The effects of Schiff Base Vanadium Complexes on Glucose Metabolism and their Cytotoxicity in skeletal (C2C12) and hepatic (HEPG2) Cells

A thesis submitted in fulfilment of the requirements for the degree of

Master of Science (Pharmacy)

By

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Abstract

Background

Diabetes mellitus (DM) is a leading cause of global mortality, as recognized by the World Health Organization. Current treatments face limitations in cost, efficacy, and safety, underscoring the need for alternative therapeutic options. Vanadium compounds have emerged as promising insulin mimetics, but their clinical application is hindered by toxicity concerns and side effects such as diarrhoea. This study investigates novel vanadium complexes synthesized through complexation with Schiff base ligands, aiming to enhance their efficacy while minimizing cytotoxicity.

Methods

The research employed *in vitro* assays on skeletal (C2C12) and hepatic (HepG2) cells treated with Schiff base vanadium complexes and sodium orthovanadate as a reference compound. Cytotoxicity, glucose uptake, and inflammatory responses were evaluated using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assays, glucose uptake assays, and enzyme-linked immunosorbent assay (ELISA) for insulin signalling proteins, including protein kinase B (Akt) and glucose transporter type 4 (GLUT4) and inflammatory markers such as matrix metalloproteinase-1 (MMP1), dipeptidyl peptidase-4 (DPP4), and interleukin-6 (IL-6). Citrate synthase activity assays were conducted to assess mitochondrial health, and cell media pH was measured post-treatment. Molecular docking studies were performed to explore the interaction of these complexes with protein tyrosine phosphatase 1B (PTP1B), a negative regulator of insulin signalling.

Results and discussion

The observations demonstrated compound- and cell-type-specific effects. Schiff base vanadium complex 1 consistently suppressed MMP1 and DPP4 levels in HepG2 cells, suggesting potential

in regulating hepatic extracellular matrix remodelling and metabolic functions. Complexes 2 and 3 enhanced glucose uptake in C2C12 cells in part through phosphoinositide 3-kinase (PI3K)/Akt-mediated GLUT4 translocation, confirming insulin-mimetic properties. However, cytotoxicity at higher doses highlights the need for dose optimization. Molecular docking revealed favourable interactions with PTP1B, supporting the proposed mechanism of action. Pharmacokinetic analysis indicated moderate lipophilicity but poor aqueous solubility and gastrointestinal absorption, limiting systemic bioavailability. To address these limitations, encapsulation within nanoparticle carriers is proposed to improve stability, targeted delivery, and bioavailability while reducing cytotoxicity.

Conclusions

In conclusion, the investigated Schiff base vanadium complexes demonstrate potential as safer and potentially more effective alternatives for DM treatment, warranting further optimization and delivery strategies to enhance their therapeutic potential.

Thesis Outline

This thesis is structured into four main chapters, as outlined below:

- **Chapter 1: Introduction and Literature Review**
    - Provides an overview of diabetes mellitus as a global health concern and the limitations of current treatments.
    - Reviews the literature on insulin signalling, diabetes mellitus pathophysiology, and the role of vanadium compounds as insulin mimetics.
    - Introduces Schiff base vanadium complexes and justifies the need for this study.
    - Outlines the objectives of the research.

- **Chapter 2: Materials and Methodology**
    - Describes the chemicals, equipment, and software used in the study.
    - Details the preparation of Schiff base vanadium complexes.
    - Explains the cell-based assays, including cell culture, viability, glucose uptake, and inflammatory marker analysis.
    - Outlines *in silico* assays, such as pharmacokinetic predictions and molecular docking studies.
    - Summarizes the statistical methods used for data analysis.

- **Chapter 3: Results**
    - Presents the findings from cell-based assays, including cytotoxicity, glucose uptake, inflammatory marker levels, mitochondrial health and media pH changes.
    - Reports the results of the *in silico* assays, such as pharmacokinetic properties and molecular docking interactions.

- **Chapter 4: Discussion and Conclusion**
    - Interprets the results in the context of existing literature and discusses their implications for diabetes mellitus treatment.

- Summarizes the key findings and their significance.
- Highlights the limitations of the study and proposes directions for future research.

List of Abbreviations

Abbreviation	Full Term
Akt	Protein Kinase B
BAOV	Bis(allixinato)oxovanadium(IV)
BEOV	Bis(ethylmaltolato)oxovanadium(IV)
BMOV	Bis(maltolato)oxovanadium(IV)
BPOV	bis(picolinato)oxovanadium(IV)
DM	Diabetes Mellitus
DMT1	divalent metal transporter-1
DPP4	Dipeptidyl Peptidase-4
DPPH	2,2-diphenyl-1-picrylhydrazyl
FOXO	Forkhead box 01
GDM	Gestational diabetes mellitus
GLUT4	Glucose Transporter 4
IL-6	Interleukin 6
IRS	Insulin Receptor Substrate
ISO	International Organization of Standardization
JNK-1	Jun N-terminal kinase-1
LPO	Lipid Peroxidation
MMP1	Matrix Metalloproteinase 1
MMPs	matrix metalloproteinases
PDK	phosphoinositide-dependent protein kinase
PIP2	Phosphatidylinositol 4,5-Biphosphate
PIP3	3,4,5-Trisphosphate
PKC	Protein Kinase C
PI3-K	Phosphatidylinositol-3-Kinase
PTP1B	Protein Tyrosine Phosphatase 1B
ROS	Reactive Oxygen Species
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus

TNF-α	Tumor Necrosis Factor-alpha
VAc	Oxovanadium(IV) acetylacetonate
VEt	Oxovanadium(IV) ethylacetonate
WHO	World Health Organization

Glossary of Medical Definitions

Term	Definition
Adipokines	Hormones secreted by adipose tissue that regulate metabolism and inflammation.
Atherosclerosis	Buildup of fats, cholesterol, and other substances on artery walls, restricting blood flow.
Autoimmune Inflammation	Immune system mistakenly attacks the body's own tissues, as seen in T1DM.
β cells (Beta cells)	Insulin-producing cells found in the pancreas.
Cardiovascular Disease	A group of disorders of the heart and blood vessels, often exacerbated by diabetes.
Citrate Synthase	Enzyme indicating mitochondrial function; catalyzes the first step of the citric acid cycle.
Cytokine	Signaling proteins involved in immune and inflammatory responses.
Cytotoxicity	The quality or capacity of being toxic to cells.
Dipeptidyl Peptidase-4 (DPP4)	Enzyme that degrades incretin hormones, reducing insulin secretion.
DPP4 Inhibitors	Drugs that block DPP4, prolonging incretin activity to improve glycemic control.
Gestational Diabetes Mellitus (GDM)	Diabetes first diagnosed during pregnancy.
GLUT4 (Glucose Transporter 4)	Insulin-responsive protein that facilitates glucose uptake into cells.
Hyperglycaemia	Elevated blood glucose levels.
Inflammation	Biological response to harmful stimuli, chronic in diabetes and other conditions.
Insulin	Hormone secreted by β cells to regulate blood glucose.

Insulin Mimetic	Substance that simulates the effect of insulin in cells.
Insulin Resistance	Condition in which cells fail to respond effectively to insulin.
Interleukin-6 (IL-6)	Pro-inflammatory cytokine elevated in chronic diseases like diabetes.
Lipid Peroxidation	Oxidative degradation of lipids leading to cell damage.
Matrix Metalloproteinase 1 (MMP1)	Enzyme that breaks down extracellular matrix; involved in inflammation.
Metabolic Dysfunction	Impaired regulation of metabolism often linked to insulin resistance.
Neuropathy	Nerve damage, often a complication of diabetes.
Oxidative Stress	Imbalance between free radicals and antioxidants, contributing to disease.
Pharmacokinetics	Study of how drugs are absorbed, distributed, metabolized, and excreted by the body.
Protein Tyrosine Phosphatase 1B (PTP1B)	Enzyme that downregulates insulin signaling by dephosphorylating insulin receptors.
Reactive Oxygen Species (ROS)	Chemically reactive molecules containing oxygen that cause cell damage.
Schiff Base	Organic compound with a C=N double bond; used in coordination with metals like vanadium.
T1DM (Type 1 Diabetes Mellitus)	An autoimmune disorder that destroys insulin-producing β-cells.
T2DM (Type 2 Diabetes Mellitus)	Metabolic disorder characterized by insulin resistance and β-cell dysfunction.
TNF-α (Tumour Necrosis Factor-alpha)	Cytokine that promotes systemic inflammation and insulin resistance.
Vanadium Complexes	Compounds containing vanadium.

Chapter 1 : Introduction and Literature Review

1.1 Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder affecting millions of people worldwide and remains one of the leading causes of mortality. The condition is characterized by an inability of the body to regulate blood glucose concentrations due to either insufficient insulin production, as seen in type 1 diabetes mellitus (T1DM), or due to an inability to utilize insulin effectively, which is characteristic of type 2 diabetes mellitus (T2DM). If left untreated, DM can lead to severe complications such as hyperglycaemia, cardiovascular disease, and damage to nerves (neuropathy) and blood vessels (atherosclerosis). According to the World Health Organization (WHO), in 2022, 830 million people were living with DM, and resulting in severe consequences with an estimated 6.7 million deaths attributed to the disease in 2021, according to the International Diabetes Federation (1,2).

Between 2011 and 2021, the number of adults aged 20-79 living with diabetes in South Africa rose sharply from 1.9 million to 4.2 million, making it the country with the highest number of people living with diabetes in the African region at the time (1,3). This growing burden is deeply consequential, as diabetes was identified as the second leading cause of death in the country according to Statistics South Africa (4). This increased prevalence has been attributed to lifestyle changes associated with urbanization, such as increased physical inactivity and unhealthy diets.

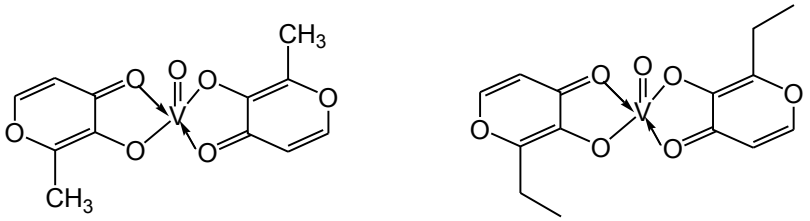
The burden of the disease is further compounded by the unequal two-tiered healthcare system in the country (5). The under-resourced public sector serves over 70% of the population, while the better equipped private sector remains inaccessible to the majority, this imbalance places pressure on both public and private healthcare systems, exacerbating inefficiencies in limiting

timely access to screening, diagnosis, and management of non-communicable diseases like diabetes (6).

Current therapies for DM, such as insulin and oral hypoglycaemic agents, offer some degree of control but come with several limitations, including high costs, adverse effects, and diminishing long-term efficacy (7). As the global burden of DM increases, there is an urgent need for novel and more effective treatment. Transition metals, particularly vanadium, have garnered significant attention in recent years due to their insulin-mimetic properties (8). Vanadium can stimulate glucose uptake in insulin target cells and has been shown to possess antioxidant properties that could help prevent DM-related complications.

The exploration of vanadium as a therapeutic agent for DM dates to the late 19th century, but its advancement has been limited by toxicity concerns, particularly with long-term use (9). Vanadium salts, which were among the first vanadium-based therapies explored, have demonstrated insulin-like effects. However, these vanadium-based metallodrugs are hydrophilic in nature and thus unable to efficiently cross cell membranes, which hindered their therapeutic potential. To address these issues, researchers have turned to appropriate organic ligands to improve the lipophilicity and stability of vanadium compounds (10).

A significant breakthrough came with the development of bis(maltolato)oxovanadium(IV) (BMOV), which demonstrated improved efficacy and reduced toxicity compared to earlier vanadium salts with its analogue bis(ethylmaltolato)oxovanadium(IV) (BEOV) (Figure 1.1) making it to phase II clinical trials (11).



Bis(maltolato)vanadium(IV) **Bis(ethylmaltolato)vanadium(IV)**

Figure 1.1: Chemical structures of vanadium metallodrugs BMOV (left) and BEOV (right), adapted from reference (12).

Building on this progress, Schiff base vanadium complexes have emerged as promising candidates for DM therapy. These compounds are characterized by the presence of an azomethine group
(-C=N-), which is a functional group formed by the condensation of a primary amine with an aldehyde or ketone, playing a role in the coordination to the metal ions and biological activity. Schiff bases have been shown to enhance the stability, bioavailability, and membrane affinity of vanadium complexes, offering the potential to reduce dosage requirements and minimize toxicity (13).

This study aimed to investigate the effects of Schiff base vanadium complexes on glucose uptake, cytotoxicity and inflammation in insulin target cells. By expanding on the existing body of literature and exploring the therapeutic potential of these novel compounds, this research seeks to contribute to the ongoing development of more effective and better-tolerated treatments for DM.

1.2 Literature Review

1.2.1 Insulin signalling and glucose transport

Understanding the intricate system designed to regulate blood glucose concentrations is essential for drug discovery research in the DM arena. This system is orchestrated mainly by two hormones, which are insulin, released by the β cells of the pancreas, and glucagon, released by the α cells of the pancreas. These hormones act antagonistically to achieve glycaemic control in a narrow, tightly regulated range which is 3.9 – 5.6 mmol/L for fasting and under 7.8 mmol/L at least 90 minutes after a meal (postprandial) (14,15). Considering that the study investigates the insulin-mimetic effects of vanadium, this literature review will briefly explore the role of insulin. The research aims to investigate how vanadium compounds interact with the insulin signalling pathway to potentially enhance glucose uptake and regulate glycaemic levels. Specifically, this study seeks to understand whether vanadium compounds can activate key components of the insulin signalling cascade, such as protein kinase B (Akt) expression and glucose transporter type 4 (GLUT4) translocation, which are pivotal for cellular glucose uptake (16).

The pancreatic β cells release insulin, facilitating glucose uptake by target cells in the liver, skeletal muscle, and adipose tissue (17). This process effectively lowers blood glucose concentration, maintaining it within the ideal range and preventing hyperglycaemia. Following insulin secretion by the β cells of the pancreas, the insulin undergoes transportation to its target cells, predominantly the skeletal muscle, liver, and adipose tissue. Insulin receptors are present in every cell, consisting of two α and two β subunits. The α subunits, located extracellularly, contain the insulin binding site, facilitating the binding of insulin to the receptor. Meanwhile, the transmembrane β subunits possess tyrosine kinase activity in their cytoplasmic domain (18).

Upon insulin binding to the α subunit, autophosphorylation occurs at multiple tyrosine residues in the β subunit, amplifying kinase activity. This autophosphorylation activates insulin receptor substrate (IRS) 1-4, serving as a docking protein which then binds to the P85 subunit of phosphatidylinositol-3-kinase (PI3-K) and recruits its catalytic subunit P110 to activate PI3-K

(19). PI-3K phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 stimulates phosphoinositide-dependent protein kinase (PDK), leading to the phosphorylation and activation of Akt. Activated Akt is pivotal in triggering essential cellular functions, including synthesising lipids, proteins, and glycogen, which are crucial for cell proliferation, survival and the anti-inflammatory response. The PI3-K pathway also plays a crucial role in glucose distribution, suppressing hepatic glucose synthesis and activating glycogen synthesis. The involvement of Akt extends to activating GLUT4 through the phosphorylation of the Akt substrate 160 kDa (AS160), which facilitates the translocation of GLUT4 to the cell membrane and promotes glucose uptake into the cell (20–22). The insulin signalling pathway, as described, is summarized in Figure 1.2 below.

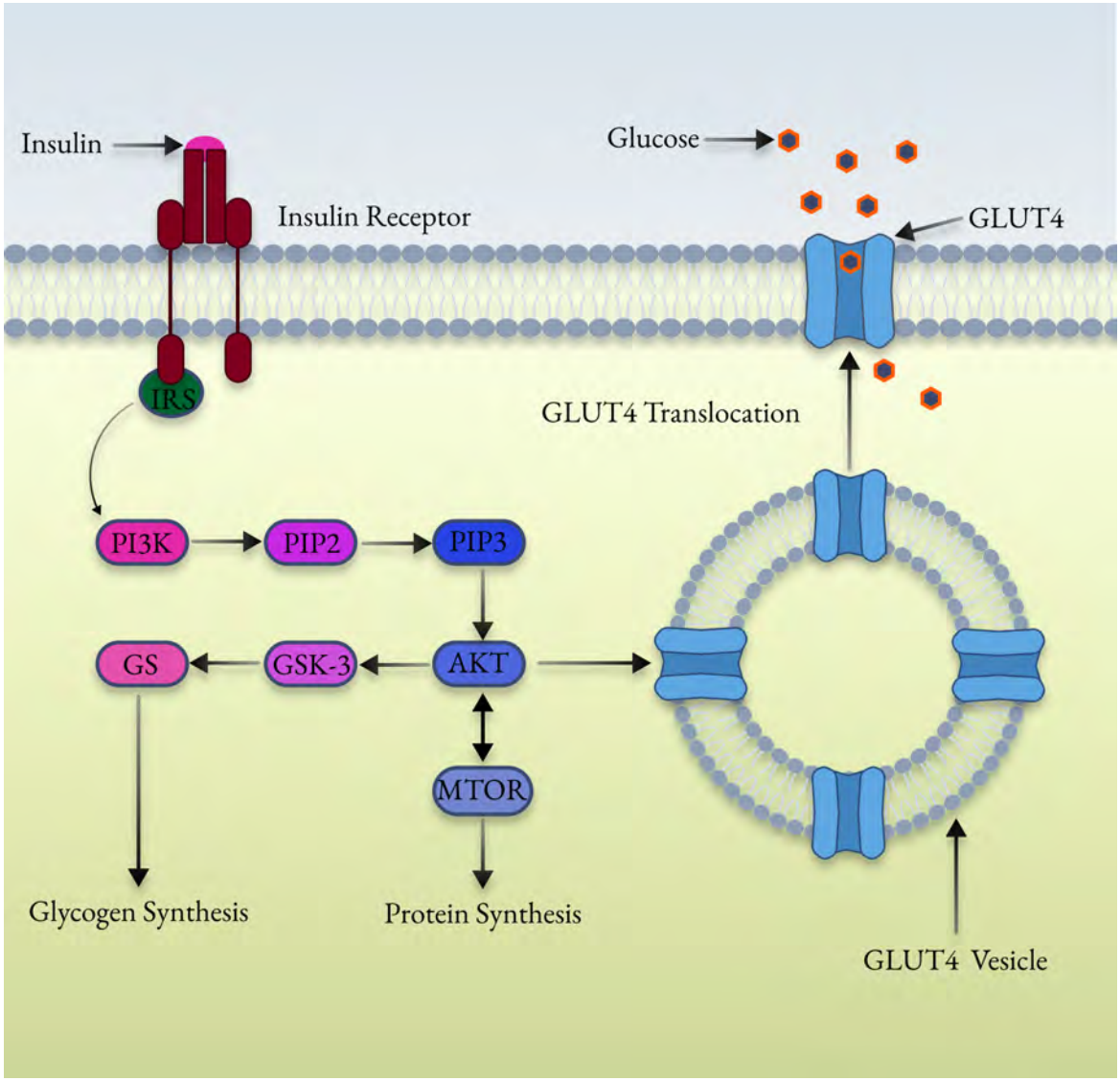


Figure 1.2: Simplified scheme of the insulin signalling pathway. Adapted from reference (23)

Figure 1.2 illustrates the key steps in the insulin signalling pathway, which is critical for regulating glucose uptake in cells. When insulin binds to its receptor on the cell surface, it triggers the activation of IRS, triggering the activation of PI3K. PI3K catalyses the conversion of PIP2 to PIP3, activating Akt. Activated Akt facilitates the translocation of GLUT4 vesicles to the plasma membrane, allowing glucose to enter the cell. Additionally, Akt influences glycogen and protein synthesis processes, further contributing to glucose homeostasis. This pathway is essential for maintaining blood glucose levels within the normal range (24,25).

Insulin signalling is intricately regulated through both negative and positive feedback pathways. In the positive feedback mechanism, specific enzymes such as Akt and P70 are activated to inhibit other enzymes, allowing the progression of the PI-3K pathway. This pathway, in turn, results in increased glycogen and protein synthesis and enhanced glucose uptake. These coordinated actions contribute to the overall effectiveness of insulin in cellular processes (26).

Conversely, negative feedback regulation involves protein tyrosine phosphatases (PTPs), a notable player being PTP1B. PTP1B functions as a negative regulator of insulin actions by catalysing the dephosphorylation of insulin receptors. This dephosphorylation renders the insulin receptor in an inactivated state, thereby reducing glucose uptake. The activity of PTP1B inhibits subsequent steps in the insulin signalling pathway, inhibiting glucose uptake by the cells (18). This action of PTP1B has emerged as a potential target site for insulin-mimetic agents. Interestingly, the antidiabetic effect of vanadium is ascribed to the inhibition of PTP1B. Inhibition of phosphorylation of the insulin receptor by PTP1B and subsequent inhibition of the steps in the insulin signalling pathway is illustrated in Figure 1.3 below.

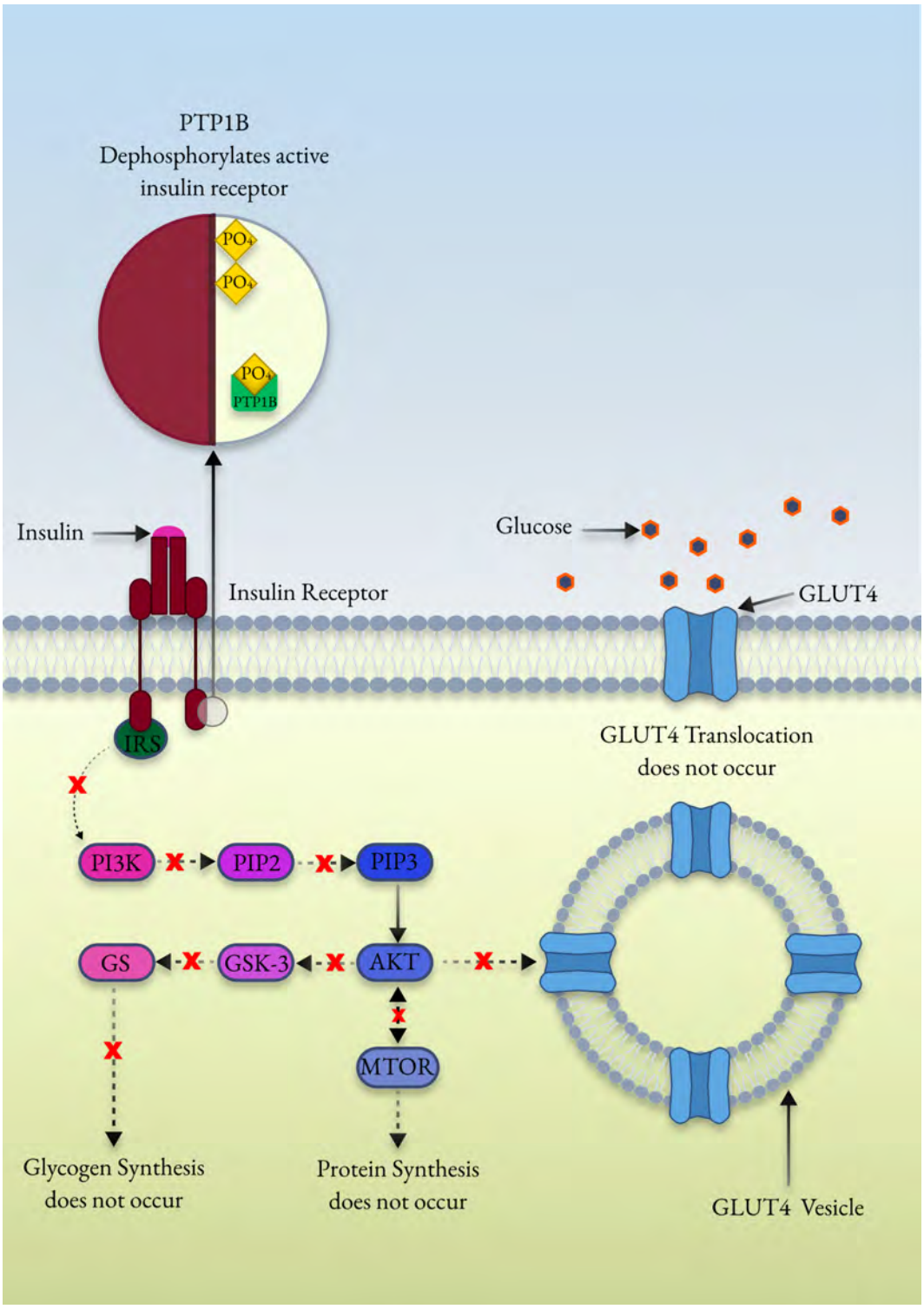


Figure 1.3: Inhibition of insulin signalling pathway by PTP1B. Adapted from reference (23)

After PTP1B dephosphorylates the insulin receptor, preventing its activation and subsequent phosphorylation of IRS, this inhibition halts the activation of the PI3K pathway, leading to the

failure of downstream processes such as the activation of Akt, glycogen synthesis, protein synthesis, and the translocation of GLUT4 vesicles to the cell membrane. As a result, glucose uptake into the cell is impaired, contributing to the ineffective regulation of blood glucose concentrations. This pathway represents a critical point of intervention for insulin-mimetic agents, such as vanadium, which can inhibit PTP1B to restore normal insulin signalling and glucose uptake (24).

