} values for vanadium complexes and acarbose obtained from DPPH scavenging activity.

Complex	DPPH (IC_{50} µg/mL)
Vanadium complex 1	**52.07**
Vanadium complex 2	**42.05**
Vanadium complex 3	**7.011**
Ascorbic acid	**7.088**

3.2.2 Ferric reducing antioxidant power (FRAP) ASSAY

Figure 3.5 shows the ferric reducing antioxidant power of the vanadium complexes and ascorbic acid at 20, 40, 60, 80 and 100 µg/mL concentrations as a function of Fe(II) dry mass.

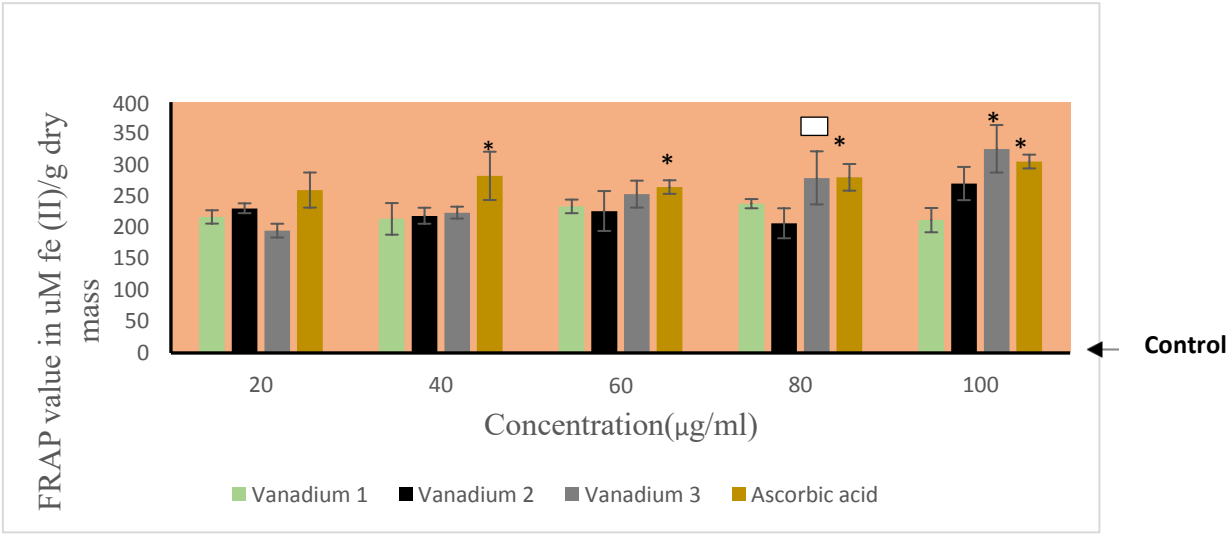

Figure 3.5: Ferric reducing antioxidant power assay of vanadium complexes and ascorbic acid at different concentrations 20, 40, 60, 80 and 100 µg/mL.

Vanadium complex 1 and vanadium complex 2 showed no significant FRAP in comparison to the control while vanadium complex 3 showed significant FRAP activity at 80 and 100 µg/mL concentrations by comparison to the control ($p < 0.05$). The reference drug, ascorbic acid, was statistically significant in comparison to the control from 40 to 100 µg/mL concentrations. Due to the nature of the presentation of the FRAP data as Fe(II) dry mass produced, IC_{50} values could not be obtained. Vanadium complex 3 is the only complex that displayed some statistical significance to the control which can be interpreted as it being the most potent of all three complexes.

3.2.3 Alpha amylase inhibition assay

Figure 3.6 shows the α-amylase inhibitory activity of vanadium complexes at various concentrations.

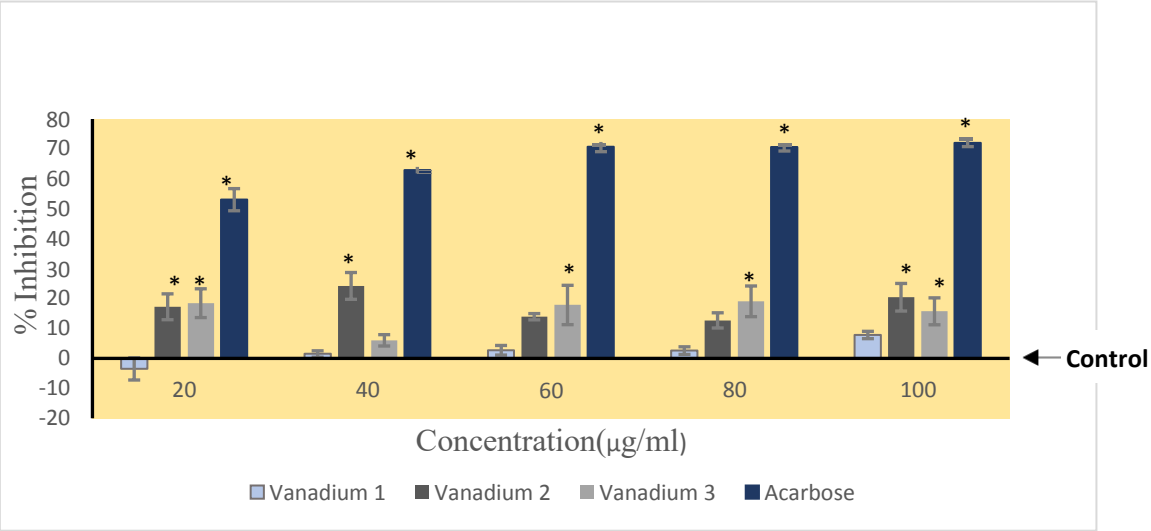

Figure 3.6: Percentage inhibition of α-amylase of vanadium complexes and acarbose by different concentrations 20, 40,60,80 and 100µg/mL.

Vanadium complex 1 shows α- amylase inhibitory activity but not statistically significant at any concentration in comparison with the control while vanadium complexes 2 shows α-amylase inhibitory activity with significant difference in comparison to the control at 20, 40 and 100 µg/mL concentration. Lastly, vanadium complex 3 shows statistical significance at all concentrations except at 40 µg/mL ($p < 0.05$). As anticipated, a statistically significant inhibition was observed for acarbose (positive control) at all concentrations ($p < 0.05$). Of the three vanadium complexes investigated, vanadium complex 1 showed more dose dependent activity and the smallest IC_{50} (Table 3.2).

Table 3.2: IC_{50} values for vanadium complexes and acarbose obtained from the α-amylase inhibition assay.

Complex	Alpha amylase (IC_{50} µg/mL)
Vanadium complex 1	**226.8**
Vanadium complex 2	**8593**
Vanadium complex 3	**1097**
Acarbose	**22.28**

3.2.4 Alpha glucosidase inhibition

Figure 3.7 shows the α-glucosidase inhibitory activity of vanadium complexes and acarbose evaluated at various concentrations.

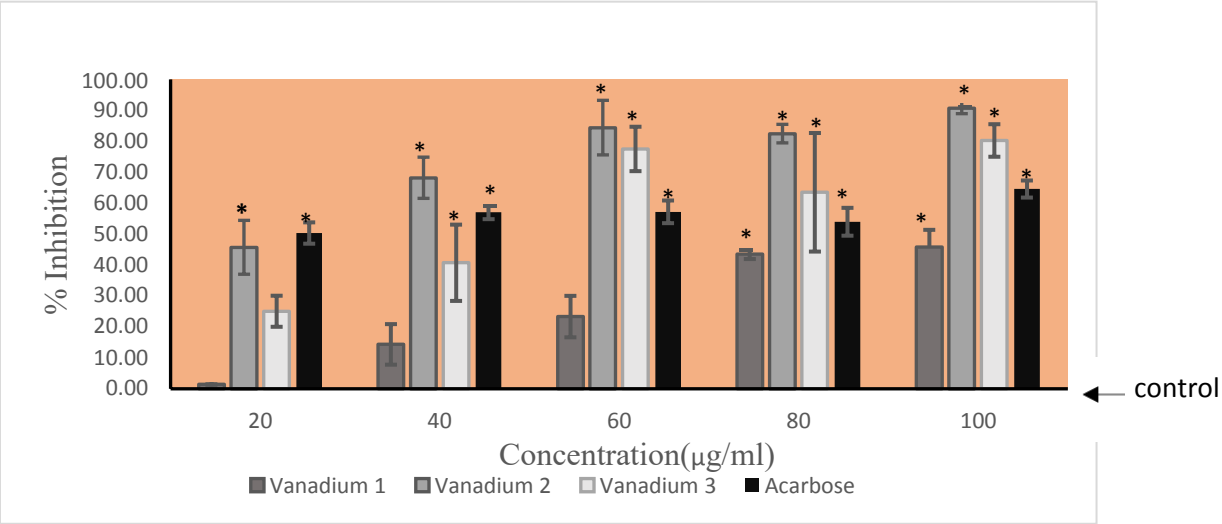

Figure 3.7: Percentage inhibition of α-glucosidase of vanadium complexes and acarbose at different concentrations 20, 40, 60, 80 and 100 µg/mL.

Vanadium complex 1 shows significant α-glucosidase inhibition at 80 and 100 µg/mL concentration while vanadium complex 3 shows significant difference at 40 µg/mL concentration and above in comparison with the control. Vanadium complex 2 shows a statistically significant difference in all concentrations ($p < 0.05$) when compared to the control, which is the same trend observed for acarbose (positive control) at all concentrations ($p < 0.05$). Of the three vanadium complexes investigated, vanadium complex 2 showed the strongest α-glucosidase inhibitory activity (Table 3.3).

Table 3.3: IC_{50} values for vanadium complexes and acarbose obtained from the α- glucosidase inhibition assay.

Complex	**IC_{50} µg/mL**
Vanadium complex 1	**52.58**
Vanadium complex 2	**20.29**
Vanadium complex 3	**31.70**
Acarbose	**9.162**

3.2.4.1 Alpha glucosidase kinetic studies

The mode of inhibition of vanadium complexes 2 and 3 against α-glucosidase was further investigated by Lineweaver Burk plot analysis (Figures 3.8 and 3.9).

The enzyme velocity (y-axis) was plotted against the substrate concentration (x-axis).

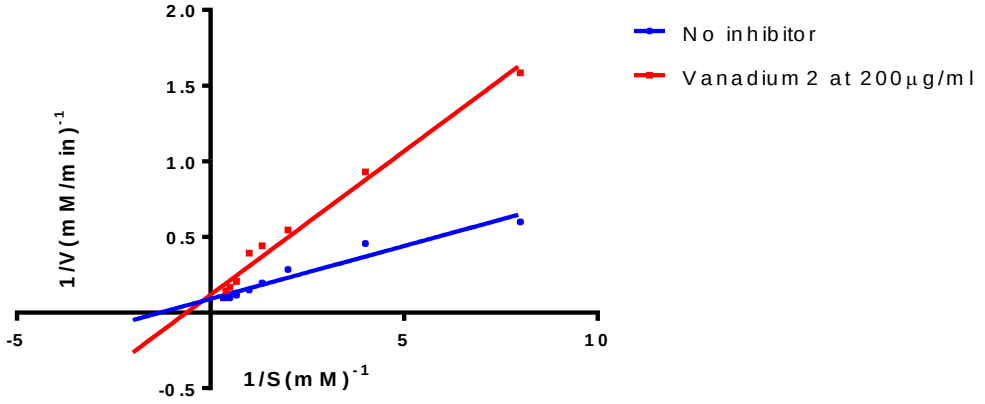

Figure 3.8: A Lineweaver Burk plot analysis showing mixed type inhibition by vanadium complex 2 on α-glucosidase enzyme.

Vanadium complex 2 showed a mixed type inhibition where Michaelis constant (k_m) increased as illustrated by the red line representing the inhibitor intersecting the x-axis at a more positive value compared to the blue line which represents no inhibitor. While maximum velocity (V_{max}) decreased (see table 3.4) . This is supported by the intersection of both graphs at the second quadrant which is typical of mixed type inhibitions(Figure 3.8). Vanadium complex 3 however, showed a non-competitive type inhibition, as km stayed the same as the blue and red lines intersect on the negative x-axis and V_{max} decreased (Figure 3.9).