1.2.2 Insulin target cells

The effects of insulin are mainly observed in skeletal muscle, liver and adipose tissue, as these act as major sites for glucose uptake, storage and utilization. Therefore, these tissues are key contributors to glycaemic control. Skeletal muscle is the major site for insulin-mediated glucose uptake from the bloodstream. This uptake is facilitated through the translocation of GLUT4 to the cell membrane, where it facilitates the entry of glucose into the skeletal muscle cell for storage as glycogen or metabolized for energy production (19). In this study, C2C12 myoblasts were used as a cell line model for skeletal muscle cells to investigate the insulin-mimetic effects of the vanadium compounds. Upon differentiation into myotubules, the myoblasts closely resemble the physiology of skeletal muscle, and are particularly suitable for studying glucose uptake and insulin signalling (28).

The liver plays an important role in glucose homeostasis as it regulates blood glucose through glycogen synthesis, glycogenolysis, and gluconeogenesis, with insulin suppressing hepatic glucose production by inhibition of gluconeogenesis and stimulation of glycogen synthesis through the activation of the PI3-K/Akt pathway (26). HepG2 cells were employed in this study, as this cell line is widely used to model and investigate insulin signalling and glucose metabolism in hepatic tissues (29). By examining the activation of the insulin signalling pathway in these cells, the results of this study sought to evaluate whether the vanadium compounds can replicate the insulin-mimetic effects in liver cells. Therefore, this study aims to provide novel insight into the mechanisms by which these compounds could contribute to improving glucose regulation.

Adipose tissue is a key player in glucose homeostasis, acting as both a storage site for excess energy and an endocrine organ that secretes hormones and cytokines involved in metabolic regulation. Insulin stimulates glucose uptake in adipose tissue by promoting the translocation of GLUT4 to the cell membrane, where it facilitates glucose entry into adipocytes. Once inside, glucose is converted into triglycerides for storage or metabolised to meet cellular energy demands. Additionally, insulin inhibits lipolysis, reducing the release of free fatty acids into the bloodstream, which helps maintain insulin sensitivity in other tissues (30,31). Dysregulation in glucose uptake or lipid metabolism in adipose tissue contributes significantly to insulin resistance and metabolic disorders, highlighting its crucial role in systemic glycaemic control. The 3T3-L1 preadipocyte cell line is a commonly used model for adipose tissue, allowing the exploration of the insulin signalling pathway and its impact on glucose metabolism in adipocytes (26).

1.2.3 Pathophysiology of diabetes mellitus

The pathophysiology of DM is closely linked to disruptions in the secretion of insulin and insulin signalling pathways. In type 1 diabetes mellitus (T1DM), autoimmune destruction of pancreatic β-cells eliminates insulin production, leaving the insulin signalling pathway non-functional. This autoimmune response is often mediated by CD8+ T-cell activation against β-cell antigens in genetically predisposed individuals. Over time, the progressive loss of β-cell mass results in insufficient insulin production, making the body incapable of lowering blood glucose concentrations effectively. Symptoms typically manifest rapidly and include polyuria, polydipsia, and weight loss. Without exogenous insulin administration, glucose remains largely unavailable for cellular metabolism despite its abundance in the bloodstream (32).

Type 2 diabetes mellitus (T2DM) is a multifactorial disease often associated with insulin resistance, where target cells in tissues such as skeletal muscle, liver, and adipose tissue fail to respond effectively to insulin. Initially, pancreatic β cells compensate by producing more insulin to maintain euglycemia. However, over time, β cell dysfunction arises, and insulin production becomes insufficient to counteract the resistance, leading to persistent hyperglycaemia (33).

Chronic low-grade inflammation, driven by metabolic stress, plays a significant role in the pathogenesis of T2DM, linking nutrient excess to insulin resistance and β cell dysfunction through mechanisms such as the activation of Toll-like receptors and endoplasmic reticulum stress (34). Risk factors for T2DM include obesity, lack of physical activity, and genetic predisposition. Excess adipose tissue, particularly visceral fat, contributes to this inflammation and releases adipokines that impair insulin signalling. In overweight or obese individuals, adaptive mechanisms fail, such as reduced β cell compensatory response, heightened glucagon secretion by α cells, increased hepatic glucose production, and systemic inflammation within adipose tissue (35). These disruptions exacerbate insulin resistance and further damage β cell function, reinforcing the metabolic dysfunction that underpins the progression of T2DM. Consequently, T2DM is increasingly understood as an inflammatory condition triggered by disordered metabolism, with therapeutic approaches focusing on restoring metabolic homeostasis to improve insulin sensitivity and β cell survival (36,37).

Gestational diabetes mellitus (GDM) refers to glucose intolerance first identified during pregnancy, typically between the 24th and 28th weeks of gestation (38). Hormonal changes during pregnancy can cause insulin resistance, which is often exacerbated in women with predisposing factors such as obesity or a family history of DM (39). If unmanaged, GDM poses significant risks to both the mother and foetus, including macrosomia, preeclampsia, and neonatal hypoglycaemia (40). Women with GDM also face a higher likelihood of developing T2DM later in life, underscoring the need for lifestyle interventions to mitigate this risk (41,42).

1.2.4 Diabetes mellitus-related inflammation and associated biomarkers

The relationship between DM and inflammation is well established, with chronically elevated blood glucose concentrations triggering inflammatory pathways that exacerbate insulin resistance, with several inflammatory makers playing crucial roles. One example is interleukin-6 (IL-6). This cytokine is a key mediator in inflammatory responses associated with DM. Elevated levels of IL-6 are often found in patients with T2DM and are linked to increased insulin resistance and β cell dysfunction (43). IL-6 contributes to insulin resistance by impairing insulin

receptor phosphorylation (44). In T1DM, IL-6 acts as an influential mediator of autoimmune inflammation and glucose homeostasis (45).

Another group of important inflammatory markers is matrix metalloproteinases (MMPs), which are enzymes involved in the degradation of extracellular matrix components (46). MMP1 is involved in tissue remodelling during inflammation and may contribute to vascular complications associated with DM. Increased activity of MMPs has been observed in uncontrolled DM conditions, suggesting its role in the progression of DM-related complications (47). Furthermore, MMP1 has been observed to influence the shedding of dipeptidyl peptidase-4 (DPP4).

DPP4 is an enzyme that deactivates incretin hormones crucial for insulin signalling. Elevated DPP4 activity is associated with increased inflammation and insulin resistance in patients with T2DM (48,49). Notably, DPP4 inhibitors have been shown to possess anti-inflammatory properties, further underlining the role of DPP4 in mediating inflammation and metabolic dysregulation in patients with DM (50).

Furthermore, Tumour Necrosis Factor-α (TNF-α) has been reported to be among the pro-inflammatory cytokines involved in DM-related inflammatory processes (51). TNF-α is primarily secreted by adipose tissue macrophages and impairs insulin signalling by promoting serine phosphorylation of IRS, therefore reducing the ability of IRS to activate the rest of the insulin signalling pathway (51). Elevated TNF-α concentrations are strongly associated with insulin resistance and T2DM, with expression particularly pronounced in individuals with obesity (52). Furthermore, TNF-α has been shown to induce lipolysis in adipocytes, therefore increasing circulating free fatty acids, which exacerbate metabolic dysfunction (53).

These pro-inflammatory agents can be clinically useful for diagnosis, prognosis, and treatment monitoring (54). The measurement and modulation of IL-6, MMP1, DPP4, and TNF-α can offer critical insights into the progression of DM, enabling a deeper understanding of its pathology. In this study, these cytokines are evaluated to assess both the therapeutic potential and safety profile of the vanadium compounds under investigation. Specifically, measuring IL-6 expression can reveal whether the compounds induce inflammation, thereby providing an indication of potential

cytotoxicity. Similarly, changes in DPP4 activity reflect the compounds' influence on inflammation and provide valuable information on their insulin-mimetic effects, as DPP4 directly impacts insulin signalling via incretin modulation. Furthermore, alterations in MMP1 levels could shed light on extracellular matrix remodelling and associated inflammation. Together, these markers provide a comprehensive view of the compounds' effects on both inflammation and glucose metabolism, highlighting their potential utility in managing DM.

1.2.5 Diabetes mellitus-related complications

Chronic inflammation is a hallmark feature of DM and plays a significant role in the progression of its long-term complications (55). Sustained inflammation exacerbates insulin resistance and causes tissue damage, contributing to the development of diabetic complications such as retinopathy, nephropathy, neuropathy, and cardiovascular diseases (CVDs) (56). In diabetic retinopathy, chronic hyperglycaemia and inflammation disrupt retinal capillary integrity and increase vascular permeability. This vascular damage leads to microaneurysms, haemorrhages, and, eventually, vision loss (57,58). Similarly, in diabetic nephropathy, persistent inflammation and hyperglycaemia damage glomerular structures, resulting in proteinuria and reduced renal function. Inflammation also promotes fibrosis in the kidneys, ultimately contributing to end-stage renal disease (59). In diabetic neuropathy, inflammation-induced nerve damage impairs neural repair mechanisms, leading to nerve degradation. This manifests as sensory loss, pain, and, in severe cases, autonomic dysfunction (60).

CVD is another significant complication linked to impaired insulin signalling. Insulin resistance promotes dyslipidaemia, chronic inflammation, and oxidative stress, which drive the formation of atherosclerotic plaques (61). These abnormalities also underpin the metabolic syndrome, which is characterized by the co-existence of several major risk factors for CDV and DM (62). Hypertension represents another critical cardiovascular manifestation of DM. Impaired insulin signalling can disrupt vascular tone and increase sodium retention, thereby elevating blood pressure (63). High blood glucose concentrations can also cause damage to the blood vessels and kidneys, which play a role in maintaining healthy blood pressure. Additionally, hyperglycaemia contributes to cardiovascular stress through its osmotic properties, with elevated blood glucose

concentration increasing blood osmolarity, leading to enhanced water retention in blood vessels and further compounding hypertensive effects (64).

Insulin resistance in the liver leads to excessive fat accumulation, which is the proposed pathogenesis of non-alcoholic fatty liver disease (NAFLD), now known as metabolic dysfunction-associated liver disease (MASLD) (65). Research shows that the dysregulation of the PI3K/Akt pathways is a key event in the development of MASLD (66). This dysregulation contributes to metabolic stress, lipotoxicity, and chronic inflammation, which further exacerbate liver steatosis and progression to more severe liver conditions such as liver cirrhosis and hepatocellular cancer (67,68).

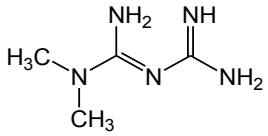
1.2.6 Current treatment options for diabetes mellitus

1.2.6.1 Exogenous insulin

Since the pathophysiology of T1DM stems from insufficient insulin production by pancreatic ß cells, a groundbreaking advancement in managing T1DM was the discovery and administration of exogenous insulin, which remains a cornerstone of T1DM treatment. Insulin formulations are categorized by their duration of action to mimic the action of endogenous insulin with a constant basal level in circulation and bolus releases during meals to mitigate postprandial blood glucose spikes (69,70). Despite its significance, insulin therapy has limitations. The risks of hypoglycaemia, drug interactions, and adverse effects like lipoatrophy, allergic reactions, and insulin oedema complicate its use (71). Drug interactions, such as those with β-blockers or Thiazolidinediones such as pioglitazone, may exacerbate hypo- or hyperglycaemia or increase the risk of cardiac failure (72). Moreover, the high cost, storage requirements, and the discomfort of self-injection associated with insulin therapy make oral hypoglycaemic agents critical alternatives in DM management (69,73).

1.2.6.2 Biguanides

Oral hypoglycaemic agents have become essential in managing DM due to the limitations of insulin therapy. Among these, biguanides, with metformin (Figure 1.4) as the leading agent, are widely regarded as the first-line treatment for T2DM (74). Metformin attenuates hyperglycaemia by inhibiting hepatic gluconeogenesis and promoting glucose uptake (75). This agent is particularly beneficial for obese patients due to its mild anorectic effect, which helps control body weight. However, metformin therapy is not without adverse effects such as the risk of lactic acidosis, which makes it unsuitable for patients with renal impairment, especially older adults (69). Other side effects include gastrointestinal disturbances such as diarrhoea and a metallic taste, which may affect patient compliance (76).



Metfromin

Figure 1.4: Chemical structure of metformin adapted from reference (77).

1.2.6.3 Secretagogues

Sulphonylureas, such as glibenclamide and repaglinide (Figure 1.5), are another class of oral hypoglycaemic agents. They function by stimulating ß cells in the pancreas to release insulin and have shown other effects beyond the pancreas, such as reducing insulin clearance in the liver, reducing glucagon secretion, and enhancing peripheral insulin sensitivity, making them effective for T2DM treatment (78). However, because T1DM patients lack functional ß cells, these drugs are ineffective for this condition. Sulphonylureas have their adverse effects, including the risk of weight gain, allergic reactions, gastrointestinal issues, and cardiovascular disease (78). They also present significant drug interactions, particularly with warfarin and β-blockers, complicating their use in patients with comorbid conditions such as atrial fibrillation, which often requires an anticoagulant, and hypertension, which is commonly treated with a β-blocker (69).

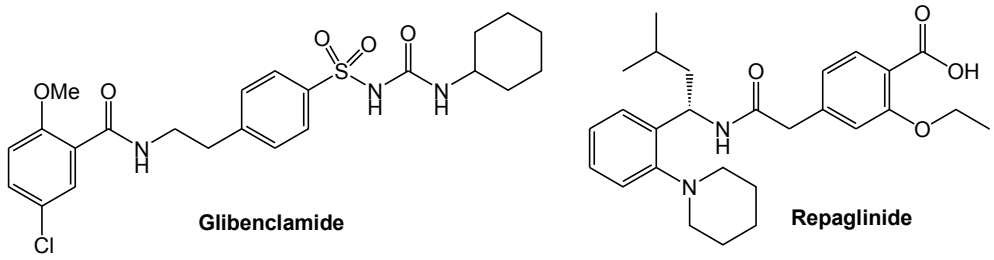


Figure 1.5: Chemical structures of glibenclamide (left) and repaglinide (right) adapted from reference (79,80).

Meglitinides, like repaglinide, share a similar mechanism to sulphonylureas but are shorter acting, which reduces the risk of hypoglycaemia (81). Despite this advantage, they are contraindicated in patients with severe renal or hepatic impairment, with studies showing them to increase the risk of hypoglycaemia in these patients (82). Meglitinides are also associated with gastrointestinal side effects, including abdominal pain, nausea, and vomiting, and there is a risk of skin hypersensitivity reactions (69).

1.2.6.4 Carbohydrate hydrolysing enzyme inhibitors

Another class of agents, α-glucosidase inhibitors (e.g., acarbose and miglitol) (Figure 1.6), is primarily used in Europe and Japan. These drugs slow the absorption of carbohydrates by inhibiting enzymes in the brush border of the small intestine, helping to control blood glucose concentrations effectively, especially in patients with high-starch diets (83). However, they have their adverse effects, such as gastrointestinal discomfort, including flatulence and diarrhoea (84). They are also contraindicated in patients with gastrointestinal disorders, such as inflammatory bowel disease and colonic ulceration (69).

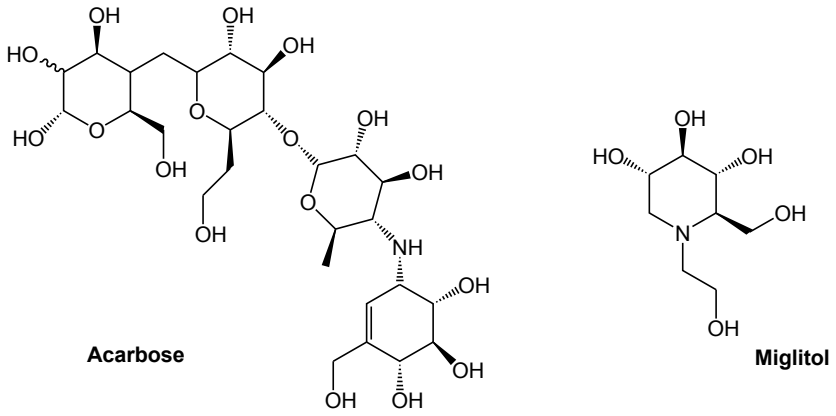


Figure 1.6: Chemical structures of acarbose and miglitol adapted from reference (85,86)

1.2.6.5 Thiazolidinediones

Thiazolidinediones, such as ciglitazone, pioglitazone, and rosiglitazone (Figure 1.7), work by improving cellular insulin sensitivity by acting as agonists for peroxisome proliferator-activated receptor gamma (PPAR-γ), which regulates the expression of genes involved in glucose and lipid metabolism (69). This mechanism makes them suitable for patients previously diagnosed with T2DM, which is inadequately managed by diet and exercise. However, their use is restricted by significant contraindications, including T1DM and impaired cardiac or liver function. Adverse effects like oedema, angina, skeletal fractures, and a heightened risk of bladder cancer make thiazolidinediones a less favourable option for many patients (87,88).

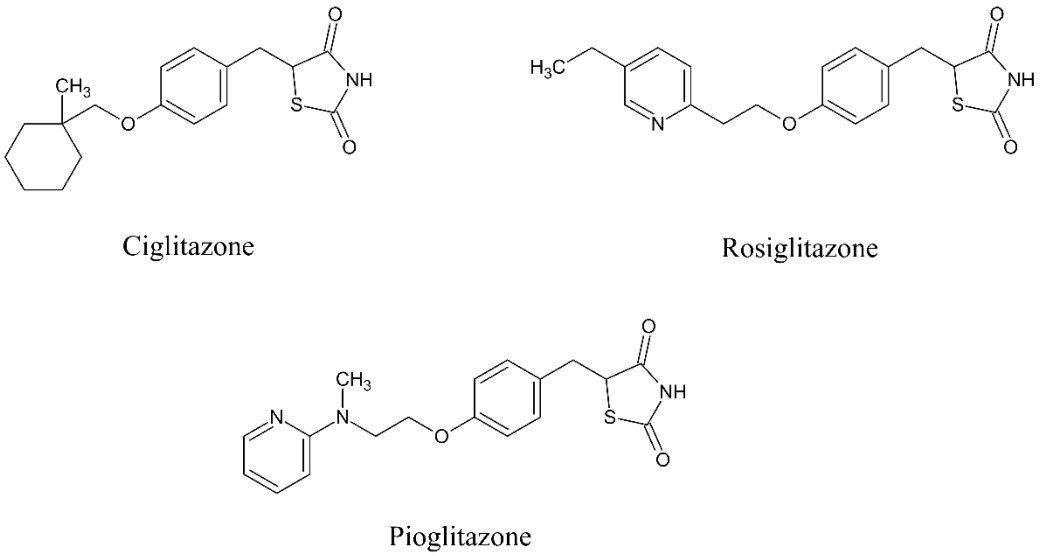
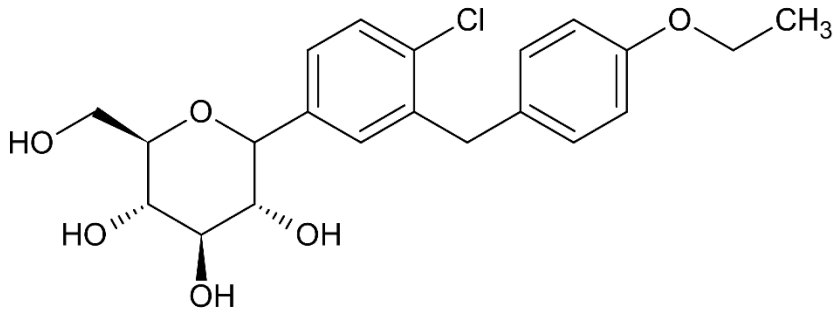


Figure 1.7: Chemical structures of ciglitazone, rosiglitazone, and pioglitazone adatapted from reference (89–91)

1.2.6.6 Sodium-glucose co-treansporter-2 (SGLT2) inhibitors

Sodium-glucose co-transporter-2 (SGLT2) inhibitors, such as dapagliflozin (Figure 1.8), offer a novel approach by promoting glucose excretion through the kidneys (92). While effective for T2DM, they are contraindicated in T1DM and patients with moderate to severe renal impairment. Common side effects include urinary tract and genital infections, which can be of clinical significance and result in further complications for patients (69,93)



Dapaglifozin

Figure 1.8: Chemical structure of dapagliflozin adapted from reference (94).

1.2.6.7 Incretin-like agents

Non-oral options include Glucagon-Like Peptide-1 (GLP-1) receptor agonists, such as semaglutide (Ozempic), which are administered subcutaneously (95). These drugs increase insulin secretion and suppress glucagon while aiding in weight loss (69). Semaglutide, in particular, has demonstrated superior glycaemic control and significant weight reduction compared to older GLP1-analogues and traditional therapies like sulfonylureas and insulin, due to these dual metabolic benefits, semaglutide has gained widespread use as a preferred second-line therapy in overweight patients with T2DM (96). However, they are contraindicated in T1DM, severe renal impairment, and gastrointestinal diseases. Adverse effects include nausea, vomiting, risk of pancreatitis, and injection-site discomfort, which may deter patient adherence (97).

1.2.6.8 DPP4 inhibitors

Finally, Dipeptidyl Peptidase-4 (DPP4) inhibitors (e.g. sitagliptin an linagliptin) prevent the breakdown of GLP-1, prolonging its insulin-enhancing and glucagon-suppressing effects (98).

These drugs are effective for T2DM but are contraindicated in severe hepatic impairment (69). Side effects may include gastrointestinal discomfort and risk of pancreatitis, highlighting the need for cautious use (99).

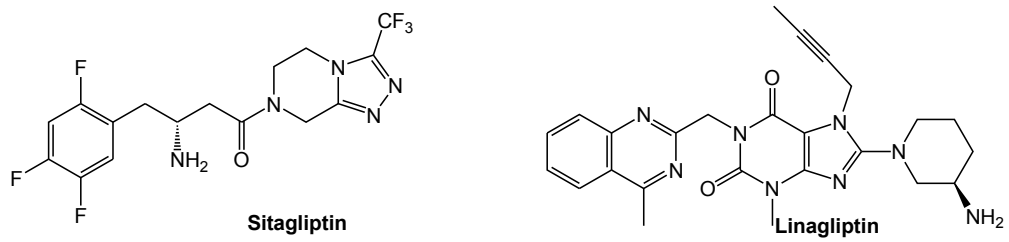


Figure 1.9: Chemical structures of sitagliptin and linagliptin adapted from reference (100,101).

The increasing prevalence of DM, coupled with the challenges of accessing medications in many developing regions and underserved communities, highlights the urgent need for more accessible, affordable, and safer treatment options. While current treatment options offer unique benefits within each drug class, they still present common drawbacks, such as the risk of hypoglycaemia, drug interactions, and contraindications, especially during pregnancy or in the presence of comorbid conditions. These limitations underscore the importance of pursuing alternative treatments that not only address the physiological aspects of the disease but also tackle systemic barriers, including access, cost, and the specific needs of individual patients. This approach can contribute to more personalized, patient-centred care.

1.2.7 Trace metals and diabetes mellitus

Interestingly, vanadium is just one example of a trace metal with potential therapeutic applications in DM management. Trace metals play diverse and significant roles in metabolic regulation, with several being studied for their ability to modulate key processes in glucose homeostasis. Another example is zinc, with its link to DM and insulin established a decade after the discovery of insulin (102). Zinc ions facilitate the efficient storage of insulin in pancreatic β-cells through hexemerization, where insulin dimers aggregate to form a hexamer around two Zn^{2+} ions. This process also triggers the crystallization of insulin, making it more stable and leading to more efficient storage in insulin-secreting vesicles (103). Along with its role in the processing

and storage of insulin, zinc has also been studied as an insulin mimetic, where it has been observed to inhibit PTP1B in a study by Bellomo and colleagues (104). Furthermore, the researchers observed a secondary mechanism of inhibition of PTP1B through the binding of zinc to the acidic surface residues, where the researchers suggest that it may involve aggregation (104).

Another trace metal, chromium, also plays a pivotal role in glucose metabolism. Chromium enhances insulin signal transmission and increases insulin sensitivity by activating insulin receptors via chromodium oligopeptides (105). Acting as a cofactor or secondary messenger for insulin, chromium facilitates glucose utilization by target cells (106). Chromium deficiency can lead to metabolic disturbances, including glucose intolerance, fasting hyperglycaemia, and increased circulating insulin concentrations (106). Notably, a study observed that chromium supplementation over a four-month period resulted in significant reductions in fasting blood glucose and HbA1c levels, highlighting its potential in the control and management of DM (105). While chromium has been recognized as an enhancer of insulin action, its role in developing T2DM is not yet fully understood. A case-control study conducted in a Chinese population between 2009 and 2014 found that plasma chromium levels were significantly lower in individuals with T2DM and pre-DM than those with normal glucose tolerance (106). Moreover, plasma chromium concentrations were inversely associated with the risk of T2DM and pre-DM, even after adjusting for potential confounding factors, suggesting a protective role of chromium against glucose metabolism disorders (107). Subsequent clinical trials evaluated the effects of chromium picolinate supplementation on patients with poorly controlled T2DM. The study revealed that the chromium picolinate significantly improved glycaemic control, evidenced by reductions in fasting glucose and postprandial glucose compared to the control group (108). Additionally, the researchers also observed reduced HbA1c levels in the supplemented group compared to the control group.

Magnesium has also been shown to support enzymatic activities essential for glucose oxidation and insulin receptor function. Clinical observations have linked low magnesium levels to insulin resistance and an increased risk of T2DM (109). A meta-analysis study further demonstrated an inverse association between higher magnesium intake and the risk of developing T2DM,

suggesting that adequate magnesium consumption may help prevent the onset of this condition (110). The benefits of magnesium supplementation for glycaemic control are well-documented. For instance, a 12-week study showed that participants with T2DM who took 300 mg of magnesium daily experienced significant reductions in both fasting and post-meal blood glucose levels (111). The antidiabetic effects of magnesium are thought to be mediated through multiple mechanisms. One proposed mechanism involves enhanced insulin sensitivity, which helps reduce insulin resistance. Magnesium also plays a critical role in glucose metabolism by facilitating the production of ATP (112). Specifically, magnesium binds to ATP to form Mg-ATP, the biologically active form required for various metabolic processes, including glucose utilization (113). Additionally, magnesium is essential for glucose transport into cells, and magnesium deficiency has been observed to impair the translocation of GLUT4 to the cell membrane, thereby reducing glucose uptake and increasing blood glucose concentrations (112).

Other trace metals such as manganese, selenium, and copper have demonstrated antidiabetic effects. Manganese supports glucose metabolism by activating key enzymes like glucokinase and mitigating oxidative stress (114,115). Selenium, through its role in antioxidant enzymes like glutathione peroxidase, protects pancreatic β-cells from oxidative damage(116,117). Copper also plays a vital role in reducing oxidative stress and inflammation, with copper-dependent enzymes such as superoxide dismutase contributing to cellular protection (118). While these metals offer diverse mechanisms of action in glucose homeostasis, vanadium stands out for its unique insulin-mimetic properties, making it a promising candidate for further exploration in DM management.

1.2.8 Vanadium

As a transition metal, vanadium exhibits oxidation states ranging from –1 to +5. This versatility enables it to engage in a wide range of coordination chemistry with various ligands in its ionic state. The coordination with ligands profoundly influences its properties and reactivity, holding significant biological and pharmaceutical implications (119). The absorption of vanadium into the gastrointestinal (GI) tract is a complex process involving both passive and active mechanisms (120). Inorganic vanadium, such as orthovanadate, can be absorbed passively, like the absorption of other metal ions. However, organic forms of vanadium, often in ligand complexes, exhibit

enhanced absorption due to the lipophilic nature of organic compounds. This property enables them to traverse membrane walls more easily than their inorganic counterparts (121). The challenge of relatively poor absorption of inorganic vanadium faced by early researchers prompted a shift in focus toward organic ligand vanadium complexes. This shift aimed to address the risk of toxicity associated with inorganic vanadium, where higher doses were required to achieve the desired effects, increasing the potential for toxicity. The improved absorption of organic vanadium compounds allowed for administering lower doses to patients, reducing the risk of toxicity while maintaining therapeutic efficacy (122). Due to its ability to mimic phosphate, vanadium can substitute for phosphate in specific enzymatic reactions, influencing various cellular processes targeted for therapeutic use. However, this property also comes with the risk of lack of selectivity, where other cellular processes not targeted for therapy may be affected (123). Vanadium is known to bind to specific proteins and metabolites. Notably, it interacts with proteins such as transferrin and albumin, influencing its transport in the bloodstream (124). The complete understanding of how these proteins affect the uptake of vanadium by cells remains an ongoing area of research.

1.2.9 Vanadium in physiological medium

Vanadium, after oral ingestion, predominantly exists in the +4 (oxovanadium(IV), VO^{2}) and +5 (Orthovanadate, VO_4^{3-}) oxidation states, exhibiting significant biological activity in physiological media (97). Upon entering the bloodstream, vanadium compounds can undergo redox reactions, transitioning between oxovanadium(IV) and orthovanadate forms. The redox cycling is influenced by the local pH, the presence of reducing agents, and other biomolecules. In physiological conditions, vanadium ions interact with various proteins, such as transferrin and albumin, which facilitate their transport (91). Orthovanadate, due to its structural similarity to phosphate as illustrated in Figure 1.9, can substitute phosphate in enzymatic processes, notably inhibiting PTPs like PTP1B (98,99). This inhibition is crucial for enhancing insulin signalling by preventing dephosphorylation of the insulin receptor, thereby maintaining its active state and promoting glucose uptake (100). Furthermore, vanadium compounds may form complexes with biomolecules, influencing cellular uptake and distribution (101). The dynamic interplay of the redox states of vanadium and its binding to biological ligands highlights its dual potential for

therapeutic benefits and toxic effects, necessitating careful consideration of its biomedical application (97).

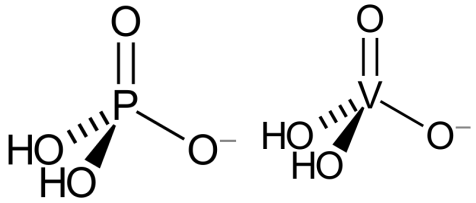


Figure 1.10: Structural similarity between phosphate (left) and orthovanadate (right)

1.2.10 Vanadium in the management of diabetes mellitus

The proposed mechanisms of action for vanadium, particularly in orthovanadate, have been elucidated, revealing its role as a potent PTP inhibitor. This inhibition arises from the tetra-coordinated structure and negative charge of orthovanadate, closely resembling the phosphate group of PTP substrates (Figure 1.10) (125). Orthovanadate mimics phosphate and fits into the PTP active site with a trigonal bipyramid geometry, stabilised by a strong network of hydrogen bonds between the oxygen atoms on the orthovanadate and specific amino acids such as serine 215, serine 216, arginine 221, glycine 220, and glycine 218 (126). As a result, orthovanadate acts as a broad-specific, reversible, and competitive PTP inhibitor (127). Some vanadium compounds, such as pervanadate, can oxidise critical cysteine residues at the active site of PTPs, leading to irreversible inhibition. This can also occur indirectly through interconversion between oxovanadium(IV) and orthovanadate redox states of the vanadium ion via Fenton-like reactions, generating reactive oxygen species (ROS) (128,129). The resulting ROS can directly oxidise cysteine residues in the critical active site of PTPs, rendering them irreversibly catalytically inactive and contributing to broad PTP inhibition (130,131).

However, the non-selectivity of orthovanadate as a PTP inhibitor poses challenges for developing orthovanadate as a drug candidate. It may inadvertently inhibit other PTP isoforms, complicating the therapeutic use of vanadium-based compounds. In the case of treating DM, non-selectivity can lead to unintended biological effects and adverse reactions. Vanadium complexes that target the less conserved sites, such as the allosteric sites of the PTP1B enzyme,

may be explored as a strategy to address resulting undesirable off-target side effects. Thus, further research is needed to overcome these challenges and refine the therapeutic potential of vanadium-based compounds (132).

In the context of PTP1B, inhibition by vanadium leads to the insulin receptor remaining phosphorylated (133). This phosphorylated state of the insulin receptor is crucial, as it mimics the upstream signalling events that typically occur upon insulin binding to the receptor. therefore, facilitating glucose uptake. This observation underscores the potential therapeutic relevance of vanadium in modulating insulin signalling pathways and, therefore, addressing conditions associated with insulin resistance (134). By mimicking phosphate, orthovanadate fits into the PTP1B active site as depicted in Figure 1.11 below, inhibiting its activity and preventing the dephosphorylation of the insulin receptor. This inhibition maintains the insulin receptor in a phosphorylated state, allowing the continuation of downstream signalling pathways. As a result, key processes such as the translocation of GLUT4 to the cell membrane and glucose uptake are facilitated, offering potential therapeutic benefits for conditions associated with insulin resistance. However, the broad specificity of orthovanadate as a PTP inhibitor highlights the need for further research to refine its use as a drug candidate, given the potential for off-target effects (24).

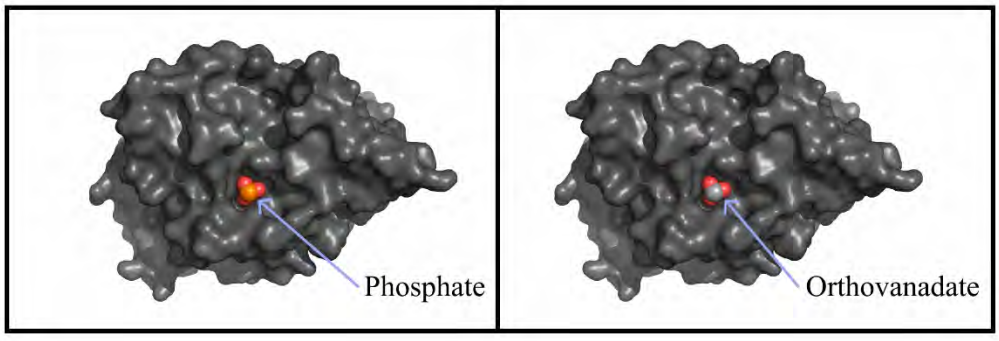


Figure 1.11: Structural comparison of phosphate (left) and orthovanadate (right) bound to the PTP1B active site, demonstrating the role of orthovanadate as an antagonist to inhibit PTP1B activity

1.2.11 Historical insights on vanadium in the treatment of diabetes mellitus

The earliest bibliographically recorded research on the potential of vanadium in treating DM dates to the end of the 19th century. In a report by Martin et al., in 1899 the researchers explored the use of inorganic orthovanadate salts, specifically sodium orthovanadate (9). They administered these salts orally to themselves and a group of patients with DM. They observed a notable reduction in glucosuria in two of the three patients with DM who received a daily dose of sodium orthovanadate without any adverse effects. This study was considered as a "Phase 0" clinical trial due to its preliminary nature. It is noteworthy to acknowledge that this research occurred before the groundbreaking discovery of insulin in 1922 by Frederick Banting. The discovery of insulin marked one of the most significant breakthroughs in DM research, revolutionising the understanding and treatment of the condition by uncovering multiple pharmacological target sites at both molecular and cellular levels. Though initially unnoticed, the early work on vanadium lays a historical foundation for the exploration of vanadium as a potential therapeutic agent in managing DM (135). Vanadium regained research interest in 1979 following a pivotal study by Tolman et al., which reported insulin-mimetic activity from vanadium salts, specifically sodium metavanadate (136). In their findings, vanadium was observed to have insulin-like properties, directly influencing glucose metabolism.

Building upon these insights, a subsequent study by Dubyak et al., in 1980 further explored the insulin-mimetic activity of vanadium (137). The research revealed that orthovanadate stimulated glucose transport and oxidation in rat adipocytes, demonstrating actions akin to insulin. These studies marked a resurgence of interest in vanadium as a potential therapeutic agent with insulin-like effects, prompting further investigations into its mechanisms of action and possible applications in DM. The insulin-mimetic effect of orthovanadate was subject to further elucidation by Tamura et al., in 1984. In their study, the authors demonstrated the ability of orthovanadate to enhance the phosphorylation of the insulin receptor (138). This enhancement

was shown to simulate the kinase reaction similarly, though not identical to the insulin action. This research provided valuable insights into the molecular mechanisms underlying the insulin-mimetic properties of vanadium, shedding light on how it influences critical signalling pathways involved in glucose metabolism and insulin action.

The following landmark discovery occurred in 1986, in a study by Heyliger et al., in this pivotal research, a solution containing 0.8 mg/ml of sodium metavanadate was administered to streptozocin-induced diabetic rats, resulting in normoglycemia (139). This study held particular significance since streptozotocin is known to destroy the insulin-producing β cells in the pancreas of rats (140). These findings by Heyliger et al., indicated that vanadium could induce glucose uptake like insulin, even without functional insulin-producing β cells. This highlighted the potential of vanadium as a promising agent for managing hyperglycaemia and provided crucial insights into its therapeutic role in the context of DM (139). Another vanadium salt demonstrating insulin-mimetic activity is oxovanadium(IV) sulphate. A study by Sakurai et al., in 1990 revealed that oxovanadium(IV) sulphate could significantly reduce blood glucose concentrations in streptozocin-induced diabetic rats (141). Up to this point, most research on vanadium had focused on vanadium salts. However, these vanadium salts were associated with toxic side effects, including gastrointestinal discomfort, hepatic and renal toxicity, as well as haematological and biochemical changes (142). Recognising the need for safer alternatives, researchers focused on organic vanadium compounds such as BMOV, which demonstrated improved safety profiles and enhanced absorbability (143,144).

1.2.12 Transport of vanadium compounds across membranes

Vanadium species are primarily absorbed through two mechanisms, with passive diffusion being the most common. The process follows Fick's law of diffusion, whereby molecules move along a concentration gradient from higher to lower concentrations until equilibrium is achieved. The nature of the compound affects this process, with hydrophilic compounds typically diffuse through aqueous pores in the cell membrane, while larger compounds or those bound to proteins like transferrin and albumin, which vanadium attaches to, cannot pass through these pores (124). In contrast, lipophilic compounds undergo lipid diffusion, passing through lipid barriers such as

cell membranes, which cover a greater area and facilitate the uptake of lipophilic molecules over hydrophilic ones (145). Additionally, vanadium species can enter cells through various channels and transporters. These include phosphate or sulphate channels, membrane citrate transporters, lactate transporters (monocarboxylate transporter, MCT1), and organic anion transporters (OCT) (146). Active absorption also occurs by recognising specific carrier proteins (such as transferrin, albumin, and IgG) by cell surface receptors, followed by endocytosis into the cells (147). Subsequently, protein pumps acidify the intravesicular environment, releasing vanadium species into the cytoplasm. This process likely involves the divalent metal transporter-1 (DMT1) (148).

Inorganic vanadium salts, such as sodium metavanadate, typically exhibit high solubility in water. However, absorption of vanadium salts in the gastrointestinal tract is poor and is reported not to exceed 2% in humans (149). The poor absorption of vanadium salts is attributed to the hydrophilic nature of salts. While this hydrophilicity grants them high solubility, it impedes their permeation through the cell membrane. Conversely, lipophilic organic compounds can easily penetrate cell membranes, potentially enhancing absorption. In a study by Reul et al., the efficacy of organic vanadium compounds was compared against oxovanadium(IV) sulphate, an inorganic vanadium salt. The most notable organic vanadium compound was oxovanadium(IV) acetylacetonate (VAc), which resulted in a plasma vanadium concentration of more than double that of vanadium sulphate (150). The authors concluded that the observed difference is attributed to the greater gastrointestinal absorption of VAc compared to vanadium sulphate. Interestingly, the study also found that rats treated with oxovanadium(IV) sulphate presented the greatest urinary excretion rate of vanadium compared to the organic compounds tested in the study. However, the increased urinary excretion rate in rats treated with vanadium sulphate may be due to the increased urine output in these rats, which could have affected the excretion dynamics. Another compound from the same study showed similar activity and potency to VAc was oxovanadium(IV) ethyl acetylacetonate (VEt), which was only moderately soluble in an aqueous solution and required sonication to obtain a complete dissolution, which was expected. Increasing the lipophilicity of the compounds is accompanied by a drastic decrease in the aqueous solubility; However, this is well compensated by the increase in the absorption of the compounds. Studies have demonstrated that organic vanadium compounds primarily influence absorption, tissue uptake, and tissue distribution (151). In a study by Peters et al., it was noted

that for Bis(maltolato)oxovanadium(IV), the organic ligand plays no role in the enzyme PTP1B except as a delivery vehicle for orthovanadate to PTP1B (152).

1.2.13 Vanadium complexes with organic ligands

Developing organic vanadium complexes has been a crucial advancement in research, aiming to address the toxic side effects associated with inorganic vanadium salts. The therapeutic potential of vanadium salts has been hindered by their toxicity, prompting efforts to use the salts at the minimum therapeutic concentration to limit the potential for toxicity. Vanadium, primarily oxovanadium(IV), permeates cells and undergoes intracellular conversion by glutathione to orthovanadate through oxidation (153). Both oxovanadium(IV) and orthovanadate exhibit biological activity. However, providing orthovanadate instead of oxovanadium(IV) theoretically spares mammalian cells the effort of converting oxovanadium(IV) into orthovanadate. Despite this advantage, oxovanadium(IV) is poorly absorbed in the gastrointestinal tract, resulting in limited bioavailability to body cells. Due to the inadequate absorption of oxovanadium(IV), various organic chelators of vanadium have been employed to bind oxovanadium(IV), encapsulating it for transport via the hydrophobic surface of biological membranes (154).

A review by Badmaev et al., in 1999 highlighted that organic vanadium compounds exhibited a therapeutic effect up to 50% greater than their inorganic counterparts (154). In one of the studies cited in this review, conducted by Yeun et al., (10) it was observed that to lower plasma glucose concentration in streptozotocin-diabetic rats, the effective therapeutic BMOV dose was 0.445 mmol/kg, compared to 0.55 to 0.64 mmol/kg for oxovanadium(IV) sulphate, as noted in a study by Ramanadham et al. (155). Additionally, Yeun et al., pointed out that the maintenance dose required to sustain the therapeutic effect for BMOV was almost half that required for oxovanadium(IV) sulphate, at 0.18 mmol/kg compared to 0.4 mmol/kg, respectively. Another study supporting the effectiveness of BMOV over inorganic vanadium was conducted by Domingo et al. (156), where they observed a similar required effective dose for BMOV. In contrast, the effective doses for sodium metavanadate and sodium orthovanadate ranged from 0.26 to 1.02 mmol/kg and from 0.20 to 0.65 mmol/kg, respectively (154).

The superiority of organic vanadium compounds is attributed to their enhanced gastrointestinal absorption, a feature not commonly observed with inorganic vanadium compounds. The improved absorption profile enables organic vanadium compounds to exert optimal biological effects while requiring lower doses than their inorganic counterparts. Consequently, administering lower doses reduces the risk of gastrointestinal side effects, which are commonly associated with inorganic vanadium compounds, due to the necessity of higher doses for therapeutic efficacy. The accumulation of unabsorbed inorganic vanadium in the gastrointestinal tract often leads to side effects such as nausea, diarrhoea, and cramps. Therefore, the enhanced absorption of organic vanadium compounds offers a more promising avenue for developing vanadium-based therapies with reduced side effects (154). BMOV has shown promise in mitigating the toxic side effects that are commonly associated with inorganic vanadium salts, making it the standard in investigating insulin-mimetic activity amongst other vanadium compounds in several studies as it has undergone extensive preclinical testing for its safety and efficacy (157,158). The favourable characteristics of BMOV and positive outcomes in studies make it an essential compound for researchers exploring the therapeutic potential of vanadium in the context of DM and related conditions.

The utilisation of BMOV highlights the ongoing efforts to develop safer and more effective vanadium-based therapies. Further, the promising characteristics of BMOV and its analogues, such as Bis(ethylmaltolato)oxovanadium(IV) BEOV, are evidenced by advancements from preclinical testing to phase II clinical trials. In Phase I, clinical trials for BEOV were conducted by Medieval, Ltd in Manchester, United Kingdom, under the auspices of Kinetek Pharmaceuticals in Vancouver and Canada (8). The results showed no adverse effects, and all biochemical parameters remained within normal limits. In the phase II trial carried out by Akesis Pharmaceuticals in La Jolla, California, United States of America 20 mg of BEOV was administered daily for 28 days to seven patients with T2DM (159). The findings revealed reductions in fasting blood glucose concentrations and improved responses to oral glucose tolerance testing, as well as a decrease in %HbA1c, a key marker for long-term glycaemic control that reflects the average blood glucose levels over the past two to three months, compared to worsening diabetic symptoms observed in two placebo controls.

These trials also provided valuable insights into the safety and efficacy of vanadium complexes (159). The outcomes of these studies have been notably positive, showcasing the potential of BMOV and its analogues as viable candidates for therapeutic applications. The successful progression into clinical trials underscores the commitment to developing safer vanadium-based treatments and highlights the encouraging results observed regarding safety and efficacy. This progress represents a significant step forward in translating research findings into potential real-world therapies for conditions such as DM. Similar to BMOV, another compound of interest is bis(allixinato)oxovanadium(IV) (BAOV), which has demonstrated insulin-mimetic properties. In a study conducted by Hirmura et al., in 2007, the insulin-mimetic activity of BAOV was investigated, and the functional mechanism of this compound was explored (160). The study concluded that the mechanism of BAOV involves the activation of the insulin signalling pathway through the enhancement of the phosphorylation of the insulin receptor.

Another vanadium complex of interest is bis(picolinato)oxovanadium(IV) (BPOV) studied by Gatjens et al., in 2003, the researchers investigated the insulin-mimetic activity of BPOV, $VOSO_4$, and BMOV. The study evaluated their mode of action by examining the release of free fatty acids from adipocytes *in vitro* in the presence of various inhibitors related to glucose metabolism. It was found that the inhibition of FFA release by these compounds was reversed with the addition of specific inhibitors, such as cytochalasin B (GLUT4 inhibitor), cilostamide (phosphodiesterase inhibitor), HNMPA-(AM)3 (tyrosine kinase inhibitor), and wortmannin (PI3-k inhibitor) (161). These findings suggest that BPOV and other vanadyl compounds primarily affect GLUT4 and phosphodiesterase, proposing a multi-target mechanism of glucose regulation by these compounds.

In a 2006 study exploring the effects of oxovanadium(IV)–picolinate complexes on insulin signalling by Basuki et al., researchers investigated compounds BPOV, bis(3-methylpicolinato)oxovanadium(IV), and bis(6-methylpicolinato)oxovanadium(IV) in 3T3-L1 adipocytes. Among these, bis(3-methylpicolinato)oxovanadium(IV) emerged as the most potent activator, significantly increasing the phosphotyrosine levels of both insulin receptor and IRS and downstream activation of kinases such as Akt and Glycogen synthase kinase-3 β (GSK3β) (162). This activation facilitated the translocation of GLUT4 to the plasma membrane, which is

crucial for glucose uptake. Further experiments demonstrated that bis(3-methylpicolinato)oxovanadium(IV) [$VO(3\text{-mpa})_2$] exhibited a notable hypoglycaemic effect in STZ-induced diabetic mice, surpassing the efficacy of the other complexes. These findings suggest that oxovanadium(IV) complexes, particularly bis(3-methylpicolinato)oxovanadium(IV) [$VO(3\text{-mpa})_2$], improve hyperglycaemia by activating the insulin signalling pathway and enhancing glucose utilization in diabetic animals (162).