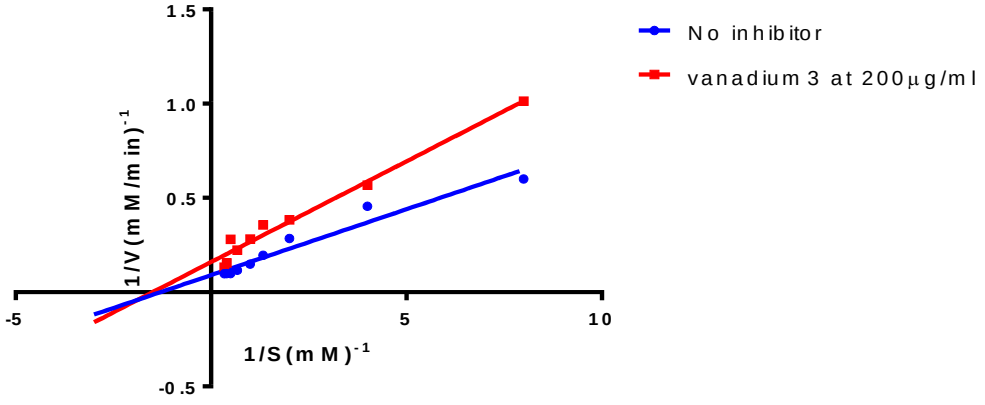

Figure 3.9: A Lineweaver Burk plot analysis showing non-competitive type inhibition by vanadium complex 3 on α-glucosidase enzyme.

A numerical representation of the Lineweaver Burk plot kinetics of α-glucosidase by the investigated vanadium complexes (Table 3.4). Again, vanadium complex 2 showed mixed type inhibition against α-glucosidase with an increase in K_m from 0.78 to 1.67 mM and a decrease in V_{max} from 11.32 to 0.69 mM/min^{-1}. Vanadium complex 3 showed a non-competitive type inhibition as K_m is almost the same with only a difference of 0.07 units and V_{max} decreased from 11.32 to 6.54 mM/min^{-1}.

Table 3.4: Alpha-glucosidase kinetic parameters (K_m and V_{max}) for vanadium complexes.

Concentration	Vanadium complex 2		Vanadium complex 3	
	K_m	V_{max}	K_m	V_{max}
No inhibitor	0.78	11.32	0.78	11.32
Inhibitor at 200 µg/mL	1.67	0.69	0.71	8.64

3.2.5 Dipeptidase peptidyl-4 inhibition

Figure 3.10 shows the DPP-4 inhibitory activity of vanadium complexes at different concentrations (20, 40, 60, 80 and 100 µg/mL) and sitagliptin prepared at IC_{50} concentration according to the assay kit's instruction provided.

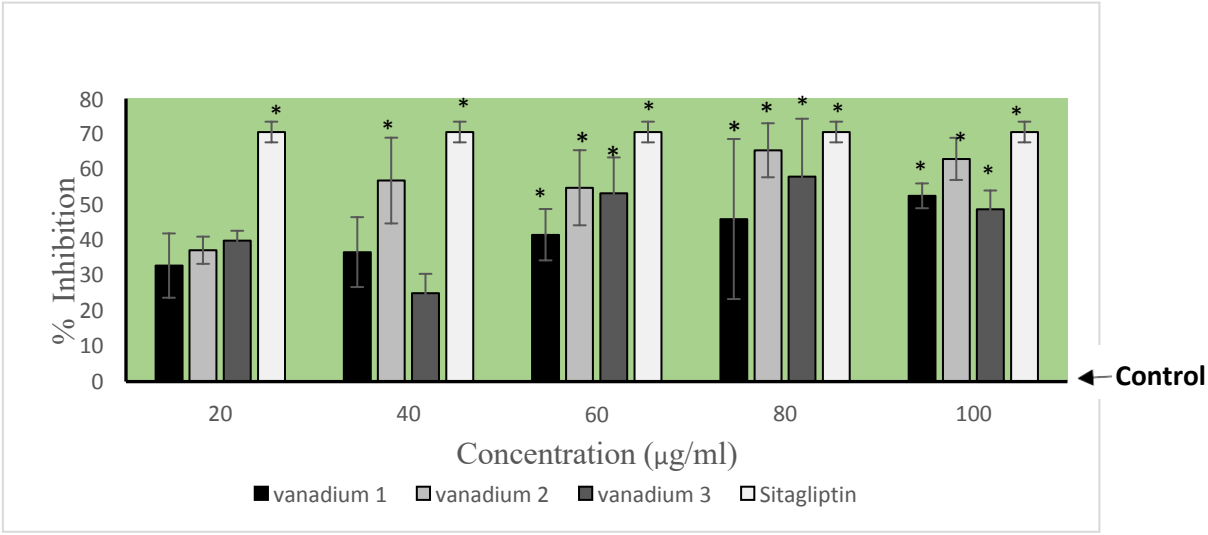

Figure 3.10: Percentage inhibition of DPP-4 by different concentrations of vanadium complexes (20, 40, 60, 80 and 100 µg/mL) and sitagliptin at IC_{50}.

From the DPP- 4 inhibition results obtained, vanadium complexes 1 and 3 showed a significantly different DPP-4 inhibitory activity from 60 µg/mL upwards, while vanadium complex 2 showed a significant difference in DPP-4 activity from 40 µg/mL concentration and upwards in comparison with the control ($p < 0.05$). As expected, the reference drug sitagliptin, showed statistically significant difference at the IC_{50} concentration ($p < 0.05$). The IC_{50} values obtained from the DPP-4 assay shows vanadium complex 3 having lower IC_{50} while vanadium complexes 1 and 2 gave similar IC_{50} values above 60 µg/mL (Table 3.5). From this data, it emerged that vanadium complex 3 was marginally potent than vanadium complexes 1 and 2.

Table 3.5: IC_{50} values for vanadium complexes 1, 2 and 3 obtained from DPP-4 inhibition assay.

Complex	**IC_{50} (µg/mL)**
Vanadium complex 1	**62.80**
Vanadium complex 2	**63.16**
Vanadium complex 3	**45.56**

3.2.6 Protein tyrosine phosphatase docking results

The Figure 3.11 represents the results from docking the inhibitor compounds and reference compound into the catalytic site of the protein tyrosine phosphatase enzyme retrieved from protein data bank (ID:1NNY). Interactions giving the least binding energy from all 50 runs was represented and images were generated using the Protein-Ligand Interaction Profiler online tool.

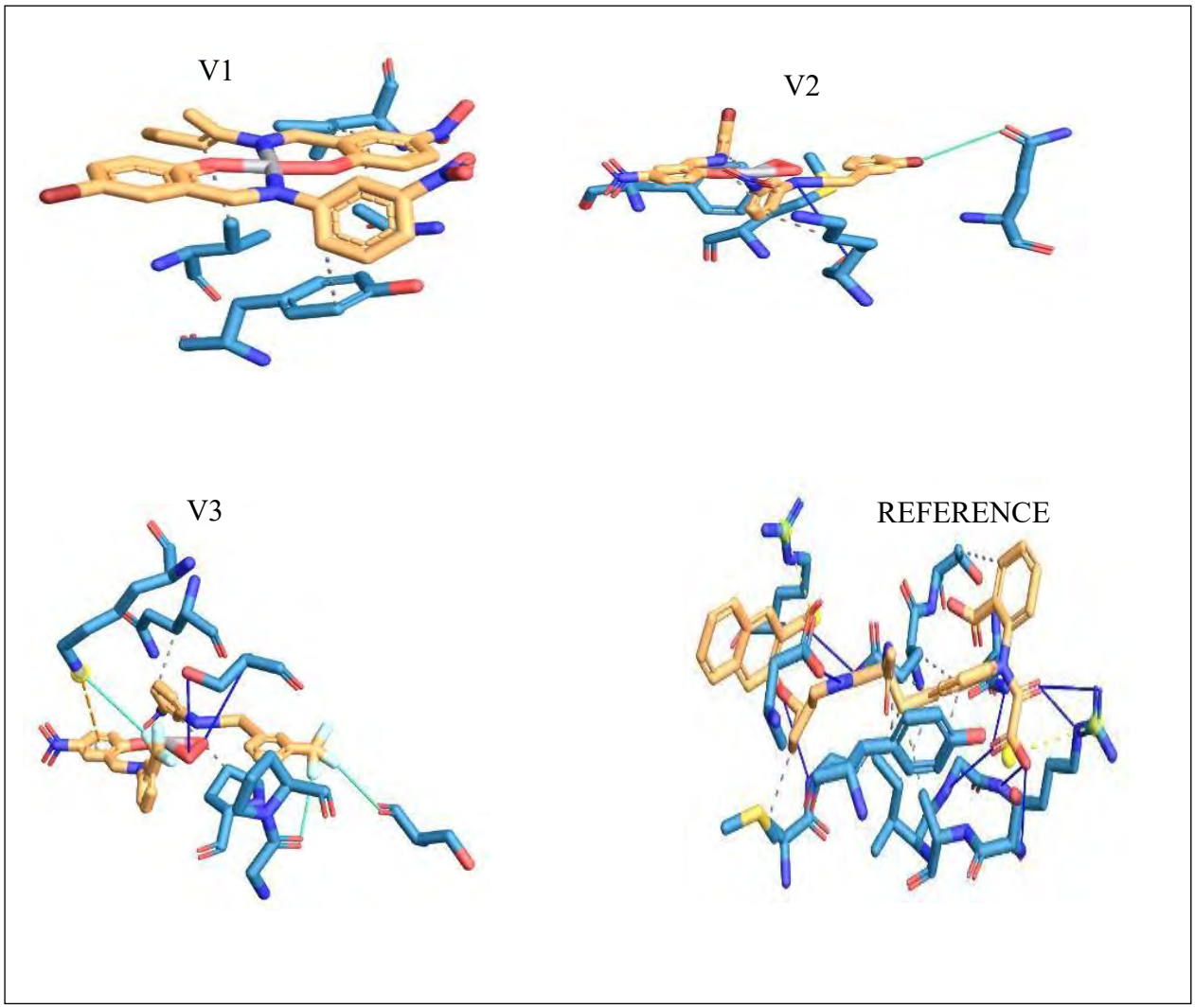

Figure 3.11: Molecular docking results for vanadium complexes and known PTP1B catalytic inhibitor (reference compound) showing predicted ligand-receptor interaction with the PTP1B enzyme.

Grey dotted lines show hydrophobic interactions with the enzyme, solid blue lines depict hydrogen bond interaction, orange dotted line represents represent π-π interactions and the

solid green line represents halogen bonds. From the molecular docking results of the PTP1B enzyme. Vanadium complex 1 gave the most favourable binding free energy of −9.89 kcal/mol to the PTP1B catalytic site compared to vanadium complexes 2 and 3 with -5.2 and -5.36 kcal/mol, respectively (Table 3.6). In terms of predicted ligand-receptor interaction, vanadium complex 1 formed four hydrophobic contacts with the amino acid residues at the enzyme catalytic site while vanadium complex 2 formed four hydrophobic interaction plus equal number (i.e. 1 each) of hydrogen and halogen bonds. The reference compound made the highest number of interactions with the enzyme. From the three complexes, vanadium complex 3 showed the most interaction consisting of three hydrophobic contacts, one hydrogen bonds, three halogen bonds and one π-π interaction (Table 3.6).

Table 3.6: Estimated bond interactions and binding free energy (kcal/mol) from docking simulation of vanadium complexes and reference compound to the catalytic binding site of PTP1B.

Bond type	**Vanadium complex 1**	**Vanadium complex 2**	**Vanadium complex 3**	**Reference compound**
Hydrophobic interactions	TYR-46, VAL-49, ALA-217, ILE-219	LYS-131, MET-133, PHE-135	GLY-78, PRO-206, PRO-210	TYR-46,ALA-217,ILE-219(X2),MET-258,GLN-262(X2), THR-263
Hydrogen bonds	-	LYS-131	SER-80	ASP-48A,SER-216, GLY-220,GLY-220,ARG-221(X3),GLY-259, GLN-262,GLN-266
Halogen bond	-	GLY-127	LYS-73, SER-203, GLY-209	-
π-Cation Interactions	-	-	LYS-73	-
Salt Bridges	-	-	-	ARG-24A,ARG-221
Binding energy	-9.89	-5.20	-5.36	-6.03
Estimated inhibition constant Ki	0.056.69 μM	153.47 μM	117.30 μM	38.16 μM

3.2.7 Acetylcholinesterase inhibition assay

Figure 3.12 shows the acetylcholinesterase inhibitory activity of vanadium complexes and the reference drug donepezil at concentrations of 20, 40, 60, 80 and 100 µg/mL.