Other organic vanadium compounds among the diverse array of organic vanadium complexes include Vanadyl Acetylacetonate (VAc) and Vanadyl Ethylacetonate (VEt). stand out for their potency and safety in correcting hyperglycaemia and impaired hepatic glycolysis in diabetic rats when compared to inorganic vanadium salts (150). These promising results with organic vanadium complexes underscore the potential for further exploration of vanadium-based therapeutics, particularly Schiff-base vanadium complexes, which offer unique structural advantages and enhanced biological activity, paving the way for their potential application in DM management.

1.2.14 Schiff bases in pharmaceutical research

Schiff bases, characterized by an azomethine group (-CH=N-), represent another class of organic compounds with significant implications in the pharmaceutical industry. A Schiff base is obtained by condensing primary amines and carbonyl compounds (163). It is a ketone or aldehyde analogue having an imine or azomethine in place of a carbonyl group, which is formed when the carbonyl group of the carbonyl compound reacts with the amine group of the primary amine. The structure has a double bond between carbon and nitrogen (C=N), with an aryl or alkyl group attached to the nitrogen atom (164). The structure of a Schiff base is shown in Figure 1.12 below:

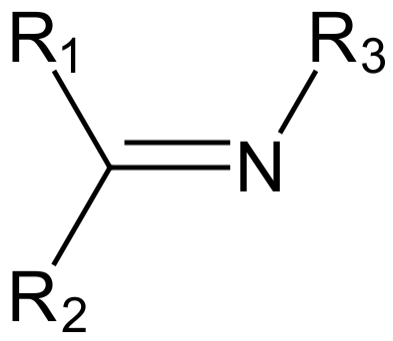


Figure 1.12: General structure of a Schiff base, where R1, R2, and/or R3 represent alkyl or aryl groups

These versatile ligands can form stable complexes with metal ions, particularly transition metals. Schiff base metal complexes have demonstrated the potential to enhance drug absorption and bioavailability (165). This class of compounds possess a range of pharmaceutical applications, including antimicrobial and antioxidant properties, making them valuable in drug design (166). The lipophilicity of Schiff bases contributes to their ability to enhance absorption and improve the stability of molecules. Schiff bases are promising components in developing novel pharmaceutical formulations due to their versatility and possession of donor atoms to bind with metals or metal ions. They stand out as unique compounds in the realm of drug discovery due to their remarkable versatility as pharmacophores, a fact underscored by a comprehensive review conducted by Kajal et al., (166). Notably, Schiff bases have been investigated for their potential as therapeutic agents across a spectrum of medical conditions, including but not limited to inflammation, pain, bacterial infections, epilepsy, tuberculosis, cancer, cell damage due to free radicals, parasitic infections, and depression. Moreover, the scope of their biological effects suggests promising avenues for further exploration and optimization, paving the way for discovering new drug candidates for treating these conditions.

Additionally, Schiff bases possess donor atoms, namely nitrogen and oxygen atoms, which can coordinate with metal ions to form stable complexes. These complexes exhibit unique chemical and physical properties, making them valuable in drug discovery. Metal complexes with Schiff base ligands can be designed to exhibit enhanced solubility, stability, and bioavailability

compared to free metal ions, thereby leading to improved therapeutic outcomes (13). Schiff base metal complexes offer several advantages. Firstly, they exhibit selectivity, allowing for the synthesizing of compounds targeted for delivery to specific locations and binding to specific receptors (167). Secondly, these compounds are modifiable, meaning their chemical structure can be altered to enhance various properties, such as stability and lipophilicity, which improves absorption through cell walls (168). Furthermore, Schiff base metal complexes demonstrate enhanced biological activities, including antimicrobial, antiviral, anticancer, antioxidant, and cardioprotective properties (166).

1.2.15 Exploring Schiff base vanadium complexes

Schiff bases have played a crucial role in the development of vanadium complexes due to their ability to impart structural features that bring desirable properties, such as improved absorption and stability, to the overall complex. This makes Schiff bases valuable ligands in the design and synthesis of vanadium-based therapeutic agents (169). Rehder et al., (2001) investigated the *in vitro* toxicity and insulin-mimetic behaviour of organic vanadium (IV, V) coordination compounds, yielding intriguing insights (170). Through a trypan blue exclusion assay, they observed that cytotoxicity increased with prolonged exposure to vanadium compounds but decreased with lower concentrations. Additionally, compounds with thio functional groups exhibited greater toxicity. Notably, only VO(H_2O)[N-2-oxido-3-methoxysalicylidene)-histidyl-serine (2–)] (VO(van-Hs)), a vanadium(V) complex containing a Schiff base ligand composed of o-vanillin and the dipeptide histidine-serine, showed non-toxicity at the highest concentration tested. Another significant finding was that ON ligation demonstrated superior insulin mimetic efficacy compared to OO or O/NS coordination, laying the groundwork for further research on Schiff-base vanadium complexes in the potential treatment of DM.

Previous work by Nejo et al., in 2009 focused on synthesizing, characterizing, and investigating the insulin-enhancing properties of various vanadium complexes with Schiff base ligands. A series of oxovanadium(IV) symmetrical tetradentate Schiff base complexes were isolated from the reaction of $VOSO_4$ with Schiff bases obtained from the condensation of 2-hydroxybenzophenone or 2-hydroxy-5-chlorosalicylaldehyde with various aliphatic diamines.

These complexes, part of a series of oxovanadium(IV) symmetrical tetradentates, demonstrated notable insulin-enhancing properties. *In vitro* studies showed that two of the compounds bis(2-hydroxybenzophenoneethylenediamine)oxovanadium(IV) ([VO(bp_2-en)]) and bis(2-hydroxybenzophenonetriethylenetetramine)oxovanadium(IV) methanol solvate ([VO(bp_2-tn)MeOH]) significantly increased glucose uptake compared to the basal glucose uptake in transformed and sensitized C2C12 skeletal muscle cells (171). In a 2010 study by Xie et al., they revealed the synthesis of two novel vanadium complexes using Schiff bases of substituted salicylaldehyde and 2-hydroxyethylenediamine (172). The IR spectra described these as binuclear complexes of six centres of coordinated vanadium, bridged by O-O atoms of homocitrate with a V_2O_2 diamond core. *In vivo* tests showed that one of the complexes, $[VO(\mu–O)(5\text{-Cl-salen-}OC_2H_5)]_2$ complex (II), exhibited remarkable antidiabetic activity by lowering the blood glucose concentration and improving glucose tolerance in diabetic rats. This result demonstrated that the ligand with a halogen atom enhanced the antidiabetic characteristics of vanadium complexes with the Schiff base. The other complex, $[VO(\mu–O)(\text{Salen-}OC_2H_5)]_2$ without the halogen substituent, however, showed no antidiabetic activity.

A subsequent study by Li et al., in 2015 continued this line of research. The authors synthesized and characterized two complexes, 2-hydroxy-N′-(4-oxopentan-2-ylidene) benzohydrazide 8-hydroxyquinoline vanadate and 4-bromo-N′-(4-oxo-pentan-2-ylidene) benzohydrazide 8-hydroxyquinoline vanadate (173). The study reported insulin mimetic activity from both compounds when tested on C2C12 skeletal muscle cells, utilizing a glucose uptake assay indicating the complexes significantly simulated glucose utilization similar to the reference drugs insulin and metformin. Using an MTT assay, the group reported the compounds to have relatively low cytotoxicity.

In 2016, Ki et al., conducted an interesting study focusing on the synthesis of oxovanadium(IV) Schiff base complexes containing a tryptamine moiety (174). The choice of the ligand was based on the fact that oxovanadium complexes possess insulin-mimetic activity, and the tryptamine moiety offers anti-inflammatory potential, targeting inflammation, which is believed to be a primary cause of T2DM. Using total internal reflection fluorescence microscopy to quantify GLUT4 translocation in human embryonic kidney 293 cells treated with the Schiff base

vanadium complex, the researchers observed that the complex effectively acted as an insulin-mimetic substance by promoting the expression of GLUT4. Furthermore, the insulin-mimetic activity of this compound was corroborated through a high cell count-based assay using quantum dot-antibody conjugates, demonstrating the ability of the compound to restore insulin signalling at the insulin receptor by inactivating Jun N-terminal kinase-1 (JNK-1), followed by phosphorylation of the receptor and activation of the tyrosine moiety of the insulin receptor substrate. The anti-inflammatory potential of the compound was evidenced by high levels of phosphorylated forkhead box 01 (FOXO). Notably, one of the key observations from this study was that the compound exhibited high effectiveness in the nanomolar treatment ranges, thereby circumventing issues of toxicity.

In 2017, Saied et al., synthesized a series of Schiff base oxovanadium(IV) complexes derived from acetohydrazide or 4-aminoantipyrine (175). They then administered these compounds to streptozocin-induced diabetic rats and observed a significant improvement in blood glucose homeostasis. Additionally, the complexes were found to modulate oxidative stress associated with DM. Notably, the antidiabetic activity of the vanadium complexes was found to be structure-dependent, particularly for (VO)(N¢-(2-hydroxybenzylidene)-2-(phenylamino)acetohydrazide)(SO_4), (VO)(2-cyano-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4yl)-2-(m-tolyldiazenyl)acetamide)(SO_4)]·4H_2O, and (VO)(2-cyano-N¢-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)methylene)-2-(phenyldiazenyl) acetohydrazonic)(SO_4) (175). In a 2019 study by Patel et al., the researchers reported vanadium (+4 or +5) complexes with the Schiff base and imidazole as co-ligands, such as [Oxovanadium(IV) acetic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide (H_2O)]NO_3 (complex I), [Oxovanadium(IV) 5-bromo-2-[(E)-(pyridine-2-ylhydrazono)methyl]phenol (H_2O)]NO_3 (complex II), and [Oxovanadium(IV) 5-bromo-2-[(E)-(pyridine-2-ylhydrazono)methyl]phenol (imidazole)$_2$]SO_4·H_2O (complex III) exhibited antidiabetic activity (176). The antidiabetic studies performed showed that complex I and complex II demonstrated significant enzyme inhibition activities. For α-glucosidase inhibition, complex I had the lowest IC_{50} value of 432 µg/ml, indicating the highest effectiveness, while complex II had the highest IC_{50} value. For α-amylase inhibition, complex I again showed the lowest IC_{50} value of 122.28 µg/ml, demonstrating the most potent inhibition.

In 2020, Patel et al., extended their research on vanadium complexes by synthesizing two new oxovanadium(V) Schiff base complexes, namely [oxovanadium(V) Nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide imidazole] and [oxovanadium(V) Nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide methylimidazole], incorporating the tridentate ligand nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide and imidazole's (177). These complexes were investigated for their antidiabetic properties, particularly focusing on their ability to inhibit α-amylase and α-glucosidase, enzymes relevant to insulin regulation. The study revealed that [oxovanadium(V) Nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide imidazole] exhibited the most potent *in vitro* α-amylase inhibition activity, with the lowest IC_{50} value of 23.669 µg/ml, compared to [oxovanadium(V) Nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide methylimidazole]. Additionally, the antioxidant potential of these complexes was assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, with IC_{50} values for complex 1 and complex 2 being 422.579 and 441.396 µg/ml, respectively, demonstrating a promising capacity for free radical scavenging. The β-glucosidase inhibition assay further supported the antidiabetic potential with IC_{50} values of 282.050 and 2021.770 for [oxovanadium(V) Nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide imidazole] and [oxovanadium(V) Nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide methylimidazole], respectively, indicating significant enzyme inhibition. These findings suggest that the newly synthesized oxovanadium(V) Schiff base complexes hold considerable promise as therapeutic agents for managing DM through dual mechanisms of enzyme inhibition and antioxidant activity.

In 2020, a study by Szklarzewicz et al., revealed 24 oxovanadium (IV, V) complexes with Schiff base ligands were synthesized and characterized (178). These ligands were derived from 5-nitrosalicylaldehyde, 5-methoxysalicylaldehyde, or 5-sulfosalicylaldehyde and their respective hydrazides. Biological activity assays were conducted, focusing on inhibiting human tyrosine phosphatases in myocyte C2C12, adipocyte 3T3-L1, and human hepatocyte HepG2 cell lines. The study found that although some synthesized complexes were unstable, they exhibited significant biological activity. Notably, [oxovanadium(IV) 5-nitrosalicylaldehyde phenanthroline]·$1.5H_2O$ (compound 3) and [oxovanadium(IV) 5-nitrosalicylaldehyde

bipyridine]·0.5EtOH·0.5H_2O (compound 2), which differ only in their co-ligands (phen or bpy), demonstrated distinct activities: complex 3 with the rigid phen ligand showed similar activity to BMOV, while complex 2 with the flexible bpy ligand exhibited greater activity and stability. This indicates that the type of ligand influences biological activity, with some V(IV) complexes with ONO ligands being three times more effective than BMOV and human insulin. Table 1 below shows an overview of the vanadium complexes, pharmacological activity, and safety of the vanadium complexes covered in this review, where it can be observed that acetone and Schiff base vanadium compounds have better safety profiles than other vanadium derivatives.

Table 1: Overview of Schiff base vanadium compounds in the treatment of DM

Vanadium Compound	**Activity and Safety**	**Citation**
VO(H_2O)[N-2-oxido-3-methoxysalicylidene)-histidyl-serine (2–)] (VO(van-Hs))	✓ Non-toxic at the highest concentration tested ✓ Superior insulin mimetic efficacy with ON ligation compared to OO or O/NS coordination	(170)
oxovanadium(IV) bis(2-hydroxybenzophenone ethylenediamine) ([VO(bp2-en)])	✓ Significantly increased glucose uptake in transformed and sensitized C2C12 muscle cells	(171)
oxovanadium(IV) bis(2-hydroxybenzophenone triethylenetetramine) methanol solvate VO(bp2-tn)MeOH	✓ Significantly increased glucose uptake in transformed and sensitized C2C12 muscle cells	(171)
[VO(μ–O)(Salen-OC_2H_5)]$_2$	✗ No antidiabetic activity observed	(172)
[VO(μ–O)(5-Cl-salen-OC_2H_5)]$_2$	✓ Remarkable antidiabetic activity by lowering blood glucose levels and	(172)

	improving glucose tolerance in diabetic rats ✓ Chloro substituent increased the insulin-enhancing properties ✓ Chlorine atom at C4 of complex increased cytotoxicity	
Oxovanadium(V) 2-hydroxy-N′-(4-oxopentan-2-ylidene) benzohydrazide 8-hydroxyquinoline complex (I)	✓ Simulated glucose utilization ✓ Low cytotoxicity	(173)
Oxovanadium(V) 4-bromo-N′-(4-oxo-pentan-2-ylidene) benzohydrazide 8-hydroxyquinoline complex (II)	✓ Simulated glucose utilization ✓ Low cytotoxicity	(173)
bis(pyridoxylidenetryptamine) vanadium(IV) complex (VOTP)	✓ Promoted GLUT4 translocation in 3T3-L1 cells ✓ Anti-inflammatory ✓ Effective in nanomolar treatment ranges	(174)
(VO)((2-hydroxybenzylidene)-2-(phenylamino)acetohydrazideHL_3)(SO_4)	✓ Significant improvement in blood glucose homeostasis in diabetic rats ✓ Modulated oxidative stress associated with DM	(175)
(VO)(2-cyanoN-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4yl)-2-(m-tolyldiazenyl)acetamide)SO_4	✓ Significant improvement in blood glucose homeostasis in diabetic rats ✓ Modulated oxidative stress associated with DM	(175)
(VO)(2-cyano-N`-((1,5-dimethyl-3oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)methylene)-2(phenyldiazenyl)acetohydrazonic)$_2$	✓ Significant improvement in blood glucose homeostasis in diabetic rats ✓ Modulated oxidative stress associated with DM	(175)
(VO)(2-cyano-N-(1,5-dimethyl-3-oxo-	✓ Significant improvement in blood	(175)

2-phenyl-2,3-dihydro-1H-pyrazol-4yl)-2-(m-tolyldiazenyl)acetamide)(SO_4)]·4H_2O	glucose homeostasis in diabetic rats ✓ Modulated oxidative stress associated with DM	
(VO)(2-cyano-N`-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)methylene)-2-(phenyldiazenyl) acetohydrazonic)(SO_4)	✓ Significant improvement in blood glucose homeostasis in diabetic rats ✓ Modulated oxidative stress associated with DM	(175)
[Oxovanadium(IV) acetic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide (H2O)]NO_3	✓ α-glucosidase inhibition activity ✓ α-amylase inhibition activity ✓ Structure dependant activity ✓ Most potent in the study	(176)
[Oxovanadium(IV) 5-bromo-2-[(E)-(pyridine-2-ylhydrazono)methyl]phenol (H2O)]NO_3	✓ α-glucosidase inhibition activity ✓ α-amylase inhibition activity ✓ Structure dependant activity	(176)
[Oxovanadium(IV) 5-bromo-2-[(E)-(pyridine-2-ylhydrazono)methyl]phenol ($ImH)_2$]SO4·H_2O	✓ α-glucosidase inhibition activity ✓ α-amylase inhibition activity ✓ Structure dependant activity	(176)
[Oxidovanadium(V) Nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide imidazole]	✓ ß-glucosidase inhibition activity ✓ α-amylase inhibition activity ✓ DPPH scavenging activity ✓ Structure dependant activity	(177)
[Oxidovanadium(V) Nicotinic acid (2-hydroxy-3-methoxy-benzylidene)-hydrazide methylimidazole]	✓ ß-glucosidase inhibition activity ✓ α-amylase inhibition activity ✓ DPPH scavenging activity ✓ Structure dependant activity	(177)

1.3 Study Rationale

This study was undertaken to address critical gaps in the development of alternative therapeutic agents for the treatment of DM that may safer and more effective. With the challenges of current treatment options, such as suboptimal efficacy, high costs and adverse effects. Schiff base vanadium complexes, as investigated in this study, represent novel compounds with significant therapeutic potential due to their apparent insulin-mimetic properties and improved safety profiles compared to existing vanadium-based potential therapeutic agents. Therefore, this study aims to contribute new knowledge to the field by investigating the biological effects of the Schiff base vanadium complexes on glucose uptake, cytotoxicity, and inflammation. Furthermore, by examining these biological activities, the study contributes to a broader landscape of therapeutic innovation. Specifically, the findings may inform early-stage drug development and optimization strategies, supporting the long-term goal of advancing these compounds towards regulatory evaluation and clinical application in DM therapy

1.4 Objectives of the study

a) *In vitro* screening of vanadium compounds on cell viability in:
    i) Skeletal muscle cell line (C2C12)
    ii) Hepatic cell line (HepG2)
b) *In vitro* screening of vanadium compounds on glucose utilisation in:
    i) Skeletal muscle cell line (C2C12)
    ii) Hepatic cell line (HepG2)
c) *In vitro* screening of vanadium compounds to determine the expression and translocation of GLUT4 in:
    i) Skeletal muscle cell line (C2C12)
d) *In vitro* screening of vanadium compounds on expression of Akt, IL-6, MMP1 and DPP4 in:
    i) Skeletal muscle cell line (C2C12)
    ii) Hepatic cell line (HepG2)

e) *In vitro* screening of vanadium compounds on concentration of IL-6, MMP1 and DPP4 in:
    i) Skeletal muscle cell line (C2C12) Media
    ii) Hepatic cell line (HepG2) Media
f) *In vitro* screening of vanadium compounds for citrate synthase activity in:
    i) Skeletal muscle cell line (C2C12)
    ii) Hepatic cell line (HepG2)
g) *In vitro* screening of vanadium compounds for media pH in:
    i) Skeletal muscle cell line (C2C12)
    ii) Hepatic cell line (HepG2)
h) *In silico* screening of vanadium compounds for interactions with:
    i) PTP1B

Chapter 2 : Materials and Methodology

2.1 Chemicals, equipment and software

2.1.1 Chemicals

The chemicals and reagents were purchased from Sigma-Aldrich, St Louis, USA: Dulbecco's modified eagle's medium, eagle's minimum essential medium, foetal bovine serum, penicillin-streptomycin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, human insulin, bovine serum albumin, triton X-100, rabbit polyclonal anti-phospho-Akt antibody, anti-Akt2 antibody produced in rabbit, GLUT4 antibody, MMP1 antibody, DPP4 antibody, IL-6 antibody, citrate, sodium phosphate dibasic, monopotassium phosphate, sodium chloride, potassium chloride, potassium iodide, hydrochloric acid, sodium acetate, sodium orthovanadate, paraformaldehyde, mannitol, sucrose, acetyl-coenzyme A, 5,5′-dithiobis-(2-nitrobenzoic acid), 2-amino-2-(hydroxymethyl)-1,3-propanediol, trypsin-EDTA, paraformaldehyde, oxaloacetic acid, bovine serum albumin, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, tween 80 and horseradish peroxidase.

The Medicinal Chemistry group led by Prof. SD. Khanye from Rhodes University synthesised and provided the three Schiff base vanadium complexes. Briefly, the insoluble ligands were used without isolation by adding the vanadium salt and refluxing the resulting mixture for ca. 2h. The preparative procedure for the complexes of Schiff base SB-1: ((3-bromophenylimino)methyl)-4-nitrophenol.SB-2: ((3-(trifluoromethyl)phenylimino)methyl)-4-nitrophenol is as follows. (SB) no. 1 is representative. To a solution of 5-bromo-2-benzaldehyde (0.201 g, 1.00 mmol) in hot ethanol (10.0 ml), 3-nitrobenzenamine (0.138 g, 1.00 mmol) was slowly added. An orange precipitate was formed immediately. The reaction was then neutralized by the addition of sodium

acetate trihydrate (0.15 g, 1.10 mmol), and the mixture was stirred for 15 min. $VOSO_4 \bullet 4H_2O$ (0.12 g, 0.5 mmol) dissolved in 5.0 ml of water was then added dropwise to the suspension, and the mixture was refluxed for ca. 2h. After the mixture was cooled to 25 °C, light brown (for vanadium complex 1), beige (for vanadium complex 2), and brown (for vanadium complex 3) precipitates were collected by filtration, washed twice with water and ethanol, and dried in vacuum at 70 degrees Celsius overnight. The isolated yields were 71.1, 67.2, and 76.5%, respectively. All the oxovanadium (IV) Schiff base complexes were recrystallized from approx. 10 ml of dichloromethane-ethanol (1:1 vol) mixture.

The three synthesized Schiff base vanadium complexes are characterized by the presence of an azothemine group (-CH=N-), which is highlighted in red in the molecular structures below. The synthesized complexes have a structure where the Schiff base ligands are linked to the vanadium(IV) coordination sphere. Complexes 1 and 2 are stereoisomers which only differ in the positioning of the bromine (Br) and Nitro (NO_2) groups, whilst complex 3 features trifluoromethyl (CF_3) groups in place of bromine, setting it apart from complexes 1 and 2. The molecular structure of the Schiff base vanadium complexes 1, 2 and 3 are shown in Figures 2.1, 2.2, and 2.3, respectively.

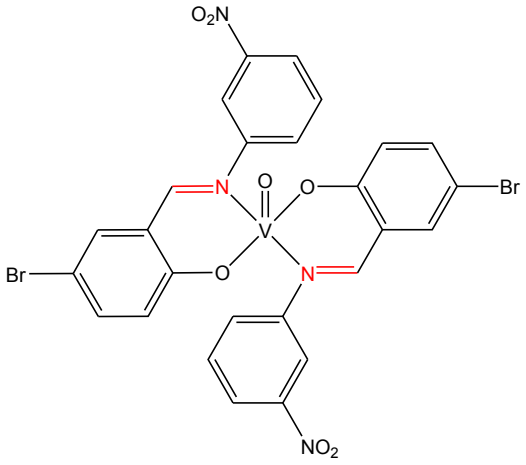


Figure 2.1: The molecular structure of vanadium complex 1 ($C_{26}H_{16}Br_2N_4O_7V$, 707 g/mol)

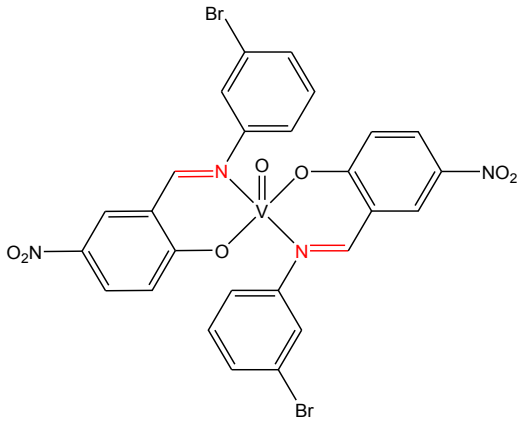


Figure 2.2: The molecular structure of vanadium complex 2 ($C_{26}H_{16}Br_2N_4O_7V$, 707 g/mol)

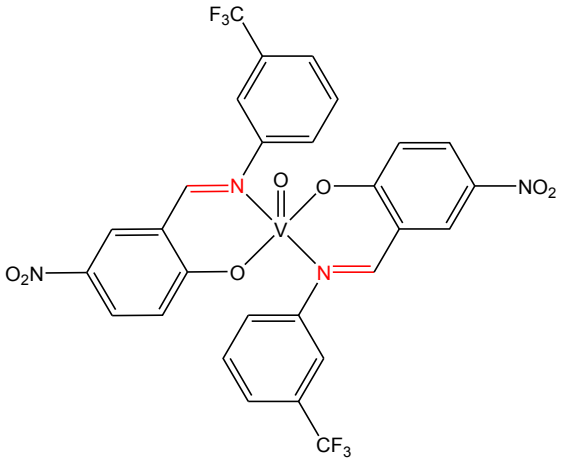


Figure 2.3: The molecular structure of vanadium complex 3 ($C_{28}H_{16}F_6N_4O_7V$, 685 g/mol)

These are referred to as Complex 1, complex 2, and Complex 3, respectively. Each vanadium complex came as dry powder.

2.1.2 Equipment

The equipment was made available by the Rhodes University Endocrinology Research Lab (RUERL): vortex mixer (Edinson, New Jersey, United States, laminar airflow hood (Vivid Air, Durban, South Africa), microplate shaker (Thermo Fischer Scientific, New Hampshire, United States), pH meter (Lasec, Cape Town, South Africa), Bio-Rad ® automated cell counter (Lasec, Cape Town, South Africa), Heal Force ® Incubator (Heal Force Group, Shanghai, China),

Inverted Microscope (Best Scope, Beijing, China), Contour Plus ® glucometer (Ascensia Diabetes Care, Basel, Switzerland), Contour Plus ® test strips (Ascensia Diabetes Care, Basel, Switzerland), RT 2100-C ® Microplate reader (COMECTA S.A Optic Ivymen System, Barcelona Spain) and a BioTek Epoch ® microplate absorbance reader (Agilent Technologies, California, United States).

2.1.3 Software

GraphPad Prism (Version 10.4.0; GraphPad Software Inc. California, United States) was used for statistical analysis and data visualization.

2.2 Searching for references

To ensure a comprehensive, structured, and balanced understanding of the existing literature, a narrative literature review was conducted. A narrative literature review synthesizes the results of individual studies (both qualitative and quantitative) without focusing strictly on statistical significance, and instead aims to provide conceptual and contextual insights (179). This approach was appropriate for the present study, as the literature on vanadium and its insulin mimetic effects involves a wide range of experimental designs and endpoints. In such cases, purely quantitative synthesis may overlook important mechanistic or theoretical developments (180).

A systematic search strategy was employed to minimize selection bias. Searches were conducted across major academic databases, including PubMed, Scopus, and Google Scholar. Keywords such as “Vanadium”, “Diabetes mellitus”, “Insulin-mimetics”, “Vanadium complexes”, and “Schiff bases”, combined using Boolean operators (AND, OR, NOT), were used to refine results and retrieve relevant literature.

To further improve the comprehensiveness of the review, reference chaining (examining the reference list of key articles) was also employed. This iterative process allowed the inclusion fo additional studies that may not have appeared in the initial search results, ensuring broader coverage and deeper understanding of the field.

2.3 Preparation of compounds

Stock solutions of complexes 1 – 3 and sodium orthovanadate (Na_3VO_4) with a concentration of 1000 µg/ml were prepared by weighing out 0,001g of each compound and then dissolved in 0.1ml DMSO to which cell media was added up to 1ml to make a concentration of 1000 µg/ml. The compounds were further diluted with the required media (DMEM) to freshly prepare the desired concentrations (20, 10, 5 µg/ml) using Equation 1 below:

Equation 1

2.4 Cell-based assays

2.4.1 Cell culture and differentiation

The culturing of cells was performed in a sterile environment. In separate culture flasks, C2C12, and HepG2 cell lines were maintained in DMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2 environment at 37 °C. The media was changed daily, and the extent of confluence was determined using an inverted microscope to observe the percentage of the flask surface covered by adherent cells. Once the cells reached confluence, the media was discarded, and the flasks were rinsed with warm phosphate buffer saline (PBS) to remove any residual media. Trypsin was then added to the flasks, which were incubated for (2-5) minutes in a 5% CO_2 environment at 37 °C. After the cells detached from the flask surface, media was added to suspend the cells and seeded into the required well plates for assays. Additional media was added back to the flasks to produce the next batch of cells. For C2C12 myoblasts, differentiation into myotubes was induced by changing the medium to DMEM supplemented with 2% FBS and 1% penicillin-streptomycin for four days. The media was changed daily, and the extent of differentiation was assessed using a microscope to observe the formation of multinucleated myotubes. The workflow for the cell culture and preparation of cells for assays is illustrated in Figure 2.4 below:

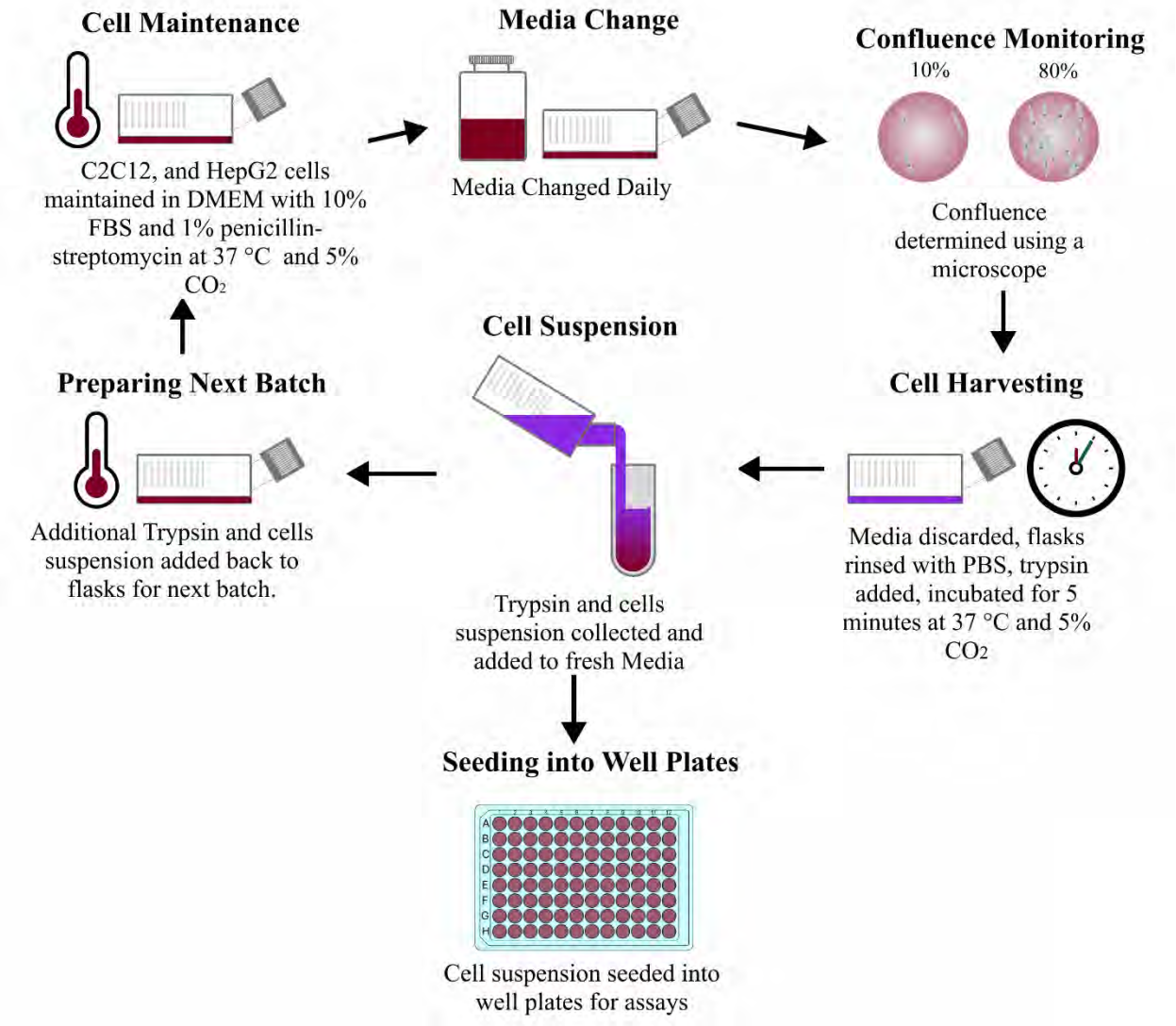


Figure 2.4: Cell culture workflow

2.4.2 Cell treatment

C2C12 and HepG2 cells were seeded in 24 or 96-well plates at a density of approximately 500 cells/well for the 96 well plates and 2000 cells/well. After reaching approximately 80% confluence, C2C12 cells were differentiated into myotubes. Subsequently, both the differentiated C2C12 cells and HepG2 cells were treated with the compounds at desired concentrations (20, 10, 5 µg/ml) in DMEM (for C2C12) and MEM (for HepG2) with untreated wells serving as controls. Insulin at a concentration of 0.05 µg/ml and sodium orthovanadate at concentrations of 20, 10, and 5 µg/ml served as positive controls. The wells containing media only served as a blank. The

plates were incubated at 37 °C for 24 hours. The workflow diagram for the cell treatment for assays is illustrated in Figure 2.5 Below:

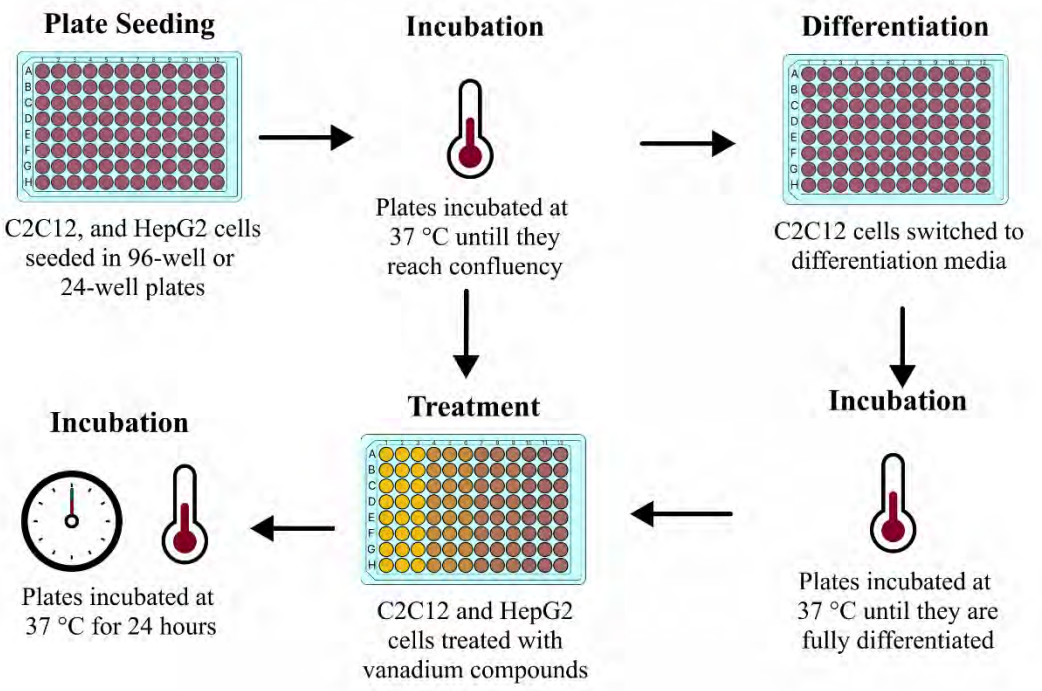
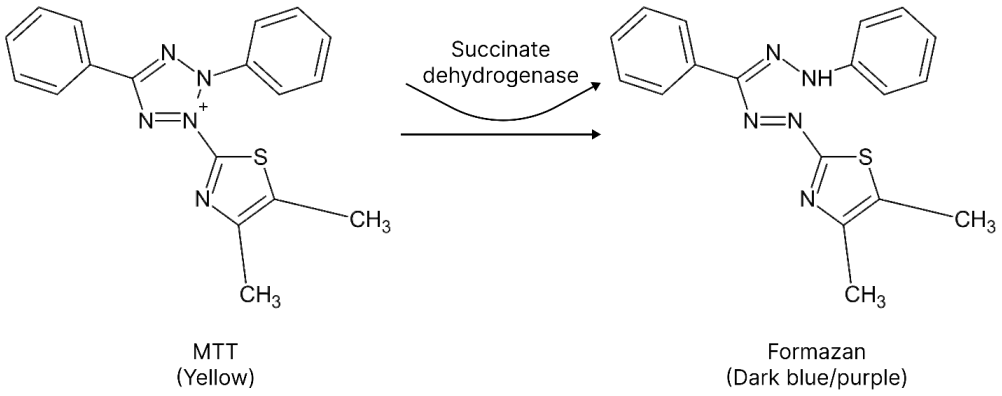


Figure 2.5: Cell treatment workflow

2.4.3 Cell viability assay

The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is employed to assess cell viability. This colorimetric assay measures cellular activity by reducing yellow MTT to purple formazan granules intracellularly. MTT, a mono-tetrazolium salt, features a positively charged quaternary tetrazole ring core with four nitrogen atoms surrounded by three aromatic rings, including a thiazolyl ring and two phenyl moieties. The reduction of MTT occurs at the core tetrazole ring, and this chemical reaction is illustrated in Scheme 2.1. Within the mitochondria of metabolically active cells, MTT is reduced by succinate dehydrogenase, producing lipophilic purple formazan. Due to its lipophilic structure and positive charge, MTT can permeate the cell membrane and mitochondrial inner membrane of viable cells. In contrast,

dead cells lose their ability to convert MTT into formazan. Therefore, the formation of the purple colour indicates the presence of viable cells. The total formazan produced is directly proportional to the number of viable cells, making the colour intensity a direct measure of cell viability. The absorbance is then measured using a spectrophotometer at 570 nm.



Scheme 2.1: The reduction of MTT into formazan (181)

Following treatment with the vanadium compounds, the medium was then aspirated from the 96-well pates containing C2C12 and HepG2 cells, and 200 µl of 5 mg/ml thiazolyl tetrazolium bromide (MTT) dissolved in a mixture of PBS (10%) and FBS-free media (90%) was added into each well. The plates were incubated at 37 °C for 3 hours in the dark. After incubation, the medium was aspirated, and 200 µl of DMSO was added to each well and mixed gently. The plates were incubated for 5 minutes to dissolve the formazan crystals. Subsequently, the plates were read at a wavelength of 550 nm using a microplate reader. The relative percentage viability of the cells was calculated using Equation 2 below:

Equation 2

The relative percentage viability was plotted against the concentrations of compounds. The workflow for the MTT cell viability assay is illustrated in Figure 2.6 below.

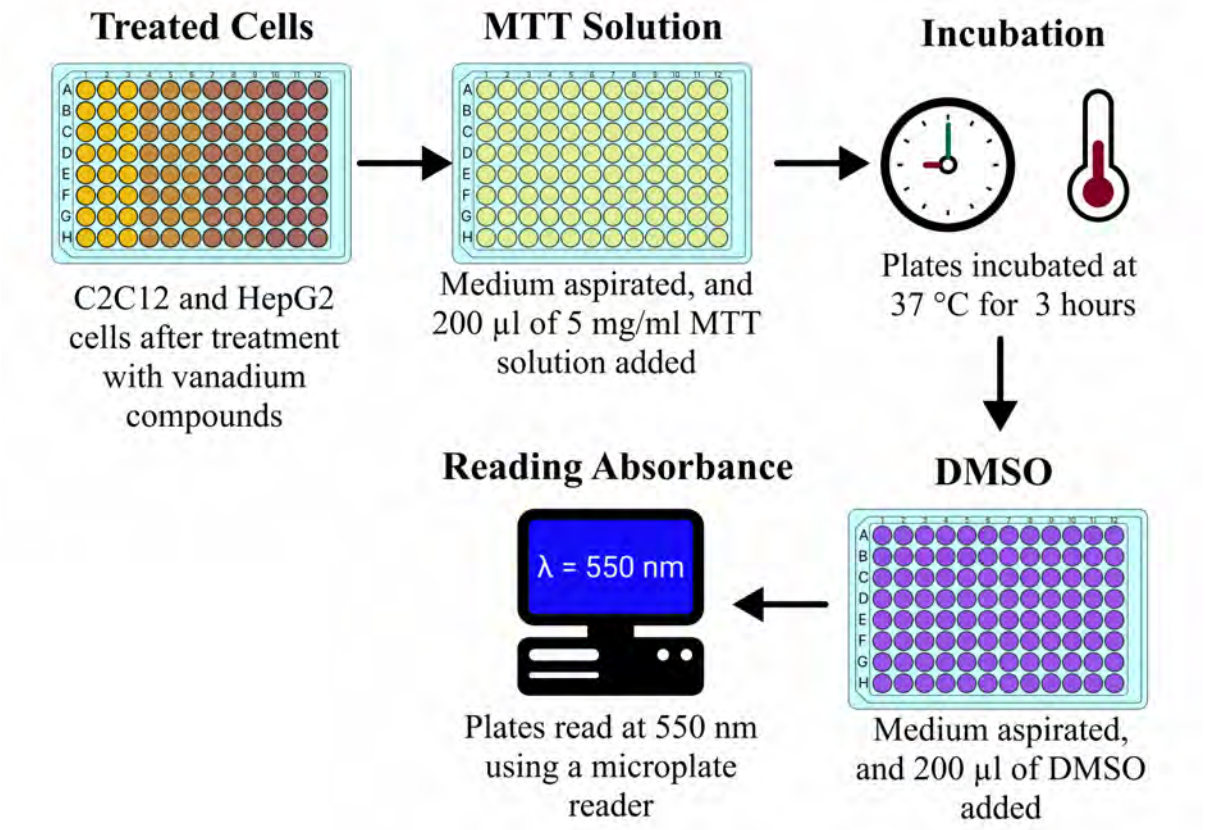


Figure 2.6: MTT cell viability assay workflow

2.4.4 Glucose uptake assay in healthy cells

A glucose uptake assay assessed the insulin-mimetic activity of Schiff base vanadium complexes by recording changes in glucose concentration in the treated media after a 24-hour treatment period. Reduced glucose concentration in the media indicates increased glucose uptake by the cells. An initial glucose baseline was taken at time 0 of the treatment of 24 well plates seeded with C2C12 and HepG2 cells with the vanadium compounds and insulin. Following treatment, glucose concentrations in the medium were assessed using a Contour Plus ® glucometer with the appropriate test strips. A calibration curve was established using serial dilutions of standard media to correlate glucometer readings with actual glucose concentrations in the media, facilitating the accurate determination of glucose concentrations. The trendline produced an R^2

value of 0.9972, as shown in Figure 2.7, with the equation of the line shown in Equation 3 below:

Equation 3

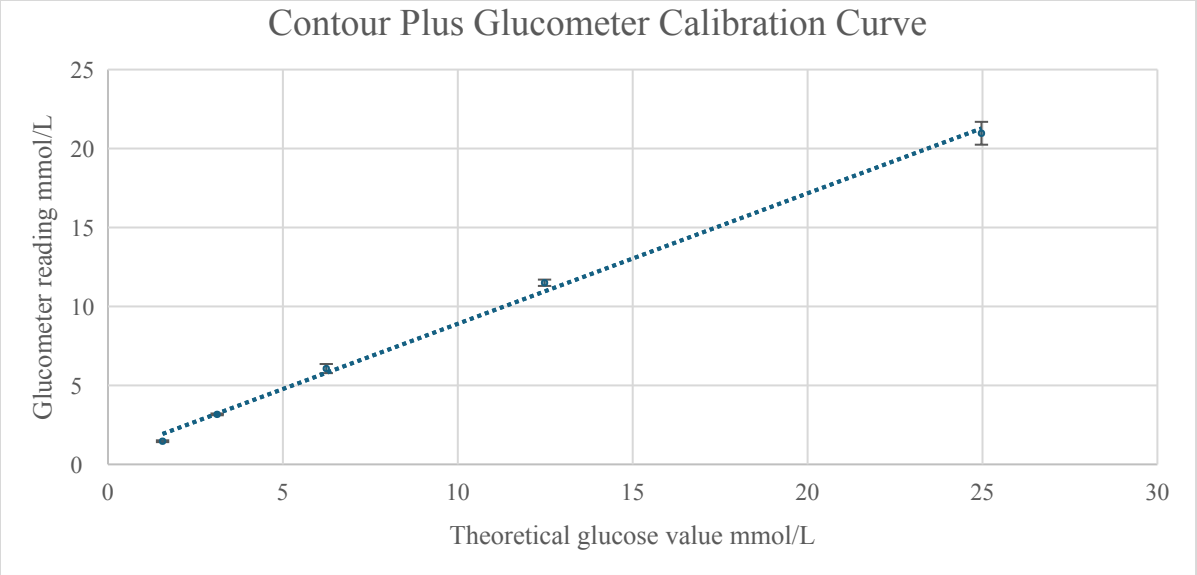


Figure 2.7: The calibration curve of glucometer readings against theoretical glucose value. The trend line was produced as a linear line of best fit for the plot points presented at the mean ± SD represented with error bars, (n = 3)

The glucose concentrations were then converted into relative percentage glucose uptake using Equation 4 below:

Equation 4

The relative percentage uptake was plotted against the concentrations of compounds used. The workflow for the glucose uptake assay is illustrated in **Figure 2.8** below:

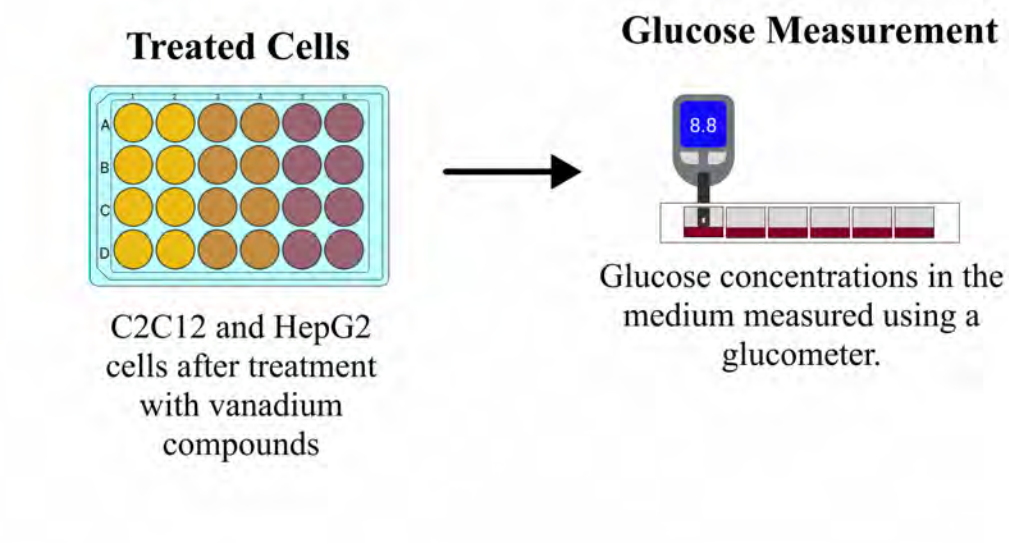


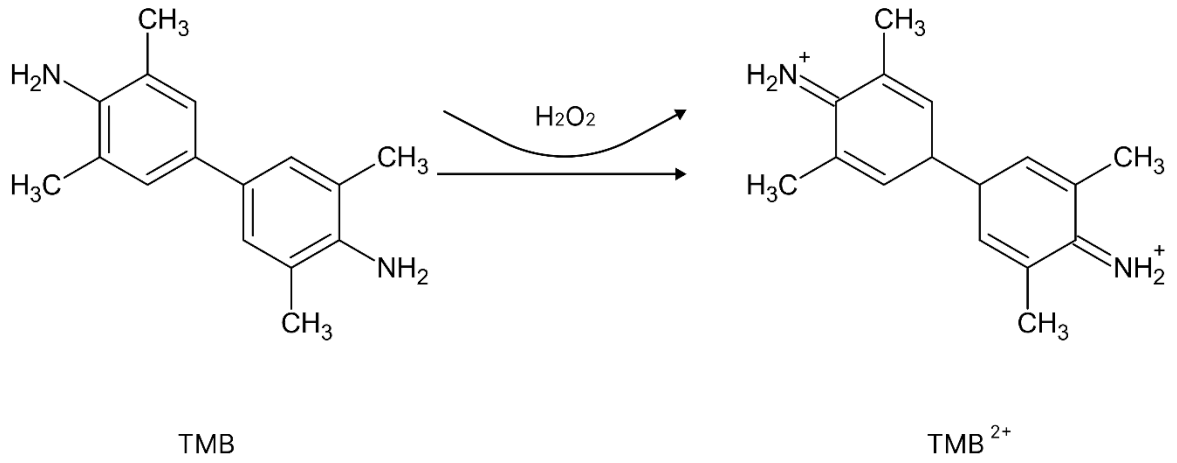
Figure 2.8: Glucose uptake assay workflow

2.4.5 Glucose uptake assay in palmitic acid-induced insulin-resistant cells

Palmitic acid-induced insulin resistance is characterized by its inhibitory effect on the insulin signalling pathway, specifically at the insulin receptor substrate (IRS), effectively terminating the cascade of downstream insulin signalling. To induce insulin resistance, 24 well plates seeded with C2C12 and HepG2 cells were pre-treated with palmitic acid at a concentration of 196 µM for 24 hours. A stock solution of palmitic acid was prepared by dissolving 51.2 mg of the fatty acid in 1 ml ethanol. 40 µl of the stock was then conjugated with 1.96 ml 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to achieve the desired working concentration of 4mM diluted stock of Palmitic acid. The diluted stock solution was then sterilized with a 0.22 µM filter and then added to the cell culture medium to make a final concentration of 196 µM. Cells were incubated in the palmitic acid-containing medium in a humidified 5% CO_2 atmosphere at 37 °C for 24 hours. Following treatment, the cells were washed twice with PBS to remove excess fatty acids before proceeding with treatment with the vanadium compounds. Glucose readings were taken at time 0 of incubation with vanadium treatments and again after 24 hours.