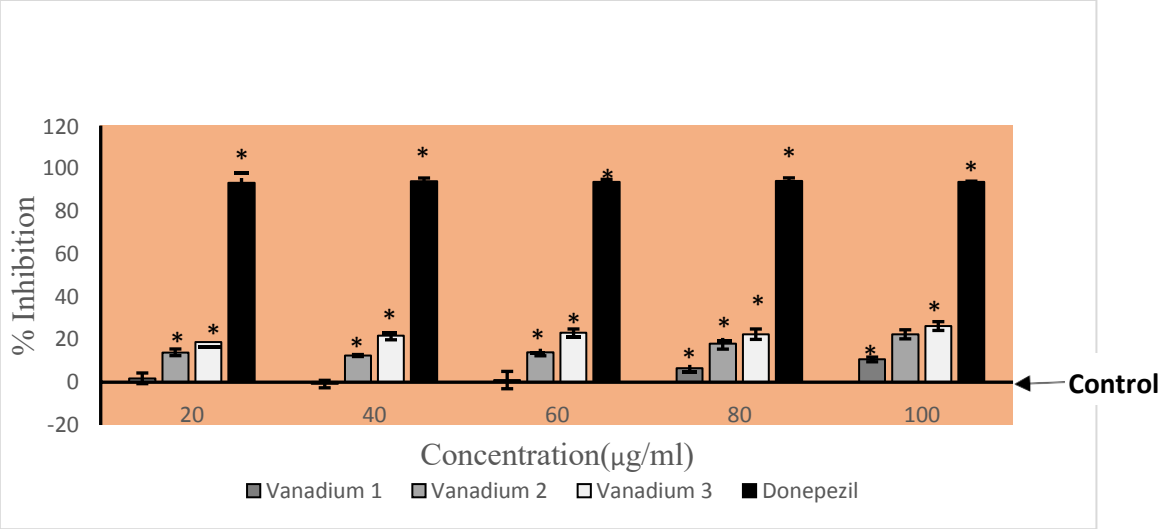

Figure 3.12: The results from the inhibition of acetylcholinesterase by vanadium complexes and donepezil.

Vanadium complex 1 shows significantly different acetylcholinesterase inhibition at 60, 80 and 100 µg/mL concentrations in comparison with the control. While vanadium complexes 2 and 3 displayed significant difference across all concentrations in comparison with the control ($p < 0.05$). As with the reference drug donepezil, a significant difference was observed at all concentrations ($p < 0.05$). Vanadium complex 3 showed the strongest and most potent acetylcholinesterase inhibitory activity of the three vanadium complexes (Table 3.7)

Table 3.7: IC_{50} values of vanadium complexes obtained from acetylcholinesterase inhibition assay.

Complex	IC_{50} µg/mL
Vanadium complex 1	**76.32**
Vanadium complex 2	**19.37**
Vanadium complex 3	**11.05**
Donepezil	**6.477**

3.2.7.1 Acetylcholinesterase kinetic studies

The mode of inhibition of the most effective two vanadium complexes against acetylcholinesterase was investigated by Lineweaver Burk plot analysis (Figures 3.13 and 3.14).

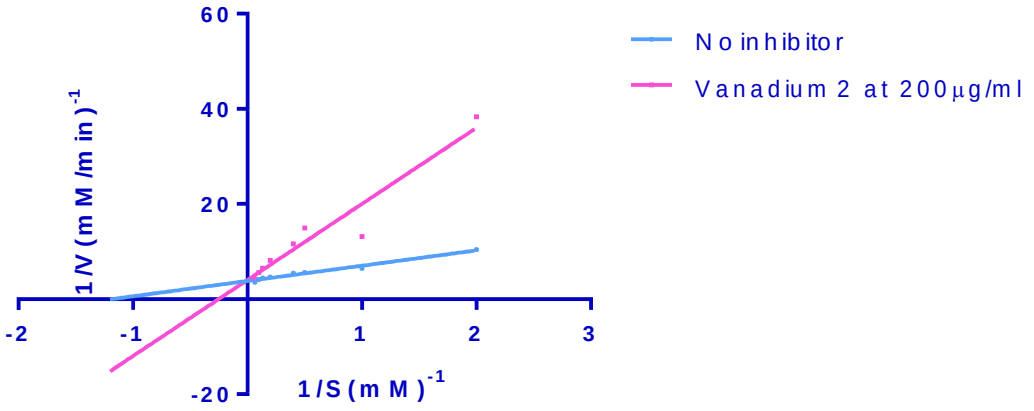

Figure 3.13: A Lineweaver Burk plot analysis showing competitive type inhibition by vanadium complex 2 on acetylcholinesterase enzyme

The enzyme velocity (y-axis) was plotted against the substrate concentration (x-axis). Vanadium complexes 2 and 3 showed a competitive type inhibition. There is an increase in Michaelis constant (k_m) as can be seen with the purple line which represents the inhibitor intersecting the x-axis at a more positive x-value than the blue line which represents no inhibitor. While maximum velocity (V_{max}) stayed the same as can be seen with both lines intersecting at the y-axis which is typical of competitive type inhibitions (Figures 3.13 and 3.14).

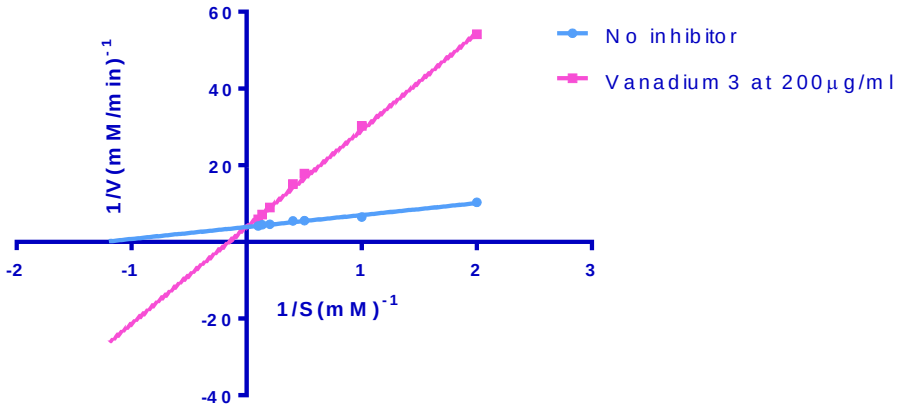

Figure 3.14: A Lineweaver Burk plot analysis showing competitive type inhibition by vanadium complex 3 on acetylcholinesterase enzyme**.**

A numerical representation of the Lineweaver Burk plot kinetics of the investigated vanadium complexes (Table 3.8). Again, vanadium complexes 2 and 3 showed competitive type inhibition against acetylcholinesterase with an increase in K_m in comparison to no inhibitor. The K_m of vanadium complex 2 increased from 0.85 to 3.99 mM and 0.79 to 5.91 in vanadium complex 3 while V_{max} remained the same for both complexes.

Table 3.8: Acetylcholinesterase kinetic parameters (K_m and V_{max}) for vanadium complexes 2 and 3.

Concentration	**Vanadium complex 2**		**Vanadium complex 3**	
	K_m	V_{max}	K_m	V_{max}
No inhibitor	0.85	0.26	0.79	0.25
Inhibitor at 200 µg/mL	3.99	0.25	5.91	0.24

3.2.8 The inhibition of advanced glycation end products (AGES)

Figure 3.15 shows the AGES inhibitory activity of vanadium complexes and the reference drug aminoguanidine at concentrations of (20, 40, 60, 80 and 100 µg/mL after 96 hours of incubation.

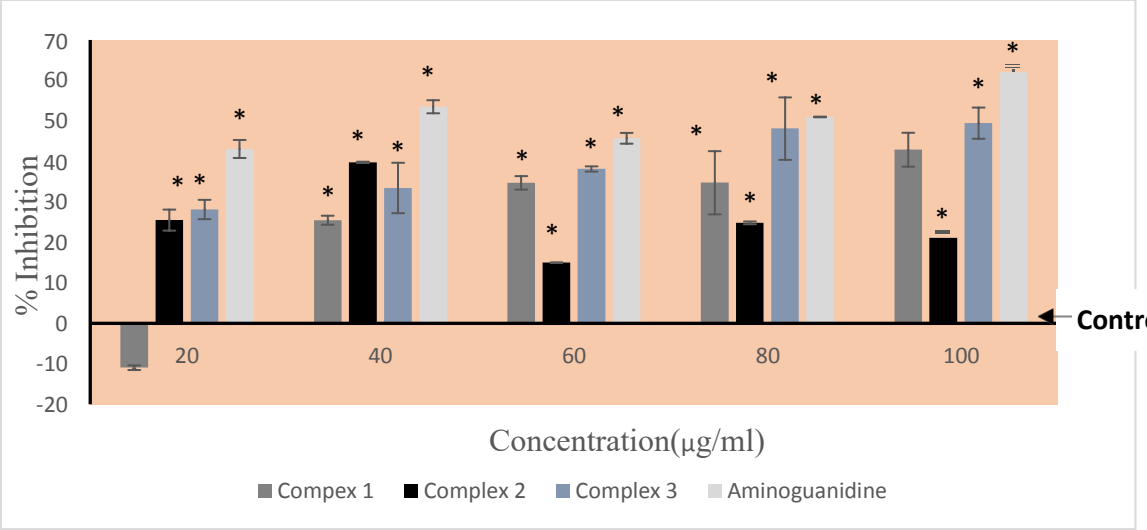

Figure 3.15: Percentage inhibition of advanced glycation end products by different concentrations of vanadium complexes and aminoguanidine.

Vanadium complex 1 showed AGES inhibitory activity with significance from 40 to 100 µg/mL concentration in comparison with the control ($p < 0.05$). Vanadium complexes 2 and 3 displayed significant difference across all concentrations in comparison with the control ($p < 0.05$). As expected, the reference drug aminoguanidine, showed a significant difference at all concentrations ($p < 0.05$). Vanadium complexes 1 and 2 showed slightly higher AGES inhibition compared to complex 3 (Table 3.9).

Table 3.9: IC_{50} values of vanadium complexes obtained from advanced glycation end products inhibition.

Complex	IC_{50} µg/mL
Vanadium complex 1	**35.72**
Vanadium complex 2	**35.85**
Vanadium complex 3	**56.27**
Aminoguanidine	**10.4**

3.2.9 Matrix metalloproteinase-1 inhibition assay

Figure 3.16 is showing the MMP-1 inhibition results of the vanadium complexes at various concentrations 20, 40, 60, 80 and 100 µg/mL and the reference drug ilomastat at IC_{50} (only concentration supplied by the manufacturer)

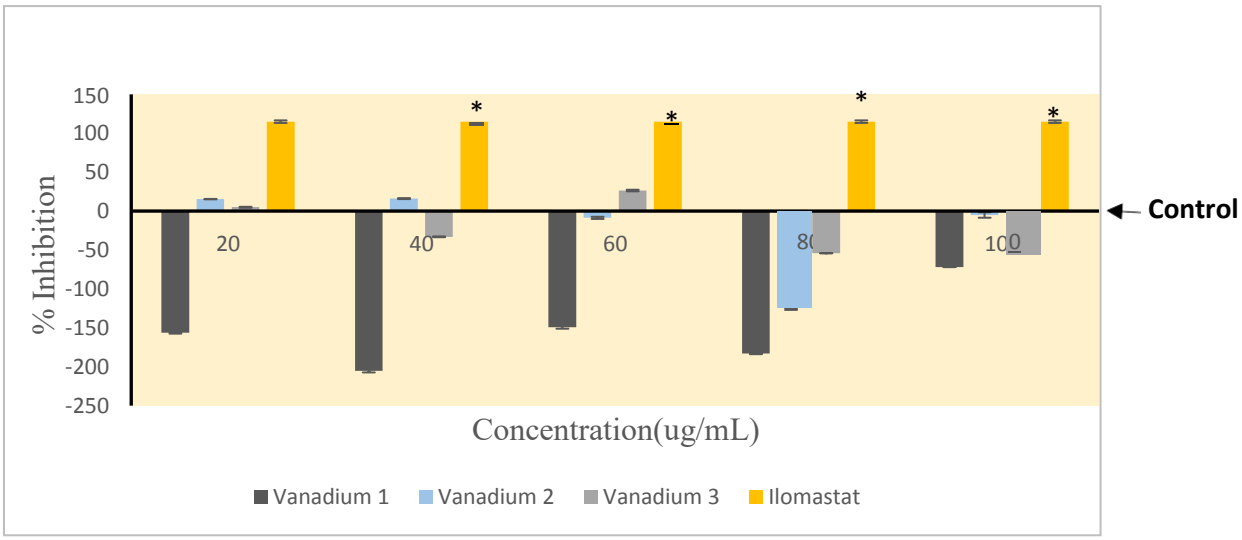

Figure 3.16: Percentage inhibition of matrix metalloproteinases-1 by vanadium complexes at various concentrations and reference inhibitor

Vanadium complexes 1 and 2 showed less than 20% inhibition at 20 and 40 µg/mL concentrations and 20 and 60 µg/mL, respectively. All other concentrations for vanadium complexes 1 and 2 as well as vanadium complex 3 at 20, 40, 60, 80 and 100 µg/mL concentrations gave negative inhibitions or what might be interpreted as an enzyme activation. The reference inhibitor ilomastat showed inhibition against MMP-1 showing a significant difference by comparison with the control ($p < 0.05$). The IC_{50} values for the vanadium complexes were not determined because there was no inhibition observed.

3.2.10 Beta amyloid anti-aggregation assay

Figure 3.17 shows the results of Aβ disaggregation potential of the vanadium complexes at 100 µg/mL concentration. Rifampicin was included as the standard at 100 µg/mL and the negative control (NC) contained buffer instead of the active compounds.

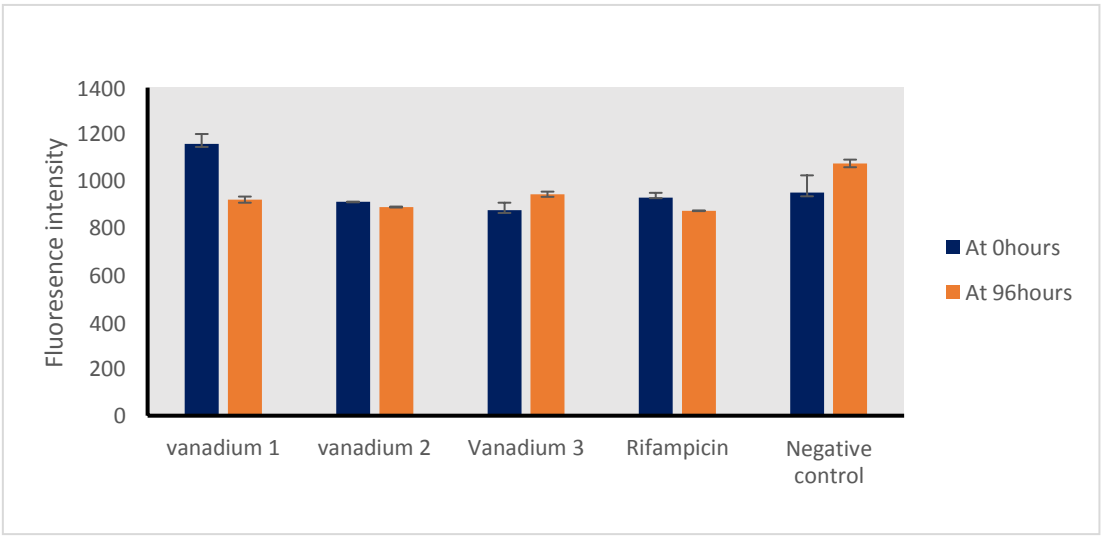

Figure 3.17: Beta amyloid disaggregation of vanadium complexes, rifampicin (100 µg/mL) and the negative control observed over a period of 96 hours. No significant difference in comparison with the control ($p < 0.05$).

Anti-aggregative ability of the investigated compounds was observed over 96 hours measured as a function of decrease in fluorescence intensity. An increase in fluorescence was observed for vanadium complex 3 and the NC which correlates to an increased peptide aggregation. On the other hand, vanadium complexes 1 and 2 as well as the standard drug rifampicin showed a

slight decrease in fluorescence intensity which may indicate a decreased peptide aggregation. Overall, the amyloid anti-aggregative activity of the complexes including the standard rifampicin showed no significant difference in comparison with the control ($p < 0.05$).

3.3 *In silico* pharmacokinetic properties of vanadium complexes

The evaluation of drug-likeness of the synthesized vanadium compounds based on Lipinski parameters was determined using online Swiss ADME prediction tool and the results presented in Table 3.10.

Table 3.10: Predicted Lipinski's properties for vanadium complexes 1-3.

Compounds	**Molecular weight(g/mol)**	**Donor Hydrogen Bond**	**Acceptor Hydrogen Bond**	**Lipophilicity Log Po/wa[a]**	**#rotorb[b]**	**Rule of 5 violations**
Acceptable range	≤ 500	≤ 5	≤ 10	(−2.0 to 6.5)	≤10	≤ 2
Complex 1	707.18	0	7	2.95	4	1
Complex 2	707.18	0	7	2.95	4	1
Complex 3	685.38	0	13	3.79	6	2

[a]*Predicted octanol/water partition coefficient log P.* [b]*Predicted rotatable bonds.*

The output data for vanadium complexes 1 and 2 are the same due to their identical canonical smiles. Vanadium complexes 1 and 2 violates one out of 4 Lipinski parameters having a molecular weight (Mw) ≥ 500 g/mol. However, vanadium complex 3 violated 2 Lipinski rules, which are Mw ≥500 g/mol and more than 10 hydrogen bond acceptors (HBA). The acceptable number of violations for drug likeness is 2. Therefore, all vanadium complexes generally showed acceptable drug-like characteristics according to Lipinski (Table 3.10).

The bioavailability radar of the vanadium complexes which is a graphical representation of bioavailability is presented as a hexagon diagrams shown in Figures 3.18 – 3.20 generated using SwissADME web tool.

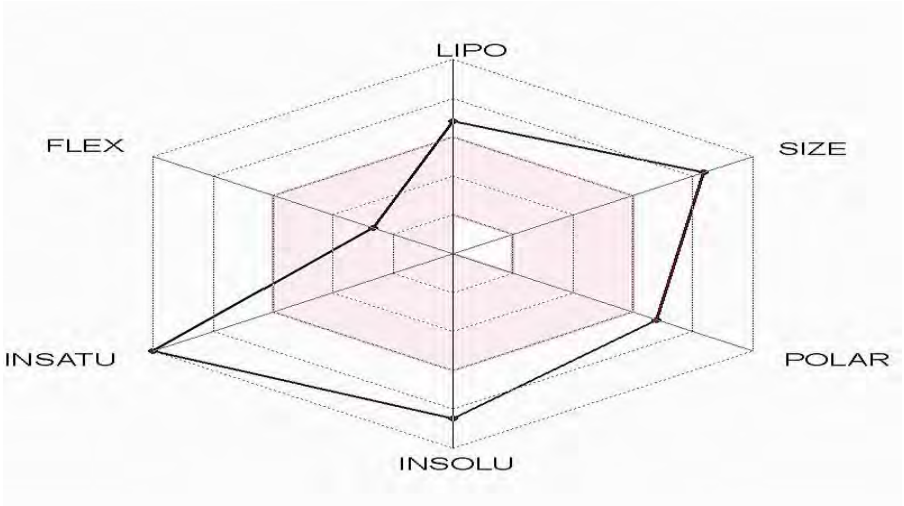

Figure 3.18: Bioavailability radar for vanadium complex 1 shows one of its properties flexibility is within the acceptable range (pink region) while other physicochemical properties are outside desirable range.

This tool makes use of the compounds canonical ‗smiles' to generate this graph with each of the vertex of the hexagon representing a parameter that defines a bioavailable drug. The pink area within the hexagon represents the optimal range for each property in which the radar plot of the molecule must fall entirely to be considered drug-like. This radar evaluates LIPO-lipophilicity, INSOLU- water solubility, SIZE-molecular weight, POLAR-polarity, INSATU-saturation and FLEX-flexibility. The acceptable ranges include (LIPO): XLOGP3 between -0.7 and +5.0, M (SIZE): MW between 150 and 500 g/mol, (POLAR): total surface area between 20 and 130 $Å^2$, (INSOLU): log S not higher than 6, (INSATU): fraction of carbons in the sp3 hybridization not less than 0.25, and (FLEX): no more than 9 rotatable bonds. Vanadium complexes 1 and 2 are isomers and a result, their canonical smiles are the same resulting in the same output from the SwissADME tool.

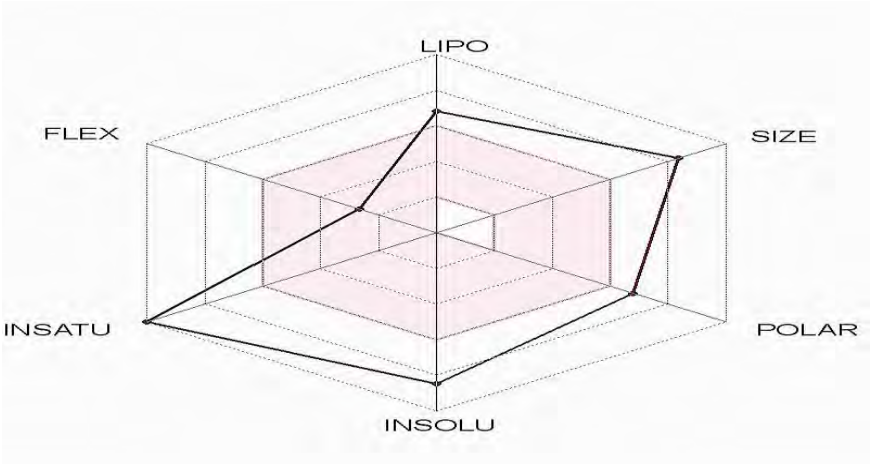

Figure 3.19: Bioavailability radar for vanadium complex 2 shows one of its properties flexibility is within the acceptable range (pink region). The rest of other parameters are outside the acceptable range (white region).

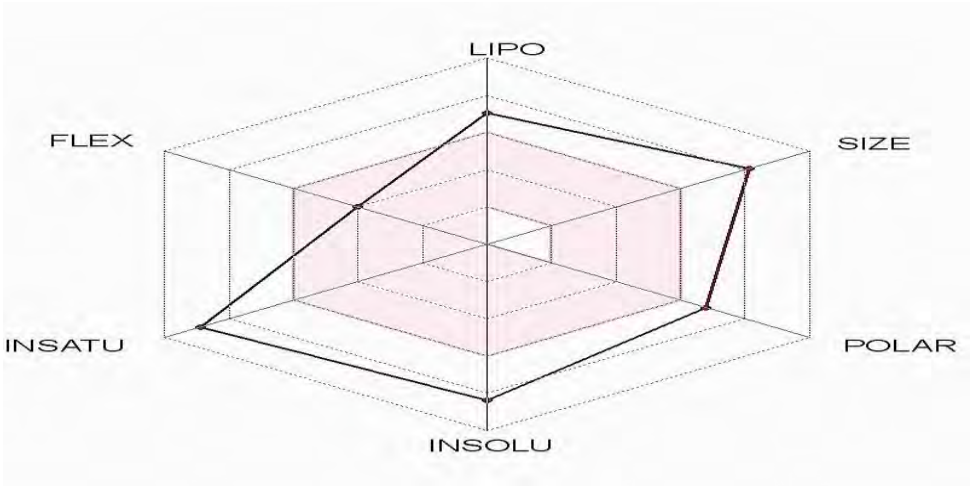

Figure 3.20: Bioavailability radar for vanadium complex 3 shows of its properties flexibility is within the acceptable range of (pink region). However, other parameters are outside the desired range (white region).

The brain or intestinal estimated permeation predictive model is a rapid method which predicts the passive gastrointestinal (GI) absorption and blood brain barrier penetration by passive diffusion of molecules. The white region is the space representing the human intestine and is occupied by molecules with greater extent of absorption by GI tract, while the yellow region represents the blood brain barrier and is occupied by molecules with highest probability to permeate through the blood brain barrier. Vanadium complexes 1 and 2 represented by molecule 1 (Figure 3.21) both lie outside (grey region) the brain and GI regions. However,

vanadium complex 3 lies outside both the BBB and gastrointestinal region and completely out of range (Figure 3.22).

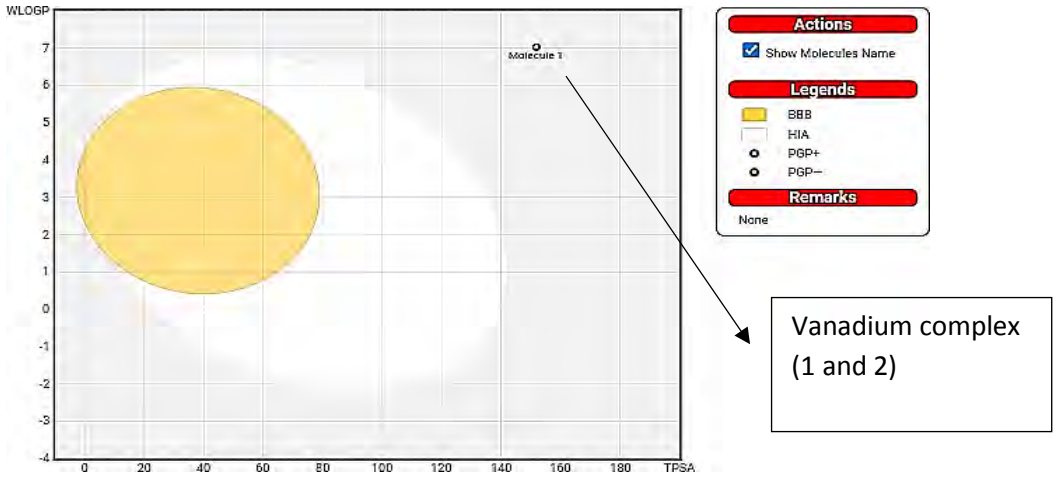

Figure 3.21: The brain and intestinal estimated permeation predictive model of the vanadium complexes 1 and 2 shows both complexes appearing outside the yellow blood brain barrier and white gastrointestinal permeability regions. Both complexes are represented by molecule 1.