2.4.6 In-cell ELISA assays

In-cell ELISA is an immunocytochemistry technique for detecting and quantifying target proteins or their modifications within cultured cells. This assay leverages highly specific primary antibodies (often derived from mice or rabbits) to capture the target antigen. A secondary antibody, conjugated with an enzyme like horseradish peroxidase (HRP), binds the captured antigen. This enzyme catalyses the conversion of a colourless substrate, typically 3,3',5,5'-Tetramethylbenzidine (TMB), to a coloured product (TMB^{2+}), as seen in Scheme 2.2. The increased absorbance of this coloured product, measured at 450 nm using a spectrophotometer, reflects the amount of target protein present within the cells.



Scheme 2.2: The reduction of TMB to TMB^{2+}

Following treatment of C2C12 and HepG2 cells in 96 well plates with the vanadium compounds, the medium was aspirated, and cells were fixed with 100 µl of 8% paraformaldehyde per well for 15 minutes at 25 °C on a microplate shaker at 300 rpm. The fixative was then aspirated, and each well was washed four times with 200 µl of PBS. For intracellular targets (Akt, IL-6, MMP1, DPP4 and GLUT4), permeabilization was performed by adding 200 µl of 2X permeabilization buffer (Triton X-100 diluted in 1X PBS) to each well. The plate was incubated for 30 minutes at 25 °C while shaking at 300 rpm. Permeabilization buffer was aspirated, and all wells were blocked with 200 µl of 1% BSA in PBS for 2 hours at 25 °C with shaking at 300 rpm. The

blocking buffer was then aspirated, and 100 µl of 0.0002% primary antibody solution specific to the target protein (Akt, IL-6, MMP1, DPP4, or GLUT4) was added to each well. The plate was incubated overnight at 4 °C. The primary antibody solution was aspirated, and the plate was washed three times with 250 µl of wash buffer (PBS with Tween 20) and 100 µl of secondary antibody solution

(anti-rabbit IgG for Akt and anti-mouse IgG for IL-6, MMP1, DPP4 and GLUT4) was added to each well, and the plates were then incubated for 2 hours at 25 °C with shaking at 300 rpm. The plates were then washed four times with 250 µl of wash buffer. Thereafter, 100 µl of HRP substrate (citric acid, disodium hydrogen phosphate, 3,3',5,5'-tetramethylbenzidine, hydrogen peroxide) was added to each well and incubated for 30 minutes at 25 °C with shaking at 300 rpm. The reaction was stopped by adding 100 µl of 0.1 M HCl to each well. Absorbance was measured at 450 nm using a spectrophotometric microplate reader. For GLUT4 translocation in C2C12 cells, the same procedure was followed, omitting the permeabilization step since the interest was GLUT4 located on the cell membrane surface. The relative percentage expression (or translocation for GLUT4) was calculated using Equation 5 below:

Equation 5

The relative percentage expression was plotted against the concentrations of compounds used. The workflow for the cell ELISA assay is illustrated in Figure 2.9 below:

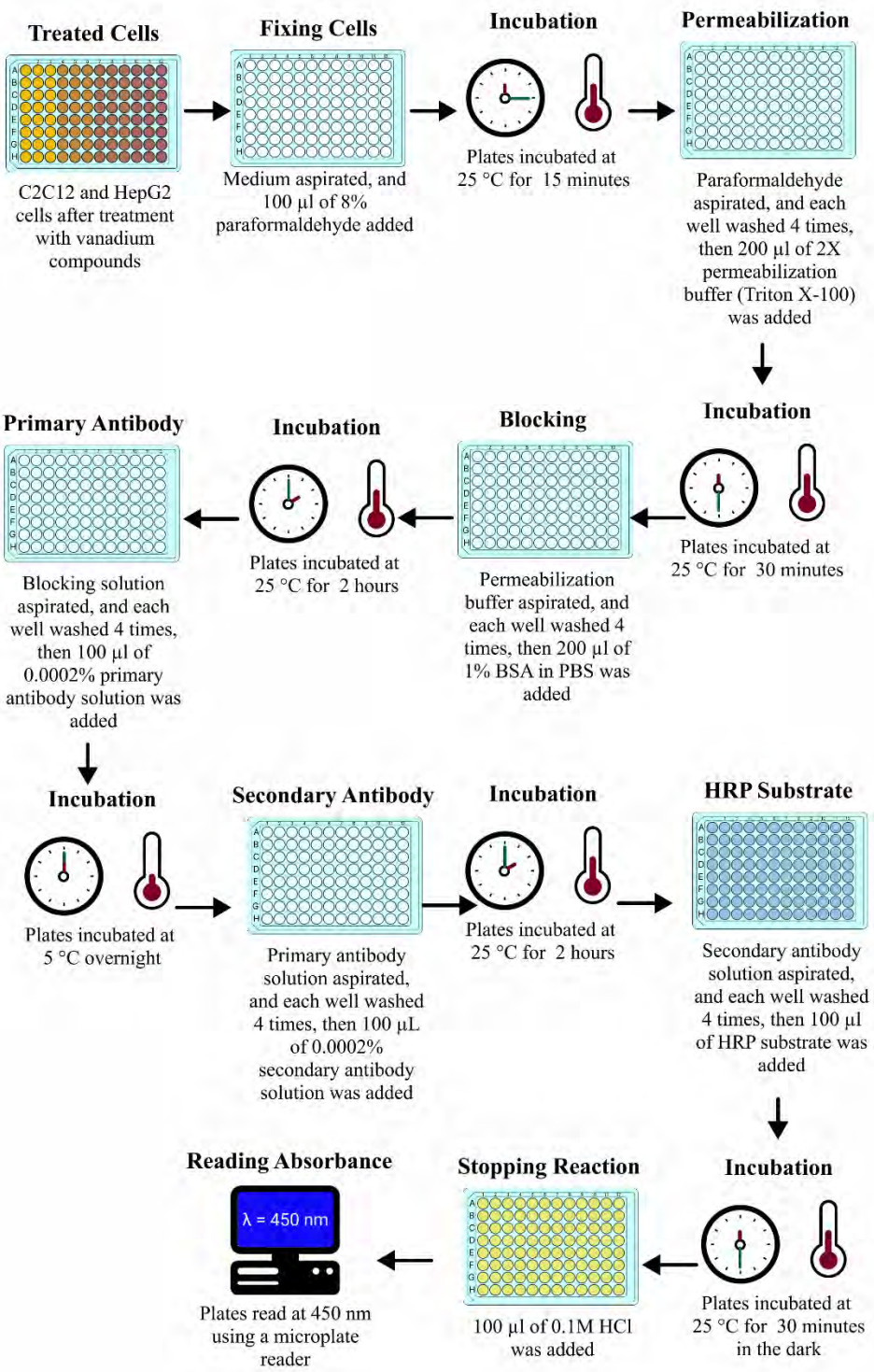


Figure 2.9: Cell ELISA assay workflow

2.4.7 Media ELISA assay

Following the treatment of C2C12 and HepG2 cells in 96 well plates with the vanadium compounds, the media was harvested and transferred into the high-binding plates. The high-binding plate was incubated at 5 °C overnight to allow proteins to adhere to the plate surface. Following incubation, the media was aspirated, and each well was blocked with 200 µl of 1% BSA in PBS for 2 hours at 25 °C with shaking at 300 rpm. The blocking buffer was then removed, and 100 µl of 0.0002% primary antibody solution specific to the target protein (IL-6, MMP1 and DPP4) was added to each well. Plates were incubated overnight at 4 °C. Post-incubation, primary antibody solution was aspirated, and plates were washed thrice with 250 µl of wash buffer (PBS with Tween 20). Next, 100 µl of secondary antibody solution (anti-mouse IgG) was added per well, and plates were incubated for 2 hours at 25 °C with shaking at 300 rpm. Then, the plates were washed four times with 250 µl of wash buffer. 100 µl of HRP substrate was added to each well and incubated for 30 minutes at 25 °C with shaking at 300 rpm. The reaction was halted by adding 100 µl of 0.1M HCl to each well, and absorbance was measured at 450 nm using a spectrophotometric microplate reader. The relative percentage expression was calculated using Equation 6 below:

Equation 6

The relative percentage expression was plotted against the concentrations of compounds used. The workflow for the media ELISA is illustrated in Figure 2.10 below:

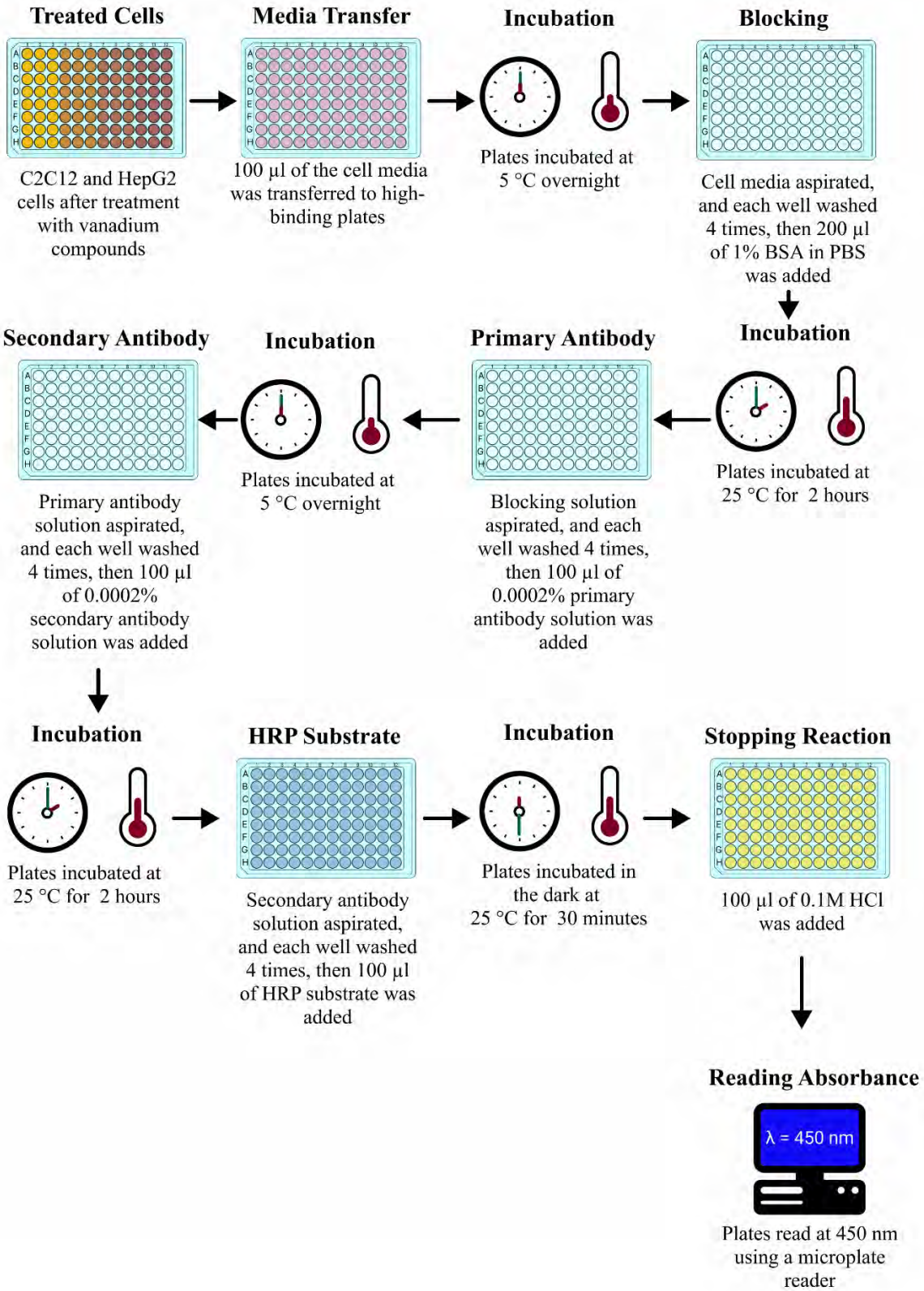
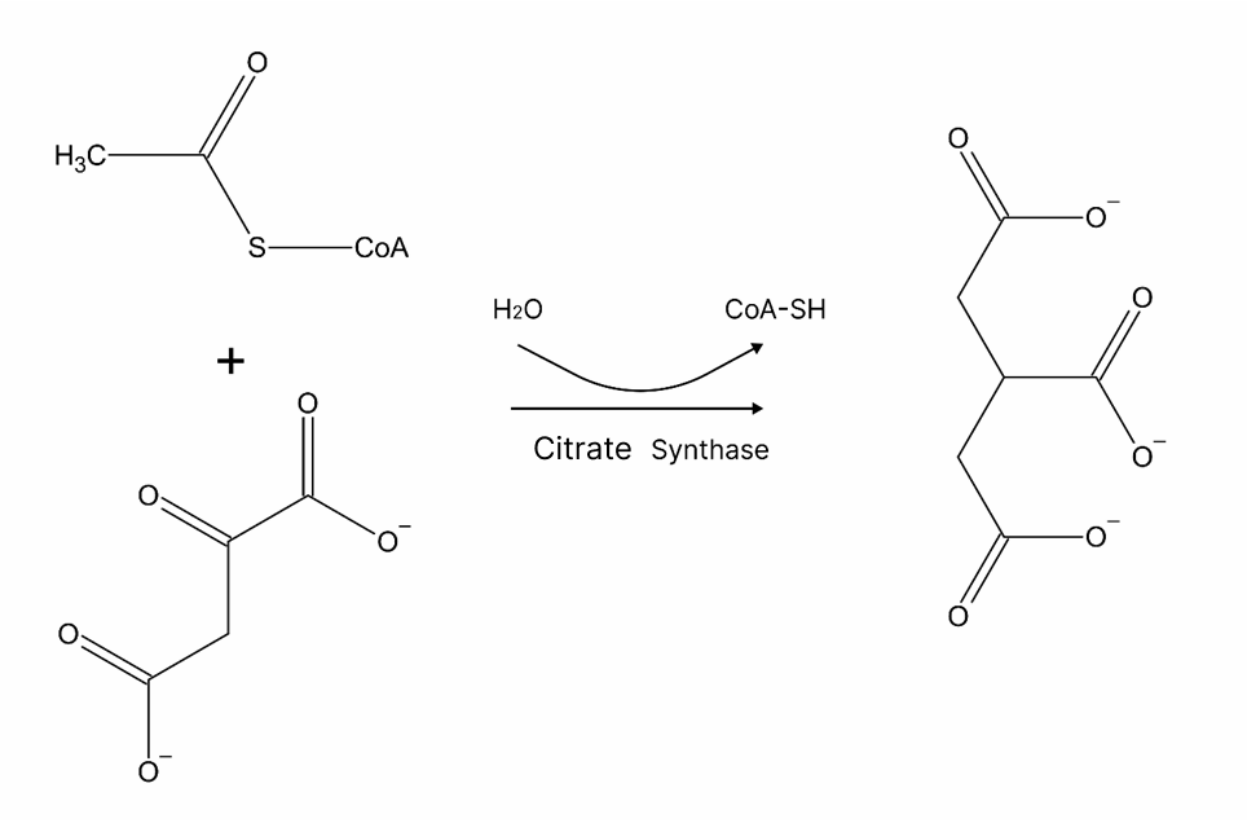


Figure 2.10: Media ELISA workflow

2.4.8 Citrate synthase activity assay

In healthy mitochondria, citrate synthase is a glycolysis enzyme which remains tightly bound within the mitochondrial matrix. However, citrate synthase can leak out into the cytoplasm in leaky mitochondria. This enzyme plays a crucial role in catalysing the condensation reaction where a two-carbon acetate residue from acetyl coenzyme A combines with a four-carbon oxaloacetate molecule to form the six-carbon citrate, which can be seen in Scheme 2.3. The activity of citrate synthase can be quantified by measuring the reaction between CoA-SH and DNTB, which produces a yellow-coloured product that can be detected at 412 nm.



Scheme 2.3: Condensation reaction between acetyl-CoA and oxaloacetate-producing citrate.

Following treatment of C2C12 and HepG2 cells in 96 well plates with the vanadium compounds, each well was gently rinsed with 100 µl of warmed (37 °C) mannitol and sucrose (MAS) buffer

containing bovine serum albumin (BSA) (MAS-BSA). The MAS-BSA was then aspirated. The cell permeabilization buffer (200 µl) was added to each well, followed by a 25-minute incubation at 25 °C. The permeabilization solution (Triton X-100 diluted in MAS-BSA) was aspirated, and the plates were rinsed gently with MAS-BSA. The MAS-BSA was then aspirated. The assay reaction was then performed directly in the plate: 197.5 µl of MAS-BSA was added to each well, followed by 40 µl of reaction mixture containing 750 µl of MES-BSA, 375 µl of 6 mM acetyl-CoA, and 75 µl of 10 mM DTNB in phosphate buffer. The plate was incubated at 30 °C for 5 minutes. The reaction was initiated by adding 12.5 µl of 10 mM oxaloacetic acid to each well (final volume 250 µl). After a further incubation at 30 °C for 4 minutes, the absorbance was measured at 412 nm using a spectrophotometric microplate reader. The relative percentage activity of citrate synthase was calculated using Equation 7 below:

Equation 7

The relative percentage activity was plotted against the concentrations of compounds. The workflow for the citrate synthase activity assay is illustrated in Figure 2.11 below:

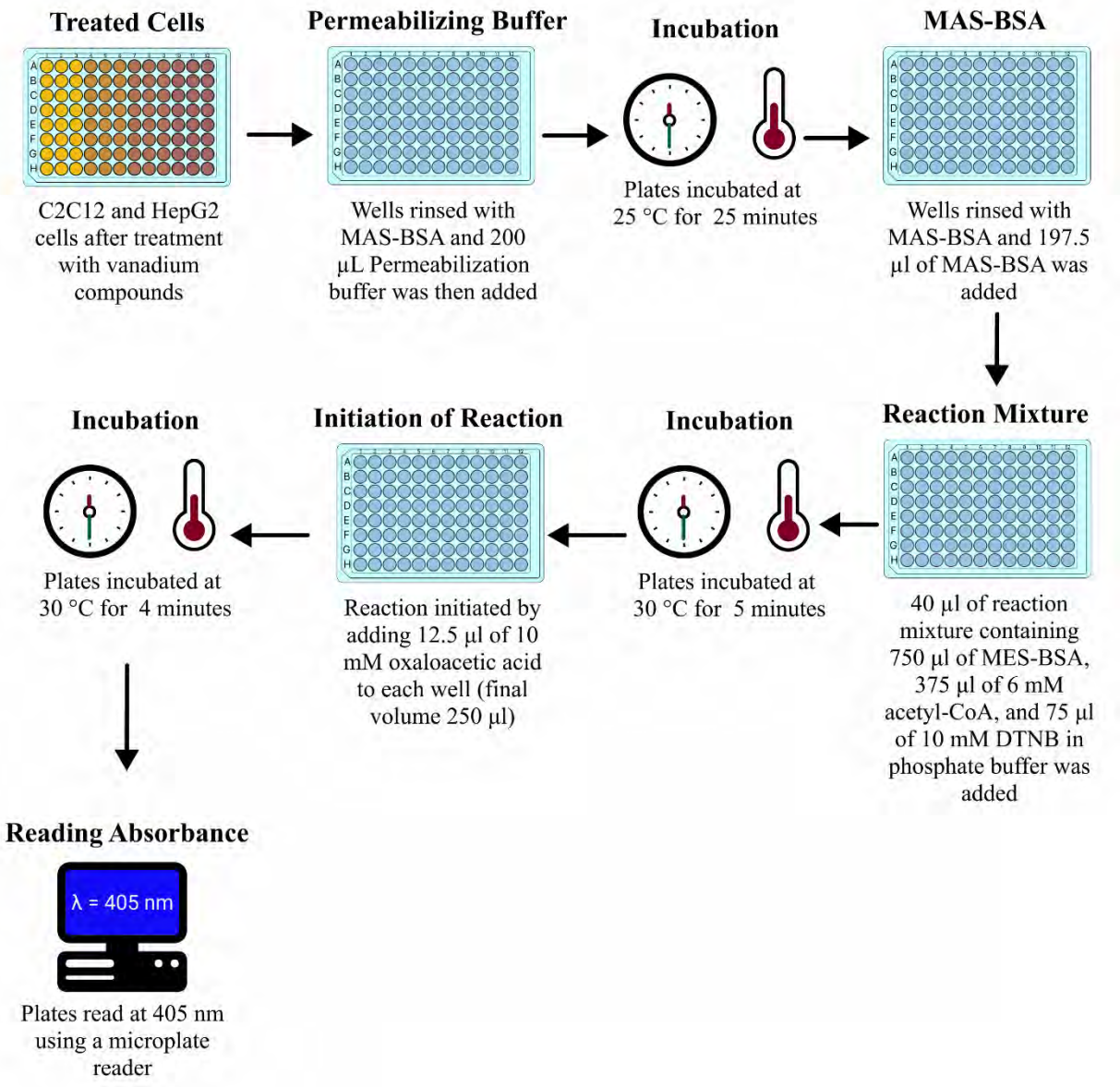


Figure 2.11: Citrate synthase activity assay workflow

2.4.9 Media pH assay

This assay assesses cell culture media pH to gain insights into cellular metabolic activity. Increased acidity (lower pH) indicates heightened glycolysis, a process of breaking down glucose to produce lactate, which acidifies the cell environment. Conversely, decreased acidity (higher pH) suggests reduced glycolysis or enhanced lactate utilization.

Following the treatment of C2C12 and HepG2 cells in 24 well plates with the vanadium compounds, the pH values in each well were measured using a pH meter. The pH values were plotted against the concentrations of compounds used. The workflow for the media acidification assay is shown in Figure 2.12 below:

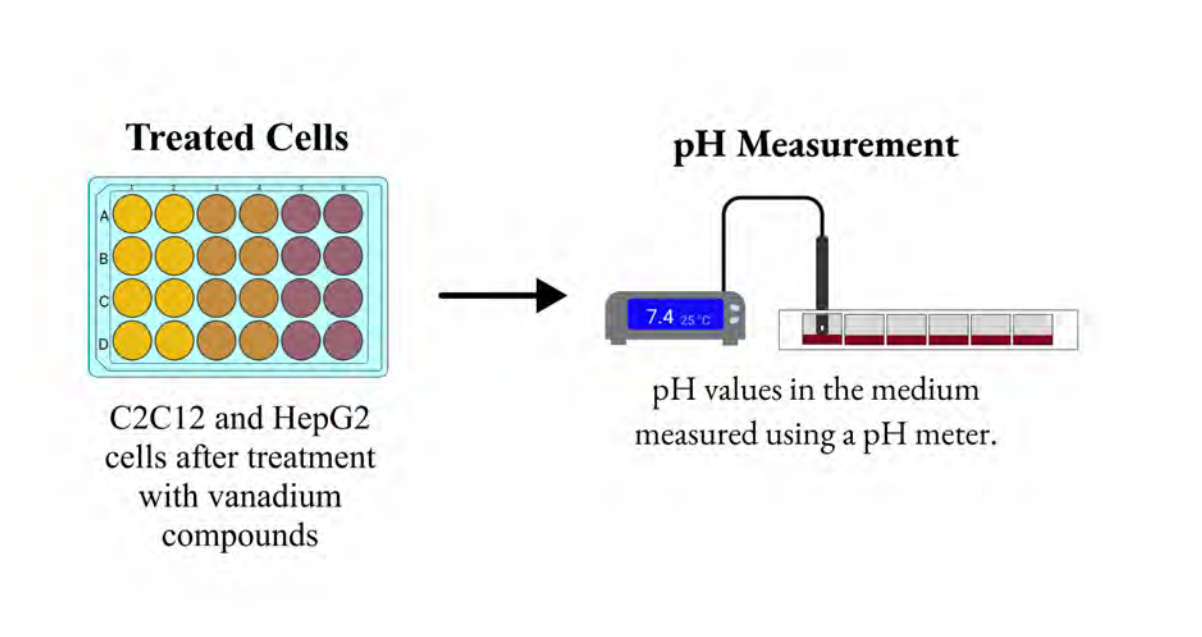


Figure 2.12: Media pH assay workflow

2.5 *In silico* assays

2.5.1 Pharmacokinetic properties

SwissADME is a web-based tool that provides crucial insights into the pharmacokinetic properties of a compound by computing a range of ADME (absorption, distribution, metabolism, excretion) parameters (182). These properties are essential for predicting the potential "druggability" and oral bioavailability of compounds during the drug discovery process. This study evaluated three Schiff base vanadium complexes using SwissADME, alongside BMOV and sodium orthovanadate for comparison. The parameters assessed include Lipinski's Rule of Five (which predicts drug-likeness), gastrointestinal absorption, water solubility, metabolism,

and bioavailability. Lipinski's Rule of Five is a guideline used to evaluate the likelihood that a compound can be orally absorbed based on molecular weight, hydrogen bond donors, hydrogen bond acceptors, and logP. Other parameters, such as gastrointestinal absorption and metabolism, provide additional insight into how a compound might perform in biological systems, particularly when administered orally (183). To assess the pharmacokinetic properties of the three Schiff base vanadium complexes, BMOV, and sodium orthovanadate, the canonical SMILES (Simplified Molecular Input Line Entry System) of each compound were obtained from their chemical structures. These SMILES strings were then entered individually into the SwissADME tool. After submission, SwissADME computed a series of pharmacokinetic parameters. The first set of parameters analysed was Lipinski's Rule of Five, which includes molecular weight, hydrogen bond donors, hydrogen bond acceptors, and logP. The results for these parameters were extracted from the SwissADME output and tabulated to identify which compounds conformed to Lipinski's criteria for drug-likeness. In addition, gastrointestinal absorption, water solubility, metabolism, and bioavailability predictions were generated for each compound. Gastrointestinal absorption estimates how well the compounds could be absorbed when administered orally, while water solubility predicts solubility in aqueous solutions. Metabolism predictions focused on interactions with cytochrome P450 enzymes, which are critical for drug metabolism, and bioavailability scores indicated the likelihood of the compounds being bioavailable after oral administration. These results were manually extracted from the SwissADME tool and tabulated to highlight these pharmacokinetic properties. The extracted data were analysed to compare the pharmacokinetic profiles of the Schiff base vanadium complexes with those of BMOV and sodium orthovanadate, enabling the identification of compounds with favourable drug-like properties and bioavailability.

2.5.2 Molecular docking studies

Molecular docking is a widely used computational method that predicts the preferred orientation of a ligand when bound to a protein. In this study, molecular docking simulations were used to investigate the binding modes of three Schiff base vanadium complexes, as well as BMOV and sodium orthovanadate as reference compounds, to the catalytic domain of the protein tyrosine phosphatase 1B (PTP1B) enzyme. These docking studies were performed to offer insights into

the inhibitory potential and binding affinities of these vanadium complexes against PTP1B. The docking process involved preparing both the protein and ligands for simulation, running docking experiments, and analysing the results to identify key interactions between the compounds and the active site of PTP1B.

2.5.2.1 Protein and ligand preparation

The crystal structure of the PTP1B enzyme (PDB ID: 1NNY; 2.40 Å resolution) was retrieved from the Protein Data Bank (184). Before docking, all heteroatoms and water molecules were removed from the protein structure. Polar hydrogen atoms, Kollman charges, and solvation parameters were added using the AutoDock Tools (ADT) suite (185). For ligand preparation, the structures of the Schiff base vanadium complexes, BMOV, and sodium orthovanadate were first drawn using ChemDraw software (186). The 2D structures were then converted to 3D molecular structures. To prepare these ligands for docking, they were converted into the pdbqt format using Open Babel software (187), ensuring that polar hydrogen atoms were retained. Gasteiger charges and torsional angles were also added to the ligands using AutoDock Tools.

2.5.2.2 Molecular docking simulation

Docking simulations were performed using AutoDock 4.2.6. (185). The active site of the PTP1B enzyme was defined by creating a grid box centred around the binding site with dimensions of 60 × 60 × 60 points and a grid spacing of 0.450 Å. A total of 50 docking runs were carried out for each Schiff base vanadium complex, as well as for BMOV and sodium orthovanadate, which were used as reference compounds. The docking parameters were set to use the Lamarckian genetic algorithm with 2,500,000 energy evaluations and a maximum of 27,000 generations. The population size was fixed at 150, with mutation and crossover rates of 0.02 and 0.8, respectively. Binding energy values were interpreted comparatively hence ligands with lower binding energies (more negative ΔG values) were considered to have stronger binding affinities to PTP1B. Out of the 50 poses generated per ligand, the ligand conformation with the lowest binding free energy from the most populated cluster was selected for further analysis. To verify the accuracy of the docking protocol, a re-docking procedure was performed using the native ligand co-crystallized

with the PTP1B structure. The resulting root mean square deviation (RMSD) tolerance between the experimental and re-docked pose was below 2 Å, confirming the reliability of the docking configuration. The docking results were initially visualized using PyMOL to analyse the binding poses of each ligand within the PTP1B active site. LigPlot was employed to generate 2D representations of the hydrogen bonds and hydrophobic contacts between the ligands and the active site of the enzyme to investigate the interactions further.

2.6 Data and Statistical Analysis

To ensure data reproducibility, each experiment was repeated at least twice, and within each experiment, each condition was assessed in triplicate wells. The data are presented as mean ± standard deviation (SD) on separate column graphs for clarity. Statistical analysis was conducted using GraphPad Prism version 10.4.0 to assess significant differences between the control and test groups. A one-way analysis of variance (ANOVA) was followed by Dunnett's multiple comparison test to pinpoint statistically significant differences between the control and treatment groups, and the results of these analyses are presented on the corresponding graphs, with asterisks (*) denoting statistically significant differences ($p < 0.05$), (**) for ($p < 0.005$) and (***) for ($p < 0.001$) between the control and treatment groups. The results for the ANOVA are described for each assay, and “F” represents the F-statistic from the ANOVA test, which quantifies the ratio of variation between group means to the variation within the groups. The “p” represents the p-value, indicating the probability that the observed differences occurred by chance under the null hypothesis.

Chapter 3 : Results

3.1 Cell-based assays

3.1.1 MTT cell viability assay

The effect of Schiff base vanadium complexes, with sodium orthovanadate as a positive control, on cell viability was evaluated in C2C12 and HepG2 cells at concentrations of 5, 10, and 20 μg/ml. Cell viability refers to the number of live, healthy cells in a sample. The control was considered to have 100% cell viability. A percentage viability below 80% is generally considered cytotoxic.

For C2C12 cells, treatment with the three Schiff base vanadium compounds resulted in varied cell viability compared to the control, as displayed in Figure 3.1. Compound 1 showed a slight dose-dependent decrease in viability. Compounds 2 and 3 did not show a clear dose-dependent trend. Sodium orthovanadate showed a significant increase in viability at all concentrations. A one-way ANOVA was performed to assess the statistical significance of the effects across compounds and concentrations at the $\alpha = 0.05$ level. A significant difference among the groups was observed ($F = 2.48$, $p = 0.018$), indicating that cell viability effects depend on both the compound and its concentration at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the control revealed that sodium orthovanadate produced a statistically significant increase in viability at all concentrations. None of the Schiff base vanadium compounds showed significant differences from the control at any concentration.

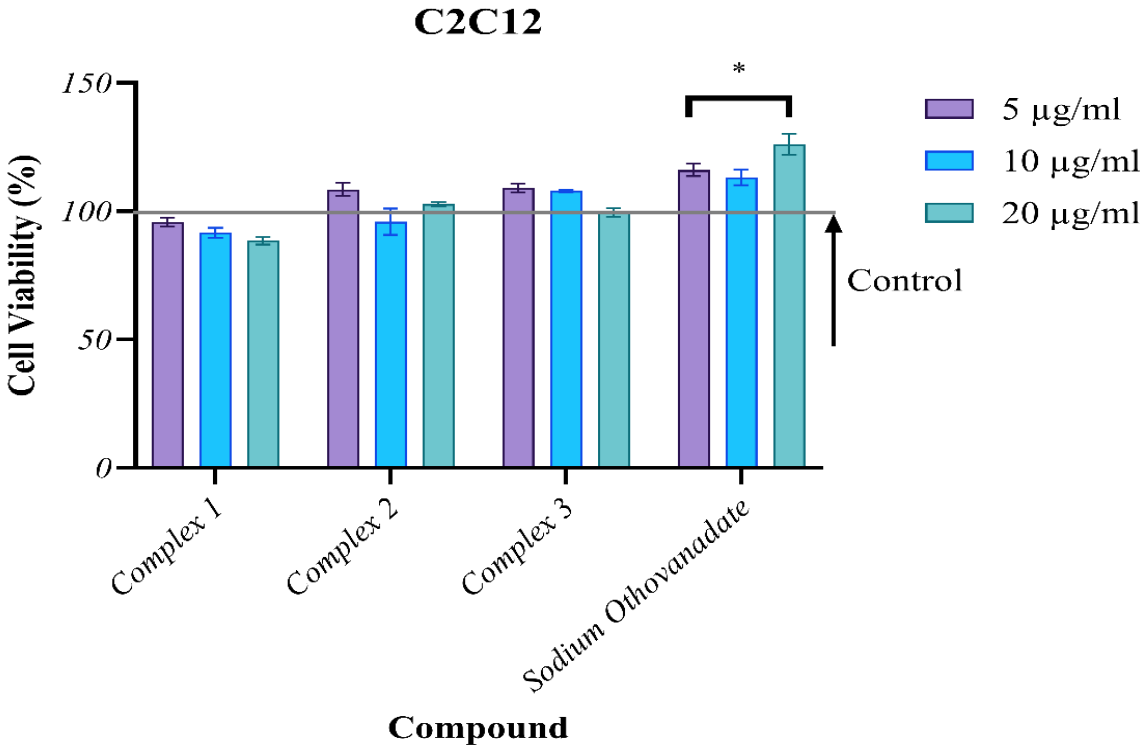


Figure 3.1: Percentage cell viability for C2C12 cell lines after 24 hours of exposure to Vanadium compounds at concentrations of 5, 10 and 20 µg/ml. The horizontal line at 100% represents the untreated control. The data are presented as mean ± SD represented with error bars, (n = 3) and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$

For the HepG2 cells, treatment with the Schiff base vanadium compounds demonstrated a clear dose-dependent response pattern, as displayed in Figure 3.2. Compounds 1, 2, and 3 all showed higher viability at lower concentrations with a progressive decrease as concentration increased. The dose-dependent effect was most pronounced at 20 µg/ml, where all compounds showed their lowest viability values. The standard, sodium orthovanadate showed consistently reduced viability across all concentrations. The one-way ANOVA for HepG2 cells ($F = 1.62$, $p = 0.098$) did not show a statistically significant interaction effect among compounds and concentrations on viability at the $\alpha = 0.05$ level. Dunnett's post hoc comparisons against the control also revealed no significant differences for any treatment at any concentration.

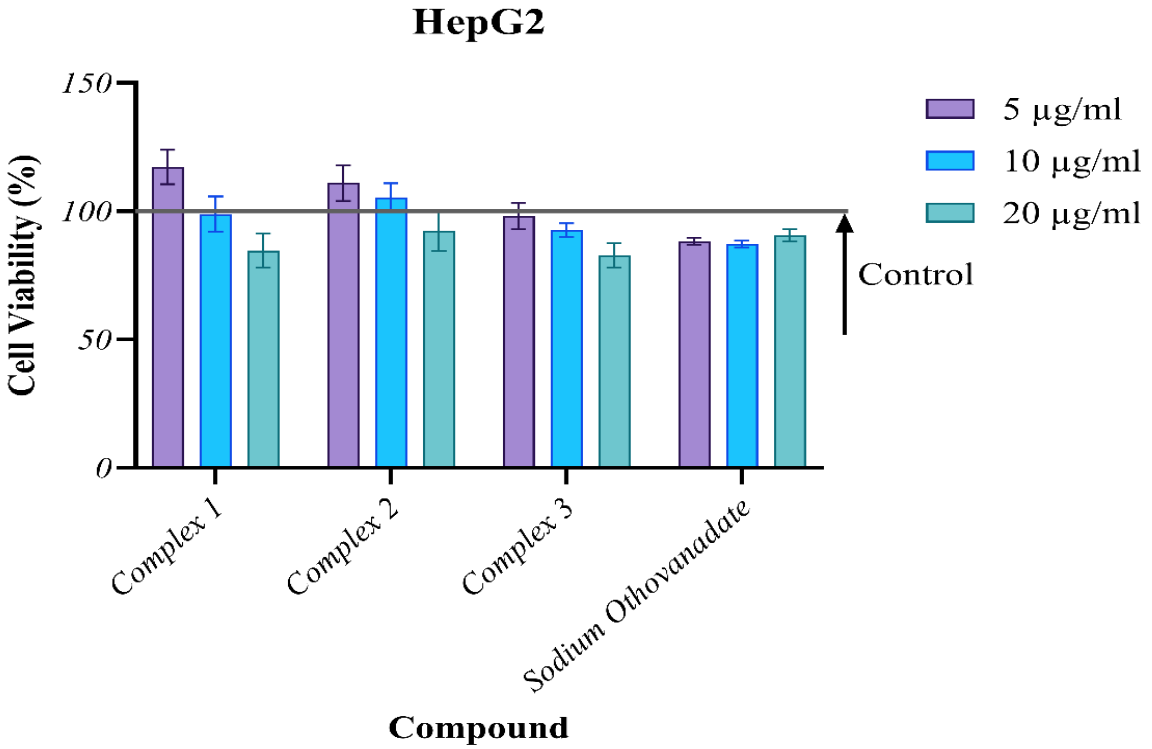


Figure 3.2: Percentage cell viability for HepG2 cell lines after 24 hours of exposure to Vanadium compounds at concentrations of 5, 10 and 20 µg/ml. The horizontal line at 100% represents the untreated control. The data are presented as mean ± SD represented with error bars, (n = 3)

3.1.2 Cellular glucose uptake in normal cells

The effect of Schiff base vanadium complexes on cellular glucose uptake was evaluated in C2C12 and HepG2 cells at concentrations of 5, 10, and 20 µg/ml, alongside insulin at a concentration of 0.05 µM and sodium orthovanadate at concentrations of 5, 10, and 20 µg/ml, both of which served as reference drugs. Glucose uptake is expressed as the percentage of glucose consumed from the culture media.

For C2C12, as displayed in Figure 3.3, the insulin-treated control showed a slightly higher glucose uptake compared to the untreated control. Schiff base vanadium complexes 2 and 3 and Sodium Orthovanadate generally showed an increased glucose uptake compared to the untreated control at the 5, 10, and 20 µg/ml concentrations. Schiff base vanadium complex 1 showed

similar glucose uptake to the control at 5 and 10 µg/ml and decreased uptake at 20 µg/ml. A one-way ANOVA test was conducted to assess the statistical significance of the effects across compounds and concentrations. The results ($F = 1.36$, $p = 0.21$) indicate that the differences in glucose uptake were not statistically significant overall at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the untreated control revealed statistically significant increases in glucose uptake for Schiff base vanadium compounds 2 and 3 and sodium orthovanadate at the 10 µg/ml concentration. Additionally, Schiff base vanadium complexes 3 and the sodium orthovanadate showed significant increases at the 5 and 20 µg/ml concentrations. Schiff base vanadium complex 2 also showed a significant increase at the 5 µg/ml concentration.

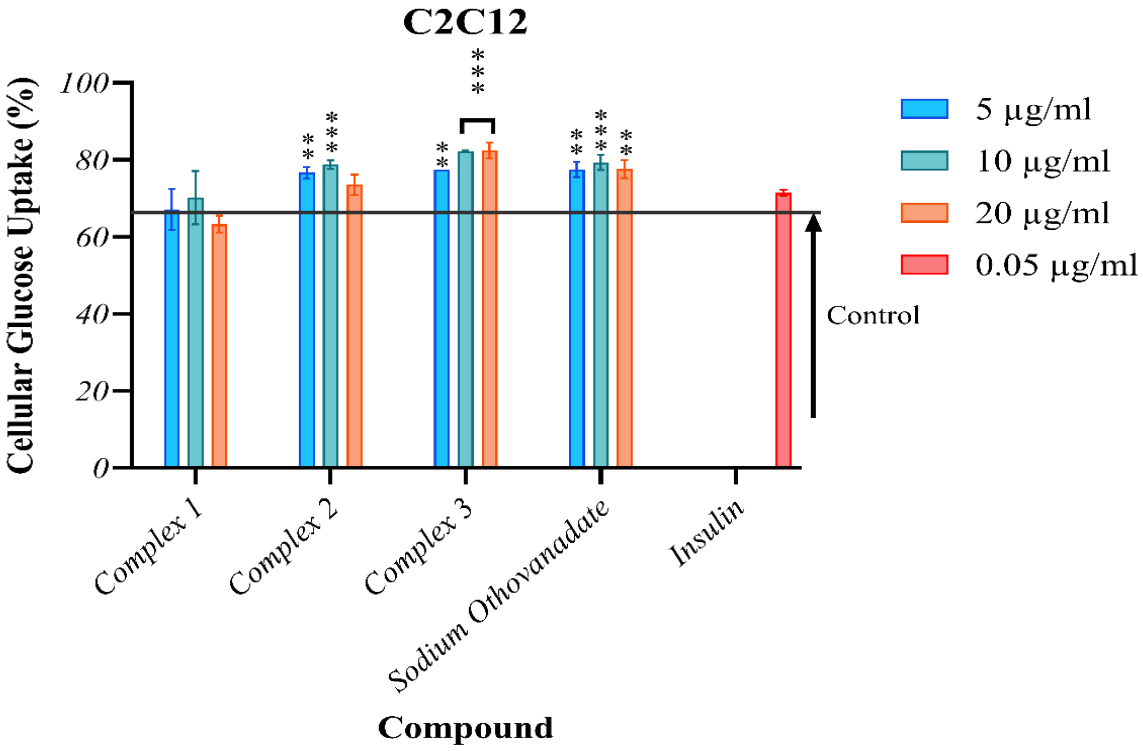


Figure 3.3: Cellular glucose uptake after exposing C2C12 cells to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line and the asterisk (**) represents the statistical difference between the test compounds and the control at $p < 0.01$ and (***) $p < 0.001$

For HepG2, as displayed in Figure 3.4, the insulin-treated control showed a slightly higher glucose uptake compared to the untreated control. At a concentration of 10 µg/ml, Schiff base vanadium compounds 2 and 3, as well as the standard (sodium orthovanadate), demonstrated a modest increase in glucose uptake compared to the control, while compound 1 exhibited glucose uptake comparable to the insulin control. At 5 µg/ml, all Schiff base vanadium compounds and the standard showed a slight increase in glucose uptake relative to the control, with compound 2 displaying the highest uptake among the compounds. At 20 µg/ml, the glucose uptake for all Schiff base vanadium compounds and the standard was comparable to that observed at 10 µg/ml. A one-way ANOVA revealed a statistically significant interaction effect between the compound and concentration on glucose uptake in the HepG2 cell line ($F = 11.38$, $p < 0.05$), indicating the effect of the compound depends on the concentration used at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the untreated control revealed statistically significant increases in glucose uptake for the insulin control and at 10 µg/ml for complexes 1, 2, 3, and sodium orthovanadate. It also showed significant increases for all three Schiff base compounds at 5 µg/ml and at 20 µg/ml.

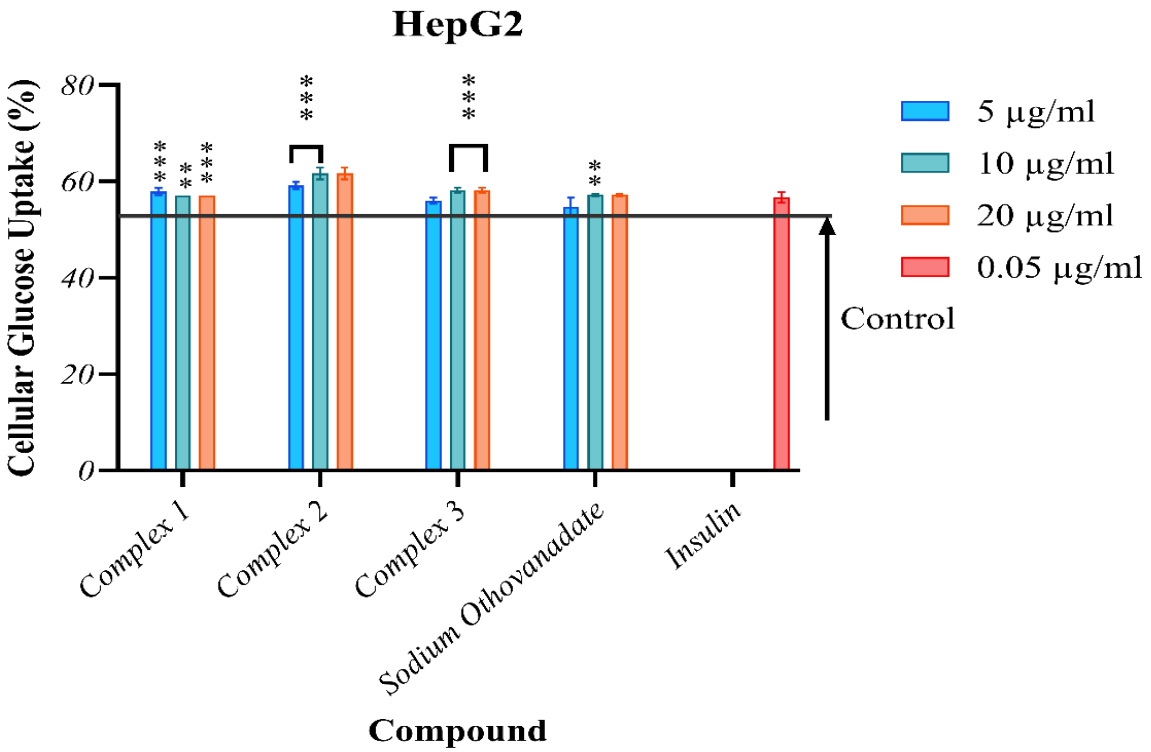


Figure 3.4: Cellular glucose uptake after exposing HepG2 cells to vanadium compounds at concentrations of 5, 10, and 20 μg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk (**) represents the statistical difference between the test compounds and the control at $p < 0.01$, (***) $p < 0.001$

3.1.3 Glucose uptake in palmitic acid-induced insulin resistant cells

The effect of Schiff base vanadium complexes on glucose uptake in an insulin-resistant state was evaluated in C2C12 and HepG2 cells at concentrations of 5, 10, and 20 μg/ml, alongside sodium orthovanadate at concentrations of 5, 10, and 20 μg/ml and insulin at a concentration of 0.05 μg/ml, which served as a positive control, cells that were not treated with palmitic acid served as a normal control. Glucose uptake is expressed as the percentage of glucose consumed from the culture media.