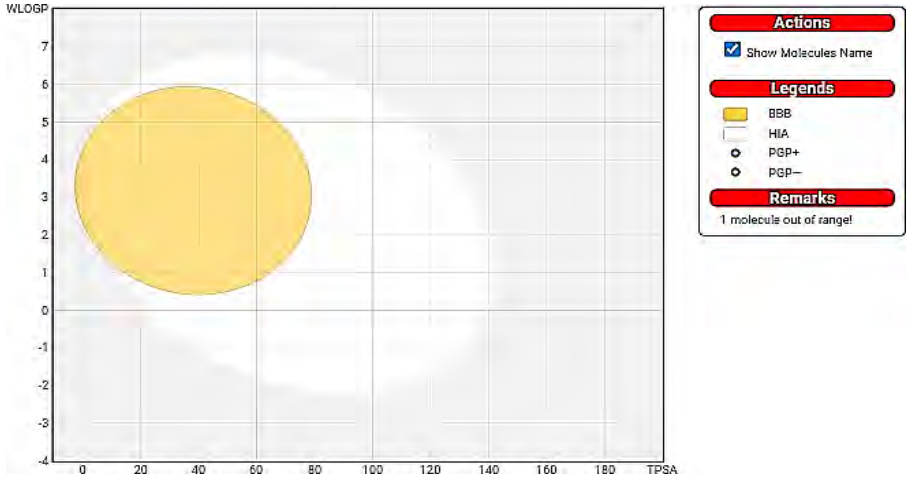

Figure 3.22: The brain and intestinal estimated permeation predictive model of the vanadium complex 3 shows the complex is outside all the regions. This complex was out of range and cannot be seen in the box.

CHAPTER 4: DISCUSSION

Vanadium compounds have been investigated over decades for their pharmacological activities against enzymes that play a role in diabetic pathology. Since then, several studies investigating their roles in other diseases, for example Alzheimer‘s diseases have emerged. The ever-rising number of cases in DM and AD as well as their devastating consequences makes them an active area of research. In addition to this fact, the current conventional drugs used in the management and control of both DM and AD are not always satisfactory in maintaining normoglycemia and are facing limited efficacy, adverse effects and poor tolerability. These have fostered the need to investigate novel compounds such as vanadium complexes as potential treatment options for diabetic and Alzheimer disease patients.

Postprandial hyperglycaemia is a risk indicator for micro and macrovascular complications among diabetic patients which makes the regulation of α-amylase activity a promising management option for type 2 diabetes (246). Due to excessive delay in carbohydrate digestion and their lack of specificity, existing amylase inhibitors such as acarbose cause gastrointestinal side effects like abdominal cramping, flatulence and diarrhoea which can affect therapeutic adherence (247,248). This creates a research gap that can be addressed through investigating new compounds such as vanadium complexes to delay carbohydrate inhibition but at the same time resulting in no gastrointestinal side effect.

From the α-amylase inhibition results, all three vanadium complexes showed very poor inhibition against α-amylase with a maximum inhibition of less than 25%. From the table of IC_{50}s (Table 3.2), vanadium complex 1 showed the lowest IC_{50} which indicates more potency compared to other two vanadium complexes. Vanadium complex 2 was the least active compound with IC_{50} about 37 times more than its isomer which is vanadium complex 1. It has been established that isomers can have different physical, chemical properties and therefore there biological actions may differ (249). Acarbose showed the strongest inhibition profile in comparison to all investigated complexes. A previous study also recorded no to generally poor inhibition by vanadium based compounds against α-amylase (213). Several studies through molecular docking and simulations have identified hydrogen bonds and hydrophobic interactions with the amino acid residues of α-amylase as the primary mode of inhibition of compounds that showed activity against α-amylase. The presence of functional groups such as carboxylate, methoxy and aliphatic chains are considered crucial for binding (250,251).

Lipophilic amino acid residues Leu162, Leu165 and Ile235 in the amylase active site may be crucial in maintaining these hydrophobic interactions with the corresponding hydrophobic groups of inhibitors (252).

Although the vanadium complexes have aromatic residues which may allow pi-stacking interactions with the amylase enzyme and possibly the reason for the slight inhibition observed, they lack functional groups such as carboxylate and methoxy moieties and aliphatic chains, which are crucial for hydrogen bond interactions and hydrophobic contacts with the enzyme.

Another study investigating the inhibitory activity of vanadium binding proteins on α-amylase also revealed H-bond interactions with Asp297 (1.87 Å), Asp340 (2.08 Å) and Arg344(3.22 Å) residues located into the active site (253). This is important because arginine residues directly interacts with the chloride ion, known to be essential for the enzymatic activity and forms direct H-bonds to catalytic residues aspartate and glutamic acid (254). It has been established that chloride ion is a catalytic activator while calcium ion is a structural stabilizer of α-amylase enzyme (255). Therefore, the interaction of inhibitors with these amino acid residues that interact with chloride ion may be crucial for α-amylase inhibition. This is further supported by a study which reported 450-fold reduction in α-amylase activity to starch binding due to a mutation in Arg 195, one of the essential amino acids that interact with calcium and chloride ion in the active sites (256).

Evidently, the goal is to address the gastro-intestinal side effects of conventional amylase inhibitors, perhaps compounds that mildly inhibit the α-amylase enzyme might be better suited as compared to candidates that strongly inhibit the enzyme. To support the above statement, a previous study has shown that only a mild inhibition of pancreatic α-amylase is recommended because mild inhibition prevents the abnormal/excessive bacterial fermentation of carbohydrates in the colon and consequent gastrointestinal adverse effects (257). The incorporation of suitable functional groups will most likely improve the activity of these compounds.

As with α-amylase, α-glucosidase inhibition attenuates hyperglycaemia by preventing the further breakdown of disaccharides to simple sugars therefore resulting in reduced post prandial glucose concentration in the blood. From our case, the results obtained from α-glucosidase assay showed that all three vanadium complexes showed a dose dependent

inhibition against the α-glucosidase enzyme. Vanadium 2 complex showing the most potent inhibition of all three complexes and vanadium complex 1 showed the least inhibition. For oxovanadium(IV) complexes as inhibitors, α-glucosidase may coordinate to the central vanadium ion at sixth (vacant) coordination position of the five coordinated VO-Schiff base complex and the formation of such a bond between the metal ion and protein side chain of the enzymes results in inhibition (258). The inhibition can be further stabilized in the active site through hydrogen bonds with catalytic residues and the establishment of hydrophobic contacts as elaborated in this study (259).

Secondly, the presence of hydrogen bond donors and acceptors seem to influence interaction of inhibitors with the protein side chains on α-glucosidase for example acarbose and acarbose-type molecules have been shown to interact with side chain of α-glucosidase with amino acid Thr 215 acting as the hydrogen bond acceptor and the side chain hydroxyl group of Ser244 acting as a hydrogen bond donor (260). The vanadium complexes have a good number of hydrogen bond acceptors which may allow interaction with the enzyme resulting in the inhibition observed. In addition to hydrogen bond interactions, hydrophobic interactions of aromatic rings of the ligands with the non-polar residues of the enzyme active site including Phe177, Leu218, Leu237, Phe300, and Phe311 may contribute to the inhibition (260). Contrary to acarbose which is a glycosidic derivative, the Schiff base vanadium complexes fall into the non-glycosidic derivatives. This is particularly important because the use of sugar mimicking drugs such as acarbose have been purported to interact with other naturally occurring sugar-binding enzymes, which can lead to gastrointestinal distress (261). This could be an advantage for the vanadium complexes in terms of minimizing gastrointestinal side effects associated with current α-glucosidase inhibitors as these complexes do not mimic sugars and may reveal more potent inhibitors with fewer adverse effects.

Due to the observed significant inhibition of α-glucosidase by the vanadium complexes 2 and 3, a further investigation was carried out to determine their mode of inhibition on this enzyme. From the kinetics data, vanadium complex 2 showed a mixed type inhibition. With a mixed type inhibition, the data lines intersect in the second or third quadrant on the Line-weaver Burk plots (Figure 3.8*)* and the value of K_m can increase or decrease with the inhibitor present (262). Mixed type inhibitors are capable of binding to both the enzyme's active site (competitive) as well as to the enzyme's allosteric site (non-competitive) and V_{max} of the reaction will always

decrease, but the K_m of the reaction can either decrease or increase, depending on whether the inhibitor has more affinity for either the active site or the enzyme substrate complex (ES) (263). Mixed inhibitors are usually more potent inhibitors than competitive or non-competitive inhibitors due to their multi-binding abilities (11). This is again consistent with the results obtained with vanadium complex 2 being the most effective of all three complexes. The clinical significance and advantage of these type of inhibitors is an increased potency resulting in less side effect as less drug is required to bring about effect.

Vanadium complex 3 showed a non-competitive type inhibition thereby reducing the V_{max} of the reaction while K_m stays the same as can be seen with the lines intersecting on the negative x-axis (Figure 3.9 and Table 3.5). Non-competitive inhibitors react with the enzyme at a site distinct from the active site. Therefore, the binding of the inhibitor does not physically block the substrate–binding site but result in a conformational change in the structure of the active site such that the active site loses its affinity for the substrate (11). As non-competitive inhibitors are chemically unrelated to the substrate, their inhibition cannot be overcome by increasing the substrate concentration (264). This type of inhibition is often long lasting and clinically significant in terms of a reduced frequency of dosing which will improve drug adherence.

The gut also releases incretin (GLP-1 and GIP) hormones which are responsible for stimulating insulin release upon a meal ingestion. These peptides play a role in increasing insulin release and suppression of glucagon and hepatic glucose production (265). GLP-1 and GIP are inactivated by DPP-4 by cleaving the two N-terminal amino acids i.e. proline or alanine at the penultimate position. In recent years, researchers have successfully applied various approaches to synthesize potent DPP-4 inhibitors as antidiabetic agent without side effects like weight gain, cardiovascular risks, retinopathy (266,267). Therefore, exploring this target is an avenue for the development of better drug candidates.

The results from the DPP-4 inhibition assay show vanadium complex 3 as the most potent inhibitor of all three complexes. Comparing the structures of vanadium complex 3 to that of sitagliptin (Figure 4.1), a trifluoro carbon (CF_3) substituent attached to a cyclic ring is common to both structures (circled). The presence of this CF_3 group in vanadium complex 3 may be a key player for its interaction with the DPP-4 enzyme.

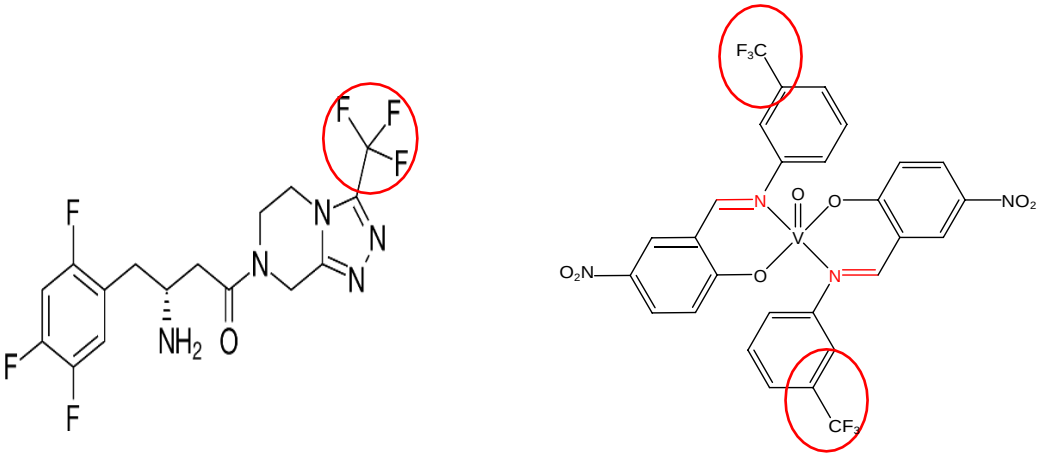

Figure 4.1: Comparison of the structural similarities between sitagliptin (Left) and vanadium complex 3 (Right).

A study reported that a CF_3 substituted moiety improved potency of DPP-4 inhibiting amides by nearly four-fold with bioavailability of 76% (268). Results from docking simulations have suggested the CF_3 group interacts with the side chains of residues Arg358 and Ser209 in the S2 extensive subsite (269). In addition, sites 1 and 2 of chain B show favourable affinities to polar molecules with hydrogen bond donors and acceptors, as well as hydrophobic and aromatic groups (180). This could explain why all the vanadium complexes showed considerable inhibition against the DPP-4 enzyme due to the presence of multiple benzene rings which contribute to their hydrophobicity. Therefore, it can be deduced that the inhibitory activity of vanadium complex 3 (most potent of all three complexes) may be a result of its interaction with the S2 subsite of DPP-4 enzyme and the subsites of chain B due to the CF_3 substituent and their hydrophobic nature, respectively. While vanadium complexes 1 and 2 interact only with the subsites of chain B by virtue of their hydrophobic nature.

Dipeptidyl peptidase-4 also plays a role in the promoting inflammation which may influence the progression of oxidative stress, dysglycaemia and insulin resistance and ultimately result in DM and AD (270). This inflammatory role of DPP-4 has been proposed to result from the degradation of ECM and the invasion of the endothelial cells into collagenous matrices (271). This ability is also a characteristic of matrix metalloproteinases (MMP-1), another enzyme of interest in this study. Studies have elucidated the synergistic interplay between DPP-4 and various MMPs including MMP-1, wherein MMP-1 has been said to contribute to higher circulating DPP-4 via shedding which proliferate smooth muscle cells resulting in increased vascular-related risks such retinopathy and nephropathy (270,272,273). Conversely, DPP-4

facilitates the proteolysis and invasion of endothelial cells into the extracellular matrices by MMPs (271).

In this regard, oxovanadium complexes have been shown to inhibit DPP-4 by forming hydrogen bonds with Tyr 547 residue, π–cation interactions (Arg 669 and 125, respectively) and π–π stacking (Phe 357, His 740 and Tyr 547) between halogen aryl groups and the active site of DPP-4 (274). Therefore, the inhibitory activity shown by these vanadium complexes against DPP-4 could indirectly arrest the destructive roles of MMPs that potentiate AD and DM.

For the purpose of improving adherence and ease of dosing, these vanadium complexes by virtue of their DPP-4 inhibitory activity may present as possible oral dosage forms to replace GLP-1 and GIP analogues which are only available as injectables. While DPP-4 hydrolyses incretins, which regulate postprandial insulin secretion, protein tyrosine phosphatases decrease insulin binding to its receptors. After insulin is released from the gut, the sensitivity of the receptors to insulin is important for their glucose lowering action which is where the inhibition of protein tyrosine phosphatase becomes relevant. Interestingly, vanadium-based compounds have been established as PTP inhibitors which made this target particularly of interest. The *in vitro* study was done but did not yield viable results to unequivocally indicate that the PTP1B enzyme was active. Thus, in-silico studies were also done as an alternative.

PTP1B (ID:1NNY) was selected for docking because it contains the catalytic site of the enzyme and vanadium-based compounds are known to interact with the catalytic site (222,275). The results from the docking process shows vanadium complex 3 with the highest number of interactions with the enzyme active site followed by vanadium complex 2 while vanadium complex 1 gave the least number of amino acid interactions although having the lowest binding energy which is the most favourable. (Table 3.6). Current PTP1B inhibitors face issues with selectivity, therefore it has been proposed that inhibitor compounds that occupy both the catalytic site (site A: Tyr 46, His214, Cys215, Ser216, Ala217, Gly218, Ile219, Gly220, Arg221) and the proximal noncatalytic sites (site B:Arg24, Arg254, Met258, Gly259) and/or site C (Arg47 and Asp48) tend to be more potent and selective (276). As can be seen from the interactions in Table 3.6, vanadium complex 1 interacts mainly with site A of the catalytic site making hydrophobic interactions with tyr46, Ala217 and Ile219 which are also interactions common with the reference inhibitor compound. Vanadium based compounds such as vanadate are reported to form intermediate transition states which allow them to oxidize the amide groups of the active site amino acid residues (275,277). Tyr46 defines the dept of

the catalytic cleft and contribute to absolute specificity for PTP1B enzyme (278). Considering the site of interactions observed, vanadium complex 1 is most likely a catalytic inhibitor of the PTP1B enzyme. On the contrary, vanadium complex 2 and complex 3 show more interactions as compared to vanadium complex 1, however these contacts are not within the catalytic site residues which may indicate more affinity towards allosteric site of the PTP1B enzyme. Vanadium complex 2 and 3 each make hydrophobic, hydrogen bond and halogen bond interactions with different amino acid residues. The presence of hydrogen bond interactions have been said to increase the residence time and activity of inhibitor compounds in the PTP1B catalytic site, this may also be true for interactions with the allosteric sites (279). Interestingly, compounds that target the less conserved sites, such as the allosteric site have gained more interest in recent times due to highly conserved nature of the active site and multiple charge requirements of the ligands, which makes catalytic site directed PTP1B inhibitors non-selective, non-permeable, resulting in undesirable off target side effects (280,281). In this regard vanadium complex 2 and 3 may present as better therapeutic interventions compared to vanadium complex 1.

The inhibition constant (Ki) reflects the binding affinity of a drug to the target. Smaller Ki values signify a high binding affinity which means that a smaller amount of drug will be needed to inhibit the activity of the enzyme (282).Vanadium complex 1 gave the lowest Ki nanomolar concentrations while vanadium complex 2 and 3 gave micromolar Ki values. The clinical significance of these results is a reduced dosing profile for vanadium complex 1, which will lower chances of side effects.

Uncontrolled hyperglycaemia leads to oxidative stress. Oxidative stress is involved in both aetiology and progression of DM and AD which happens as a result of an overpowered inherent cellular antioxidant defence system by reactive free radicals (85). The role of antioxidants in scavenging free radicals is of utmost importance in preventing complications of diabetes and vanadium complexes have proven to be effective in scavenging free radicals due to their excellent chemistry of acting both as an electron donor and an electron acceptor. It‘s been known that the presence of coordinated metal ions changes the structure of the Schiff base, which promotes their antioxidant activity and also substituents in the Schiff base have a great influence on the activity of these compounds (283).

From the DPPH radical scavenging activity results, the vanadium complex 3 showed the highest scavenging activity slightly greater than ascorbic acid. This trend in antioxidant activity

is further confirmed using the FRAP assay with compound 3 showing excellent antioxidant activity exceeding that of ascorbic acid at 80 and 100 µg/mL concentration. The antioxidant activity of these vanadium complexes can be attributed to the electron withdrawing effects of the co-ordination metal ions, in this case vanadium which facilitate the release of hydrogen to reduce DPPH radical as stated in a study that investigated the antioxidant activity of Schiff base metal complexes (284). This reason stated above combined with the presence of a strong electron withdrawing nitro and fluoro substituents increased the radical scavenging activity of vanadium complex 3 making it the most potent antioxidant of all three complexes. These results are consistent with findings in a study where C-3 tethered 2-oxo-benzo[1,4] oxazine analogues having electron withdrawing NO_2 substituent on the ring increased antioxidant activity and this was attributed to resonance based stabilizing effects (285).

For ascorbic acid and its derivatives, lipophilicity and the presence of multiple hydroxyl (OH) functional groups have been reported to be important determinants in their antioxidant activity (286). However, the susceptibility of ascorbic acid to thermal and oxidative degradation, together with its poor liposolubility, make it difficult to maintain its physiological value over a long period of time and to penetrate cell membrane (287). With another study concluding that ascorbic acids capacity to passively diffuse across the lipid matrix was insufficient to get the recommended dose (288). This reinforces the need for other molecular derivatives.

One of the major contributors to oxidative stress mechanisms is the advanced glycation end products. AGEs just like reactive oxygen species are culprits in the development and progression of several diabetic complications and neurodegenerative diseases such as Alzheimer‘s disease. The major concerns with the use of current AGEs inhibitors as therapeutic agents are low effectiveness, poor pharmacokinetics and undesirable side effects (289). While many studies focus on investigating the AGEs inhibitory activity of natural sources and plant extracts (141,239,290), there has been no study published investigating the anti-ages inhibition of metal-based complexes which makes this study a novel approach towards identifying drug candidates for the treatment of AGEs and its related complications. Formation of AGEs can be inhibited by interfering with the initial attachment between reducing sugars and amino groups through trapping the carbonyls and radicals formed from the glycation process, or by preventing the formation of intermediate amadori products which result in late stage of glycation (291).

After 96 hours of incubation to allow enough time for AGEs formation, the results obtained show that vanadium complex 3 gave the highest AGEs inhibition in a dose dependent manner. A similar trend was observed for vanadium complex 1. However, complex 2 shows inconsistent inhibition across all concentrations. Overall, all vanadium complexes show inhibitory activity against ages which was less in comparison with the reference compound aminoguanidine (Figure 3.15).

Several studies have attributed the antiglycation activities of investigated compounds including plant extract and synthetic inhibitors to their antioxidant activity and abilities to scavenge and trap reactive carbonyls and radicals formed during glycation (290,292,293). Likewise, the antiglycation activity of aminoguanidine, a well-known age inhibitor, has been attributed to its free radical scavenging activity (294). Therefore, compounds found to be more effective at inhibiting reactive radicals species were also found to show better activity on AGEs inhibition (295). From these claims, it can be deduced that vanadium complex 3 having the highest radical scavenging activity as seen in the antioxidant assays conducted would most likely be the most potent inhibitor of AGEs formation followed by vanadium complex 2 and lastly vanadium complex 1. This is partly consistent with the findings because complex 3 showed the most potent activity, and complex 1 showed better inhibition than complex 2. This could be a function of stereochemistry as complex 1 and complex 2 are isomers. The arrangement of complex 1 in space may be more favourable to allow for interaction with AGEs adducts formed *in-vitro* compared to its isomer and secondly increased ability to scavenge ROS may not directly infer increased ability to scavenge di-carbonyl or AGEs related radical species.
Chelation has also been reported as a fundamental mechanism of action of AGE inhibitors and AGE breakers and the presence of functional groups such as carboxyl, amino, imino, imidazole, oxo, and hydroxyl groups are known to potentiate chelating activity (296,297). However, these vanadium complexes have only the imino group present which may explain why observed percentage inhibitions were generally below 50%. The addition of more of the chelating functional groups may improve their *in-vitro* activity against AGEs. On the contrary, Schiff bases have been reported to undergo a series of reactions with di-carbonyl intermediates at the initial phase of the Maillard reaction to form AGEs. In addition, Schiff bases are prone to oxidation to produce free radicals, resulting in the formation of active carbonyl intermediate products (293,298). To support these statements, flavonoids and other polyphenol derivatives have been reported to inhibit the formation of AGEs by blocking the formation of Schiff bases that trap reactive carbonyl intermediates (291,293). This is significant and perhaps concerning

particularly because the vanadium complexes of interest are Schiff base derivatives. Although showing inhibitory activity *in-vitro*, they may potentiate AGES formation *in-vivo* which will further worsen these disease condition.

One common denominator between AGEs and MMPs is their pro-inflammatory action towards AD and DM pathology. Matrix metalloprotease play a vital role in many cellular processes and a dysfunction in the activity of these enzymes has been implicated in the pathogenesis of several diseases (299). Due to their essential role in ECM remodelling, MMPs have become targets of interest as biomarkers for the diagnosis and prognosis of diseases associated with alterations of the ECM (300). The significance of investigating the MMP-1 in this study is to tackle it‘s amyloidogenic and neuroinflammatory role in Alzheimer‘s disease.

From the results obtained from MMP-1 inhibition, all vanadium complexes tended towards an activation of the enzyme with vanadium complex 1 showing the most activation. This suggests that the compounds were unable to inhibit the action of the enzyme on the substrate. The underlying modes of MMP activation involve the dissociation of the cysteine from the catalytic Zn^{2+} ion and its replacement by water therefore exposing of the active site by sequential proteolytic removal of the pro-peptide (301). Chemical reactions, mercury-containing compounds, surfactants and reactive oxygen species have been proposed as main MMP activators *in-vitro* (299). The activation of MMP-1 by the vanadium complexes may be linked to one or more of these factors. Compounds with hydroxamic acid (RC(O)N(OH)R') motif, as well as pyrimidine and imidazole moieties have been proposed in the design of selective MMP inhibitors and should be incorporated in future when designing potential inhibitors (302). Doxycycline hyclate is the only clinical approved drug that inhibits MMPs and act by chelating Ca^{2+} and Zn^{2+} therefore altering the MMP enzyme conformation (303). MMPs stand on two opposing sides playing both physiological roles as well as undesired pathological roles. Physiologically known to be important regulators of cellular activities and several physiological processes, including reproduction, embryogenesis, tissue remodelling and wound healing. For example, in a diabetic patient, wounds take longer to heal which can increase risks of infection, therefore processes that enhance wound healing may be beneficial for such patients (304). MMPs are involved in wound healing promoting the early inflammatory phase during the granulation phase of tissue formation where optimum healing results from a balance between ECM matrix synthesis and their degradation (240). Therefore, their activation by vanadium complexes may be useful in promoting wound healing in diabetic

patients. This indirectly addresses one of the aims of this study which is to prevent diabetic related complications. Pathologically, their proteolytic action on extracellular matrix components can cause damage to the blood brain barrier endothelium and neurovasculature, promoting oxidative stress via pro-inflammatory pathways which can result in diabetic complication and Alzheimer‘s (125). Therefore, in this instance, the clinical implication of the activation of MMP-1 by vanadium complexes is seen with the results obtained (Figure 3.16) would result in neuropathological features in AD as well as diabetic complications.

The role of cholinergic deficit in Alzheimer‘s disease has been described to be the most common cause of progressive decline of cognitive functions common in dementia and associated diseases such as AD. This has translated to acetylcholinesterase inhibitors dominating the field of AD pharmacological treatment. From the acetylcholinesterase inhibition assay, vanadium complexes showed very little inhibitory activity against the enzyme with the most potent compound showing about 23% inhibition at the highest concentration (100 µg/mL). Vanadium complex 2 shows the second most potent inhibitory activity while complex 3 emerged as the least active compound.

The presence of hydrogen-bond donors, hydrophobic aliphatic and aromatic rings/ interactions are essential for AChE binding, as the active site of AChE is composed of 14 conserved aromatic amino acid residues (305). Although the vanadium complexes investigated in this study possess some hydrophobic properties due to the presence of aromatic rings and perhaps the central metal atom, the predicted properties for vanadium complexes according to Lipinski rule of 5 represented in Table 3.10 show that all three complexes have no hydrogen bond donors, which could explain their poor inhibitory activity against acetylcholinesterase enzyme. To further understand the mode of inhibition of the vanadium complexes with better activity against acetylcholinesterase, enzyme kinetics was done. From the results, vanadium complexes 2 and 3 both showed competitive inhibition against acetylcholinesterase. This suggests that the compounds compete with the substrate for the active site of the enzyme to form an enzyme-inhibitor complex, which prevents binding of substrate to the active site (306). Consequently, the activity of these complexes will depend upon their concentration in the synapse and the degree of occupation in the enzyme catalytic site (307). Hence, declining in vivo concentration as elimination takes place will reduce activity therefore a longer duration of action is desired.

Just like these investigated vanadium complexes, galantamine a known acetylcholinesterase inhibitor shows competitive binding against acetylcholinesterase (308). The inhibitory activity of galantamine has been attributed to the oxygen moiety on the methoxy group and its interaction with SER200, HIS440 and GLU 327 amino acid residues in the catalytic active site, hydrophobic interactions as well as the interaction of the positively charged amine with the hydroxyl of the Tyrosine 337 (309). The investigated vanadium complexes have oxygen, amine bonds and the presence of hydrophobic aromatic rings and these commonalities with galantamine could explain their common mode of inhibition observed.

In addition to MMPs, Aβ fibril misfolding to insoluble toxic aggregates is a common feature in Alzheimer's disease (310). There is growing evidence that matrix metalloproteinases are involved in the processing pathway of Aβ while other studies have highlighted the inducing effects of Aβ on matrix metalloproteinases (133,311). Therefore, discovering a drug candidate with desired inhibitory activity on both targets may yield a summative inhibition as desired. Although peptide-based inhibitors have taken the lead in the investigation of drug candidates for Aβ aggregate inhibition, promising studies on metal-based complexes have also emerged (190,312). Vanadium complexes such as the bis(ethylmaltolato) oxidovanadium (IV) have been reported to inhibit he formation of amyloid plaques (313). These presented a rationale for investigating these vanadium complexes against Aβ aggregation. Of all three vanadium complexes, vanadium complex 1 showed the better reduction of Aβ aggregation after 96 hours of incubation while vanadium complex 2 showed no significant difference after 96 hours. Just like the negative control, vanadium complex 3 did not prevent the further aggregation of Aβ , therefore resulting in an increased fluorescence after the period of incubation. To validate this method, rifampicin was used as the standard drug since it has been shown through pre-clinical and clinical studies to mediate inhibition of Aβ aggregation (314,315). However, the positive control rifampicin showed no statistically significant inhibition in comparison to the control which was the same as with the vanadium complexes. Ideally, the presence of Aβ aggregates prior to introducing the inhibitors and the progressive disintegration of aggregates/ or aggregation of amyloid fibrils is monitored by microscopic observation. Supplementing these microscopic images with *in-vitro* studies would have presented a broader picture.

The ability of these vanadium complexes to exhibit coordination chemistry and oxidative properties may be necessary for peptide modifications and their inhibitory action on Aβ aggregates (207). This was proven in a research study investigating the anti-aggregative

activity of Schiff base oxovanadium complexes on human islet amyloid peptides as the complexes prevented misfolding and fibril formation and also impeded peptide aggregation and disaggregated mature fibrils into monomers and oligomers through metal complex interaction with the peptide (316). Interestingly, these complexes possess similar structural similarities with the complexes investigated in this study differing only by the substituents present. The observation from this literature could present possible mode of action of the investigated vanadium complexes against Aβ formation and aggregation. See structural comparisons of the vanadium complexes below (Figure 4.2)

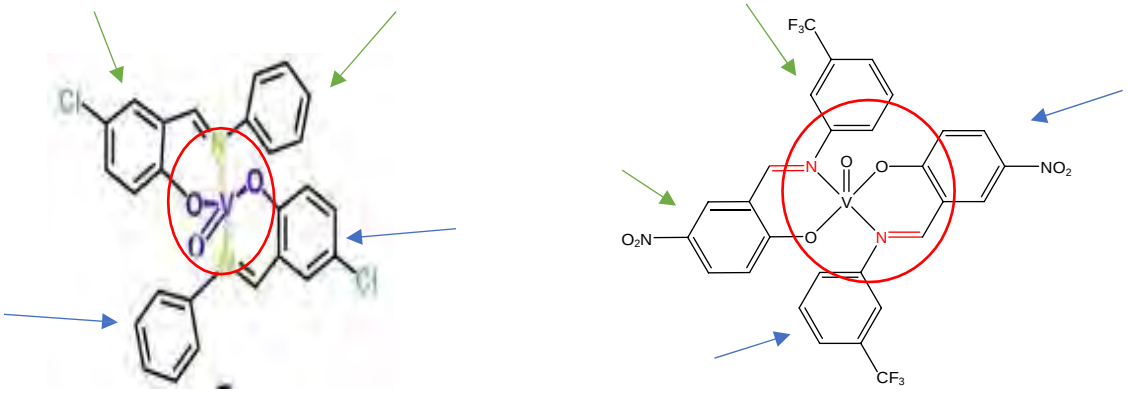

Figure 4.2: An illustration of the structural similarity between literature reported Schiff base complex (left) and vanadium complex 3 (right). Both structures have two azomethine groups, two benzene rings on opposite sides as well as the same vanadate ion backbone structure.

Furthermore, the presence of substituent groups, such as chlorine, bromine, and nitro groups, on each aromatic ring was proposed to increase the bioactivities of previously investigated vanadium complexes showing inhibition on amyloid peptide aggregation; as well as the presence of hydrophobic aromatic ligands and increased steric effect (317). All evidence stated above point to the Aβ modulatory potential of these investigated vanadium complexes and calls for more detailed exploration in the future such as in-silico studies to determine if these vanadium complexes also interact with the N-terminal residues of the peptide as reported by similar studies on metal complexes (190).

According to the predicted properties for the compounds investigated, vanadium complexes 1 and 2 violated at least one Lipinski rule which is ideal, however complex 3 violated 2 Lipinski rules (Table 3.10). With no predicted hydrogen bond donors, nitrogen and oxygen atoms present in vanadium complex 1,2 and 3 plus fluorine atoms present in vanadium complex 3 alone count towards the predicted hydrogen bond acceptors. While the rule of 5 offers a fast,

simple approach and still relevant in drug discovery, it does not estimate oral absorption in a quantitative manner, and does not definitively categorize all orally active compounds such as metal based complexes (129,318). Therefore, the definition of ‗drug-like' has expanded over the years for better correlations with absorption, distribution, metabolism, excretion, and toxicity (ADMET) including parameters such as polar surface area, rotatable bond count which co-relates to a compound's flexibility that may prove vital for access to deep-rooted binding sites in a substrate that more rigid analogues would struggle to reach (319,320). By this concept we can appreciate the relevance of Schiff base complexes investigated in this study due to their excellent flexibility.

From the bioavailability radar, vanadium complexes 1 and 2 both show slightly better flexibility as compared to vanadium complex 3 as the vertex for both isomers lies in the middle of the second hexagon while the vertex for vanadium complex 3 lies at the edge of the second hexagon from the centre. Similarly, vanadium complex 3 scores better in terms of saturation as the vertex lies closer to the centre as compared to vanadium complexes 1 and 2 whose vertices lie at the outermost edge of the hexagon. The presence of the sp^3 hybridized trifluoro carbon (CF_3) in vanadium complex 3 improves its saturation. Overall, the level of unsaturation for all compounds were more than the acceptable range and may be due to the presence of numerous aromatic rings. While aromatic features can provide an opportunity to develop π−π interactions or π−cation interactions with target receptors, saturation and reduction in the number of aromatic character of a molecule improves physical characteristics, such as solubility (321).

Other parameters such as lipophilicity, solubility, polarity and size for all the vanadium complexes are visibly the same. Overall, the investigated vanadium complexes show excellent flexibility. Their moderate lipophilicity may be as a result of the presence of multiple aromatic strutures which contribute to their hyphobicity and subsequently lipophilicity. The presence of the vanadium centre atom may be responsible for high polarity. The predicted poor performances with size is expected as the vanadium complexes have large molecular weights greater than the 500 g/mol threshold. The presence of hydrophobic aromatic molecules co-relate to low aqueous solubility. The solubility of drug candidates in aqueous medium is crucial due to the aqueous nature of the body fluids which assist in drug dissolution *in-vivo* and may sometimes affect bioavailability.

Overall, the gastrointestinal tract (GIT) and blood brain barrier (BBB) permeability of these compounds was predicted to be generally poor as can be seen with the boiled egg model which describes the potential of the vanadium complexes to passively diffuse into the GIT and brain (Fig 3.21 and Fig 3.22). The BBB has junctional proteins which significantly impedes entry of virtually all molecules from blood to brain, except those that are small and lipophilic or those that enters the brain through an active transport mechanism (134). Measures to improve the hydrophilic nature of drug candidates while maintaining some level of lipophilicity is vital to enhance their permeability through the lipid layer of cell membranes. This is crucial especially for molecules targeting the brain as their permeability into the BBB is a rate determining step; but equally important for absorption of drug molecules in the GIT. This is a desired characteristic for a multi-drug targeting candidate which is the subject of focus in this research study. With regards to physicochemical and pharmacokinetic properties of CNS acting drugs, studies investigating the chemical properties of successful CNS drugs found blood-brain barrier penetration to be optimal when the cLogP (a function of lipophilicity) values were in the range of 1.5-2.7, MW of 400 g/mol or less, sum of hydrogen bond donors and acceptors of 5 or less, polar surface area estimated at 70–90 Å and considerable aqueous solubility (322,323). From the above stated requirements, it is evident that the vanadium complexes do not satisfy the requirement for good CNS penetration and may infer poor in-vivo activity.

Overall, an increase in the concentration of the investigated vanadium complexes could result in better inhibition profile. However, it is important to aim towards smaller drug concentration to achieve inhibition which reflects on a drug‘s potency and this can also reduce potential for side effects. Nevertheless, these complexes show promising activity, therefore optimizing them according to the desired properties that enhance their ability to act as DM and AD drug candidates are encouraged.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

5.1 Conclusions

The ability of the vanadium complexes to show activity against enzymes implicated in both DM and AD shows their multi-target nature and promising potential. Unlike multiple drug therapies, single multi-target agents may present several advantages such as reduced drug interactions and potential for additive side effects, better predictable *in-vivo* characteristics and improve patient adherence to therapy which are the prime goals of this study. Overall, the vanadium complexes showed better activity with DM enzyme targets as compared to AD targets and these data are supported by numerous *in-vitro* experiments that demonstrated essentially similar effects. This is in line with existing literature because vanadium-based ligands have established themselves as good anti-hyperglycaemic candidates which is probably linked to their ability to mimic phosphate-like physicochemical behaviour and their interactions with phosphate binding sites such as phosphatases, kinases which gives them insulin-mimetic abilities. On the contrary, the vanadium complexes did not satisfy the criteria for good BBB penetration such as molecular weight (be too large to fit and interact with the enzymes) and Log P which could explain the generally poor trend observed with AD inhibition assays. Meanwhile, the complexes exhibited good antioxidant properties possibly due to their co-ordination chemistry which is an important property that allows them to easily scavenge free radicals. Data from *in-silico* studies on the PTP1B enzyme show considerable interactions with the enzyme target with hydrophobic interactions being most prominent.

Although predicted to have poor drug-like properties and generally poor pharmacokinetics according to Lipinski rule and other criteria, it should be noted that whilst molecular simulations and *in-vitro* studies are a convenient necessity in drug discovery, they may not be a true reflection of a compounds‘ potential *in-vivo*. *In-vivo* studies are encouraged especially in areas where good activity was exhibited. Moving forward, the development of more specific, inclusive drug discovery procedures is encouraged for metallo-drugs given the unique nature of their physical properties and chemical characteristics. From a clinical point of view, developing a multi-target compound that ―inhibits the desired target” to elicit the desired response whilst ―sparing other targets” when that inhibitory action is not desired is of utmost importance. Hence, this multi-target therapy may be more beneficial to patients with DM and

AD co-morbidity, which has become an increasing cohort in current times therefore justifying the importance of this research study.

5.2 Limitations and future studies

In-vitro studies are often times associated with challenges which can range from inappropriate or unreliable lab equipment, variation in environmental conditions, human error as well as conducting experiments in a resource limited setting with regards to material availability and stability as well as performance of equipment. As such, laboratory experiments should be done in replicates and repeated over time to ascertain if a specific trend is observed which increases the accuracy and confidence in the result obtained. However, in this study some assays were not conducted multiple times due to cost and limited resources such as a screening kit which allows for only one 96 well plate run considering the number of compounds and concentrations investigated. In future studies multiple experiments needs to be carried out in replicates maintaining the same conditions as much as possible. Similarly, to improve the clinical relevance of this study, stepping up the ladder by performing *in-vitro* cell line studies and *in-vivo* studies need to be done. In addition, toxicity studies to investigate the safety of these vanadium-based complexes is vital to ensure their safety for human use. Due to time and financial constraints, other targets of interest such as glucose uptake studies, tau phosphorylation inhibition and other crucial target, which are also implicated in AD and DM could not be done.

To visibly observe the level of Aβ disaggregation ability of the inhibitor compounds, observation under transmission electron microscopy would have been ideal. However, we were unable to conduct such an experiment since the transmission electron microscope was not operational following its breakdown. Furthermore, the prediction of drug-likeness and pharmacokinetic properties of metal-based compounds using organic models often do not truly reflect their capabilities for example MW requirement for drug-like properties. This has resulted in studies proposing molecular volume rather than MW as an alternative for metallodrugs which should be encouraged (324). In-silico modelling of these drug candidates should have been done prior to carrying out *in-vitro* studies to streamline better drug candidates with the best properties to target the disease of interest. This is recommended in the future.

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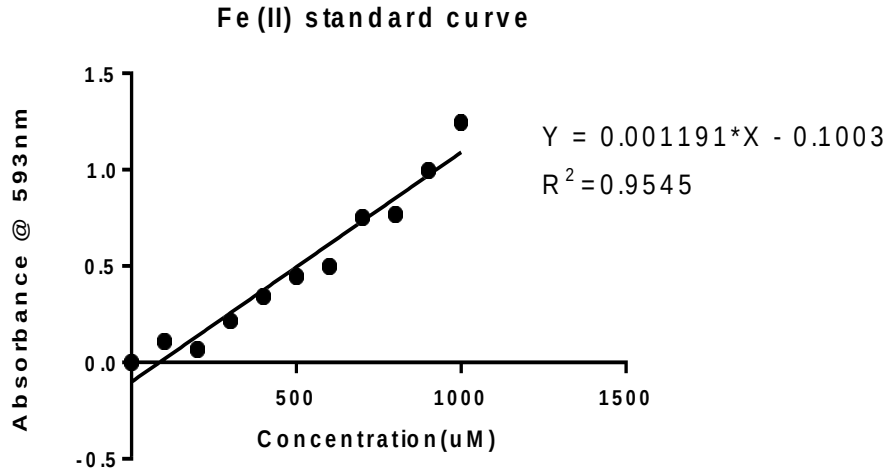
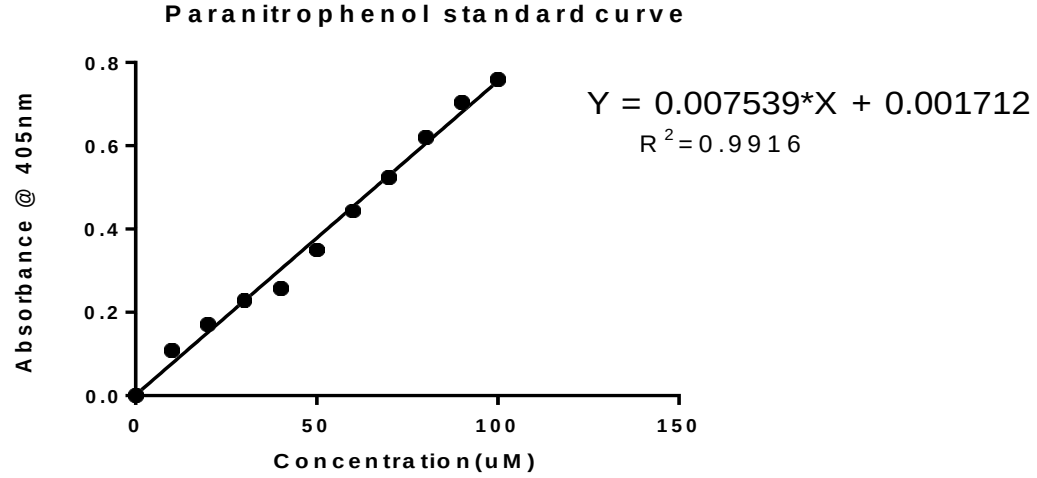
2. APPENDICES

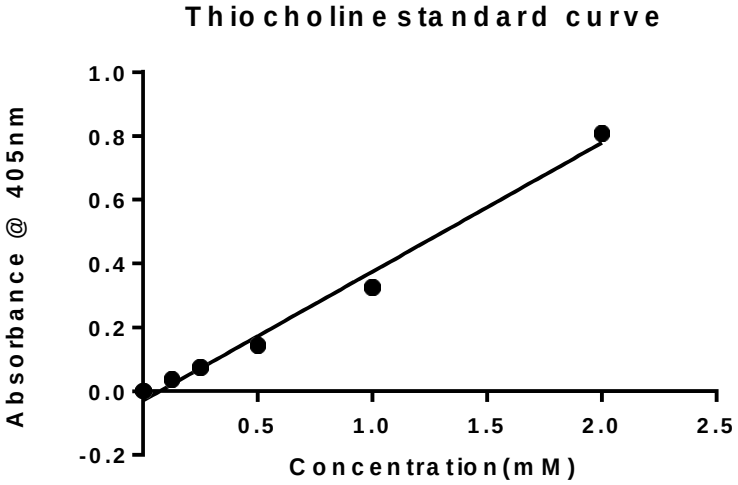
2.1 Appendix 1: Fe(II) standard curve used to determine the ferric reducing antioxidant power of vanadium complexes



2.2 Appendix 2: Paranitrophenol standard curve used to determine mode of kinetics for alpha glucosidase inhibition by vanadium complexes 2 and 3



2.3 Appendix 3: Thiocholine standard curve used to determine mode of kinetics for acetylcholinesterase inhibition by vanadium complexes 2 and 3



2.4 Appendix 4: Abstract for postgraduate conference presentation in the Faculty of Pharmacy

Miss Chiamaka Onyekwuluje

- M.Sc. Candidate

Investigating the anti-diabetic, anti-Alzheimer and antioxidant activities of Schiff base vanadium complexes in-vitro.

Chiamaka Onyekwuluje, Dr N. Sibiya, Prof SD Khanye

Pharmacology/Pharmaceutical Chemistry

Background: Diabetes mellitus (DM) and Alzheimer's disease (AD) constitute two challenging global health issues. DM is an ever-increasing epidemic with devastating chronic complications while AD remains untreatable.

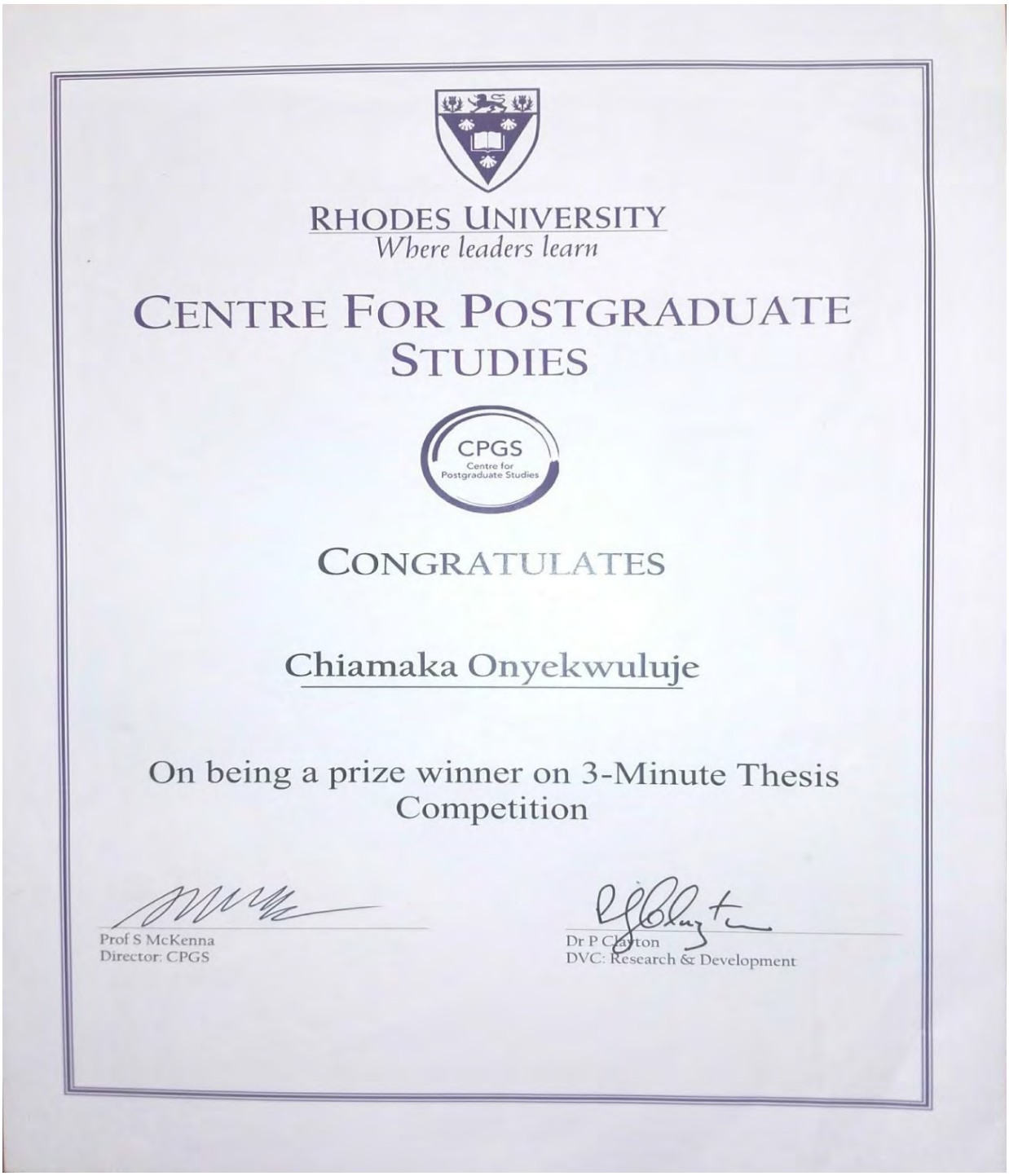
Purpose: This study is an attempt to address the issue of untoward effects and shortfalls in conventional anti-diabetic and anti-Alzheimer treatments by looking into vanadium complexes as novel curative strategies.

Method: In this study, the anti-diabetic and anti-Alzheimer inhibitory activities of three chemically synthesized Schiff base vanadium complexes were investigated on the following enzymes: α-glucosidase, α-amylase, DPP-IV, PTP-1B, aldose reductase, acetylcholinesterase, MMP-1 enzymes beta amyloid and AGES. The antioxidant capabilities of these compounds were also investigated. Standard inhibitory drugs were used to validate the protocol. The data obtained by spectrophotometric analysis were converted to percentage inhibitions. Enzyme kinetic studies were done where indicated using the Lineweaver Burk plot. One-way ANOVA was performed to test for statistical significance at p value ≤0.05 using GraphPad.

Results: Vanadium complexes showed better inhibition profile against antidiabetic enzymes with statistically significant differences from control experiment as compared to the enzymes investigated for anti-Alzheimer activity. Inhibition was clearly observed with α-glucosidase. DPP-IV and antioxidant assays while very minimal inhibition or enzyme activation was observed with other enzymes such as aldose reductase and MMP-1.

Conclusion: This study provides evidence to suggest that these Schiff base vanadium complexes may be potential drug candidates for the treatment of hyperglycaemia and oxidative stress related complications but may not be potent drug candidates in the treatment of Alzheimer's disease. Vanadium based complexes with improved structural and physicochemical properties are recommended in future studies.

2.5 Appendix 5: Certificate of recognition as a prize winner at the 2020 three-minute thesis competition. This was an activity organized for postgraduates at Rhodes to present your thesis in three minutes. I finished second place



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On being a prize winner on 3-Minute Thesis Competition

Prof S McKenna
Director: CPGS

Dr P Clayton
DVC: Research & Development

2.6 Appendix 6: Graphical Abstract