For C2C12 cells, as displayed in Figure 3.5, the insulin-treated cells showed a reduced glucose uptake compared to the normal control, confirming an insulin-resistant state. The Schiff base vanadium complexes and sodium orthovanadate showed a trend towards an increase in glucose uptake at concentrations of 5 and 10 μg/ml compared to the insulin treatment however, at the highest concentration of 20 μg/ml, the Schiff base vanadium complexes and sodium orthovanadate, start to show a reduction in glucose uptake. A one-way ANOVA revealed a statistically significant interaction between compound and concentration on glucose uptake ($F = 2.55$, $p = 0.01$), indicating that the effect of the compounds on glucose uptake depends on the concentration used at the $\alpha = 0.05$ level. Dunnet's post hoc analysis against the insulin control group showed a significant increase in glucose uptake for complex 1 across all concentrations and for complexes 2 and 3 at the 10 μg/ml concentration. Treatment with sodium orthovanadate also showed a statistically significant increase in glucose uptake at lower concentrations (5 and 10 μg/ml).

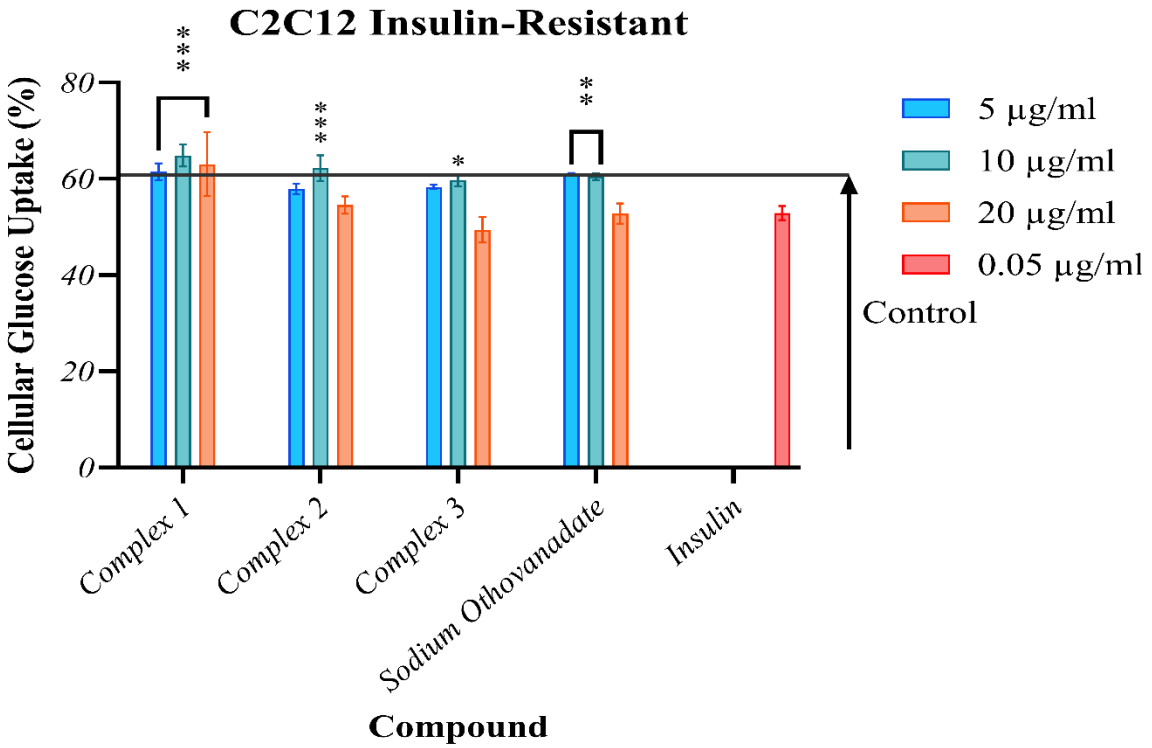


Figure 3.5: Cellular glucose uptake after exposing insulin-resistant C2C12 cells to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

For HepG2 cells, as displayed in Figure 3.6, the insulin-treated cells showed a reduced glucose uptake compared to the normal control, therefore confirming an insulin-resistant state. The Schiff base vanadium complexes (1, 2 and 3) and sodium orthovanadate generally showed an increase in glucose uptake compared to insulin, although this starts to decrease at the highest concentration (20 µg/ml) One-way ANOVA analysis revealed a statistically significant interaction effect between compound and concentration ($F = 11.51$, $p < 0.001$), indicating that the effect on glucose uptake depends on both the specific compound and its concentration at the $\alpha = 0.05$ level. Dunnett's post hoc test against the insulin control group showed statistically significant increases in glucose uptake across all compounds and concentrations.

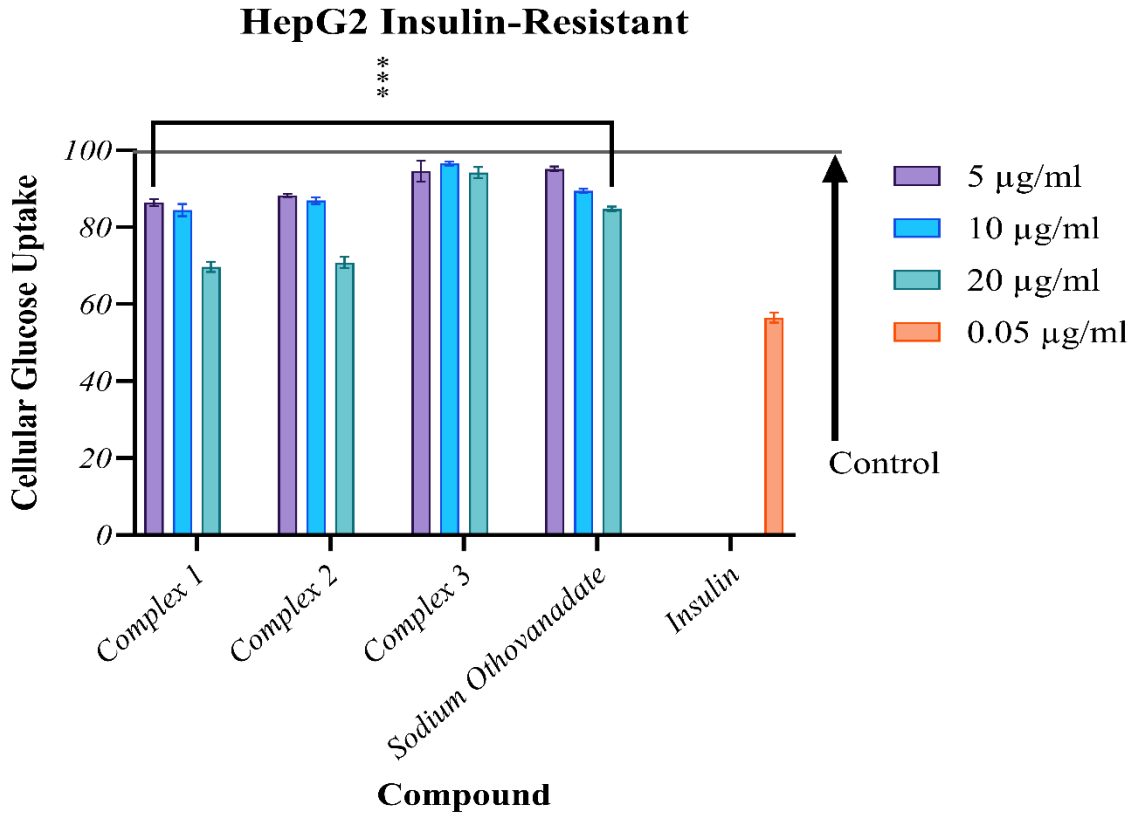


Figure 3.6: Cellular glucose uptake after exposing insulin-resistant HepG2 cells to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk (***) represents the statistical difference between the test compounds and the control at $p < 0.001$

3.1.4 In cell ELISA

3.1.4.1 Relative Akt expression

The effect of the Schiff base vanadium complexes on relative Akt expression was evaluated in C2C12 and HepG2 cells at concentrations of 5, 10, and 20 µg/ml, alongside insulin at a concentration of 0.05 µM/ml and sodium orthovanadate at concentrations of 5, 10, and 20 µg/ml both of which served as positive controls. Relative expression is expressed as a percentage compared to the untreated control.

For the C2C12 cell line, as displayed in Figure 3.7, insulin showed a slight decrease in Akt expression. The Schiff base vanadium complexes (2 and 3) and sodium orthovanadate generally showed a trend towards increased Akt expression compared to the control, although concentrations (5 and 10 µg/ml) of complex 2 showed reduced Akt expression. These effects were more pronounced at the highest concentration (20 µg/ml). A one-way ANOVA analysis suggested a possible interaction effect between compound and concentration ($F = 1.81$, $p = 0.061$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the control did not reveal any statistically significant differences in Akt expression.

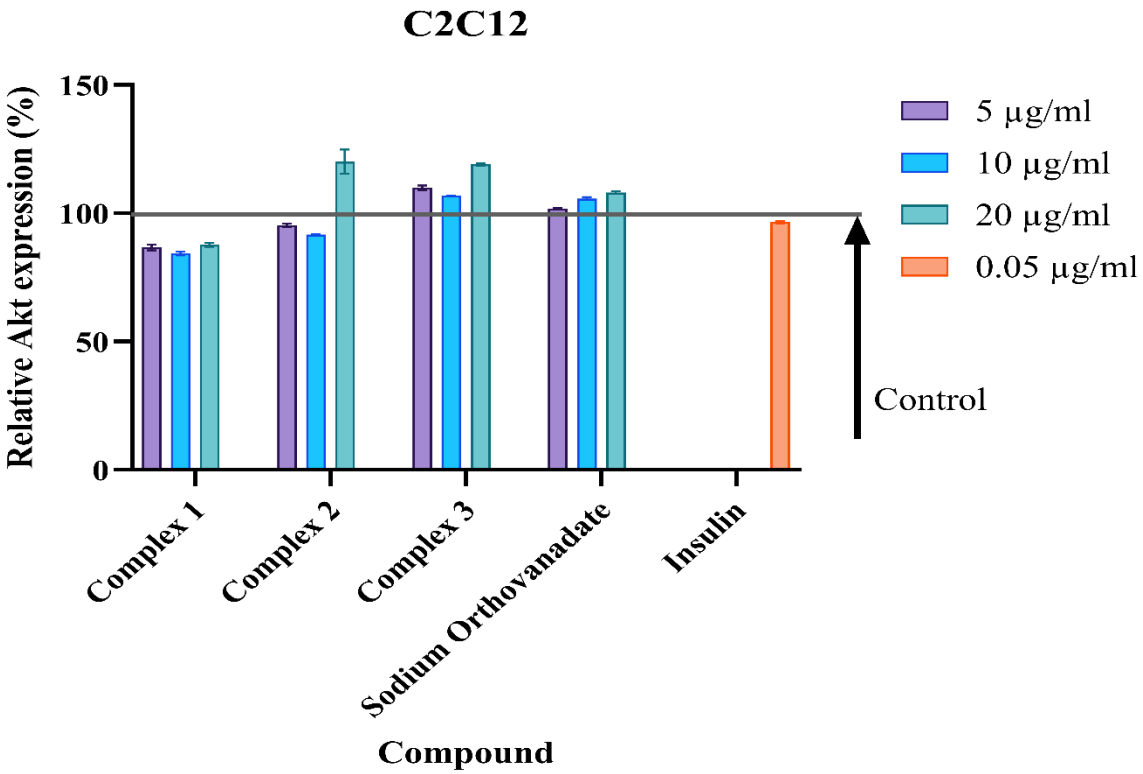


Figure 3.7: Relative Akt expression in C2C12 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line

In HepG2 cells, as displayed in Figure 3.8, insulin did not significantly affect Akt expression. Schiff base vanadium compounds (1, 2, and 3) generally decreased Akt expression in a

concentration-dependent manner, with the most pronounced reduction observed at 20 µg/ml. In contrast, sodium orthovanadate did not significantly alter Akt expression compared to the control at any concentration tested. A one-way ANOVA showed a significant interaction effect among compounds and concentrations ($F = 4.57$, $p = 0.000136$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis revealed statistically significant decreases in Akt expression compared to the control for Schiff base vanadium compound 1 at 20 µg/ml, Schiff base vanadium compound 2 at both 10 and 20 µg/ml, and Schiff base vanadium compound 3 at 5, 10, and 20 µg/ml.

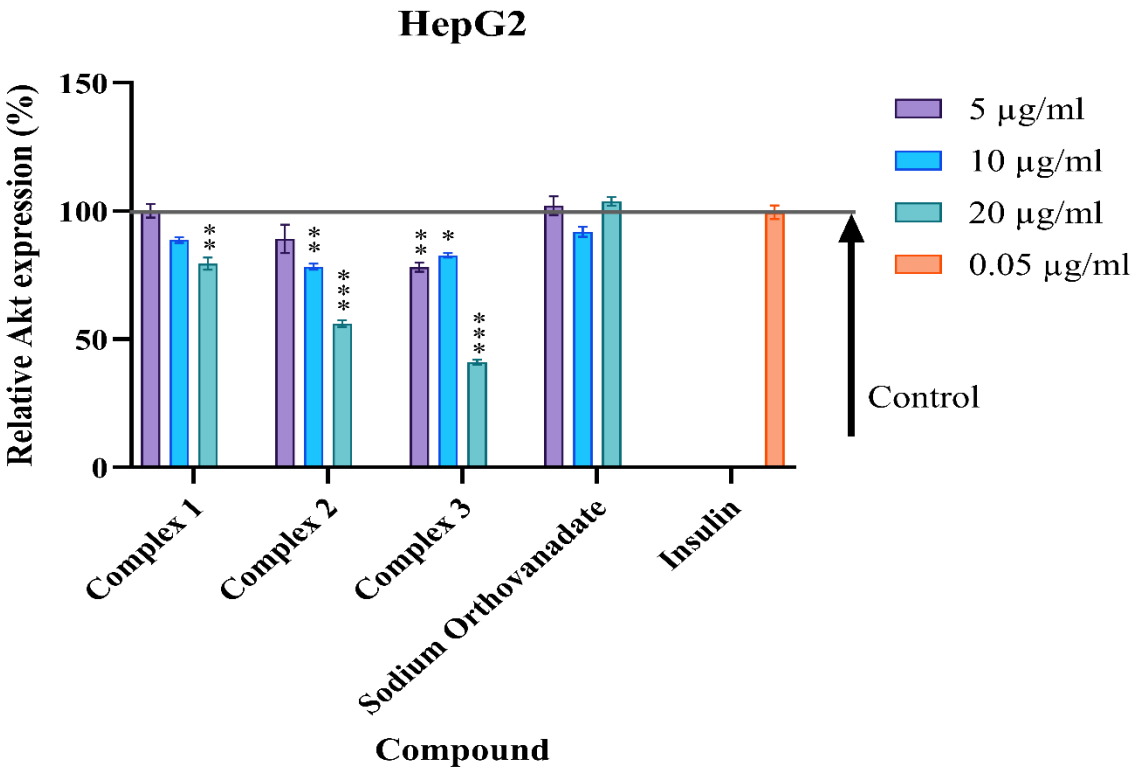


Figure 3.8: Relative Akt expression in HepG2 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

3.1.4.2 Relative GLUT4 Expression

The effect of the Schiff base vanadium complexes on relative GLUT4 expression was evaluated in C2C12 cells at concentrations of 5, 10, and 20 µg/ml, alongside insulin at a concentration of 0.05 µM/ml and sodium orthovanadate at concentrations of 5, 10, and 20 µg/ml both of which served as positive controls. Relative expression is expressed as a percentage compared to the untreated control.

As displayed in Figure 3.9, insulin significantly increased GLUT4 expression compared to the untreated control. Sodium orthovanadate also significantly increased GLUT4 expression compared to the control at 5 µg/ml. Compound 1 tended to decrease GLUT4 expression, particularly at the highest concentration (20 µg/ml). Compounds 2 and 3 showed a trend towards increased GLUT4 expression at some concentrations. The one-way ANOVA analysis did not reveal a statistically significant interaction among compounds and concentrations at the $\alpha = 0.05$ level. Dunnett's post hoc analysis confirmed significant increases in GLUT4 expression for insulin at 0.05 µg/ml and sodium orthovanadate at 20 µg/ml compared to the control.

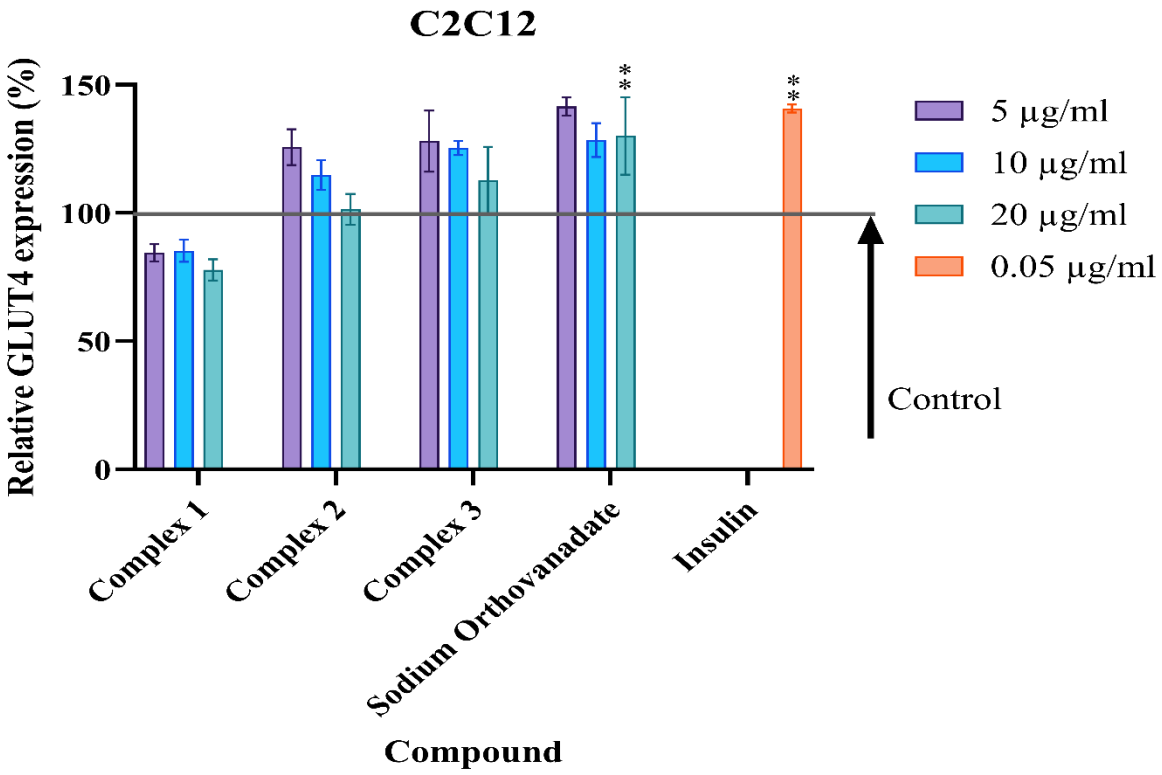


Figure 3.9: Relative GLUT4 expression in C2C12 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 μg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk (**) represents the statistical difference between the test compounds and the control at $p < 0.01$

3.1.4.3 GLUT4 translocation

The effect of the Schiff base vanadium complexes on GLUT4 translocation was evaluated in C2C12 cells at concentrations of 5, 10, and 20 μg/ml, alongside insulin at a concentration of 0.05 μM and sodium orthovanadate at concentrations of 5, 10, and 20 μg/ml, both of which served as positive controls. Relative expression is expressed as a percentage compared to the untreated control.

As displayed in Figure 3.10, Insulin increased GLUT4 translocation compared to the untreated control. The Schiff base vanadium compounds (1, 2, and 3) and sodium orthovanadate showed varied effects on GLUT4 expression compared to the control. Schiff base vanadium compound 1 generally decreased GLUT4 translocation, with this effect becoming more pronounced at higher concentrations. Schiff base vanadium compounds 2 and 3, as well as sodium orthovanadate, tended to increase GLUT4 expression, particularly at 5 and 10 μg/ml. At the highest concentration (20 μg/ml), the effects of these compounds were less pronounced than at 10 μg/ml. A one-way ANOVA analysis did not suggest a possible interaction effect among compounds and concentrations ($F = 1.19$, $p = 0.31$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the control did not reveal any statistically significant differences in GLUT4 translocation.

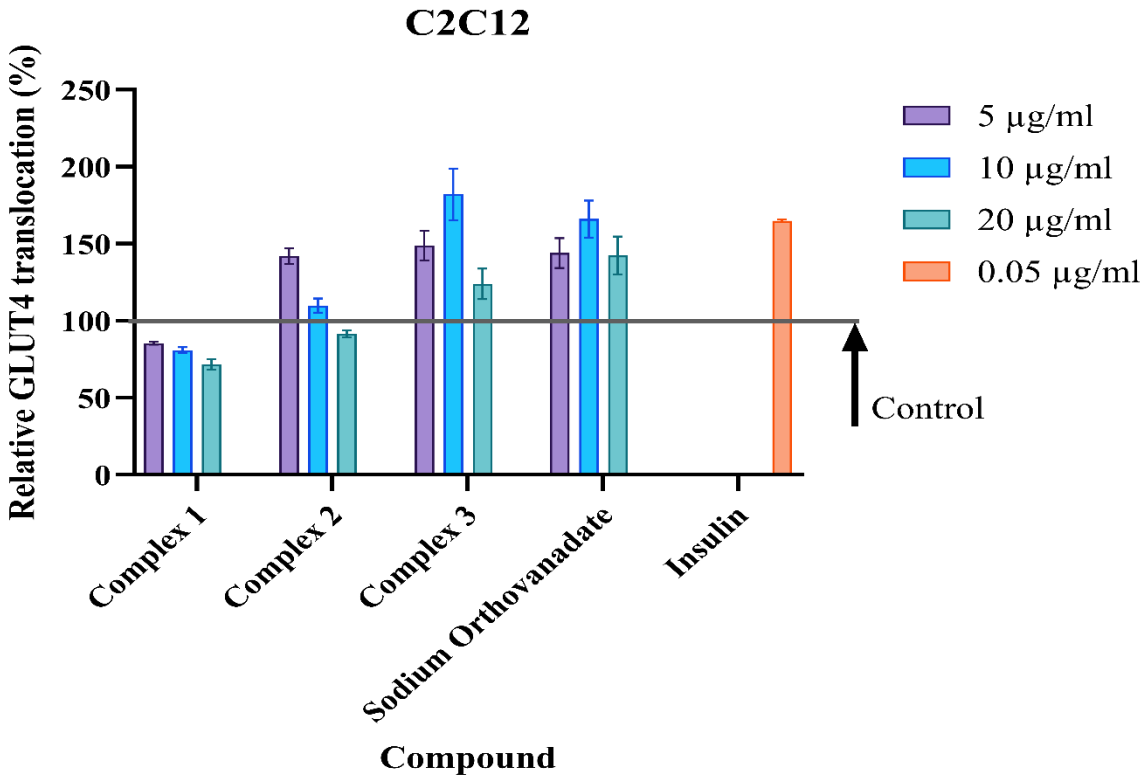


Figure 3.10: Relative GLUT4 translocation in C2C12 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line

3.1.4.4 Relative IL-6 Expression

The effect of the Schiff base vanadium complexes, with sodium orthovanadate serving as a positive control, on relative IL-6 expression was evaluated in C2C12 and HepG2 cells at concentrations of 5, 10, and 20 µg/ml. Relative expression is expressed as a percentage compared to the untreated control.

For the C2C12 cell line, as displayed in Figure 3.11, the Schiff base vanadium complexes (1, 2, and 3) and sodium orthovanadate showed varying effects on IL-6 expression compared to the control. Sodium orthovanadate and Schiff base vanadium complexes 1 and 3 generally showed a trend towards increased IL-6 expression, particularly at the 10 and 20 µg/ml concentrations. However, Schiff base vanadium compound 2 generally showed decreased IL-6 expression,

especially at higher concentrations. A one-way ANOVA analysis suggested a possible interaction effect among compound and concentrations ($F = 1.76$, $p = 0.083$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis revealed a statistically significant increase in IL-6 expression for Schiff base vanadium compound 1 at the 10 µg/ml concentration compared to the control.

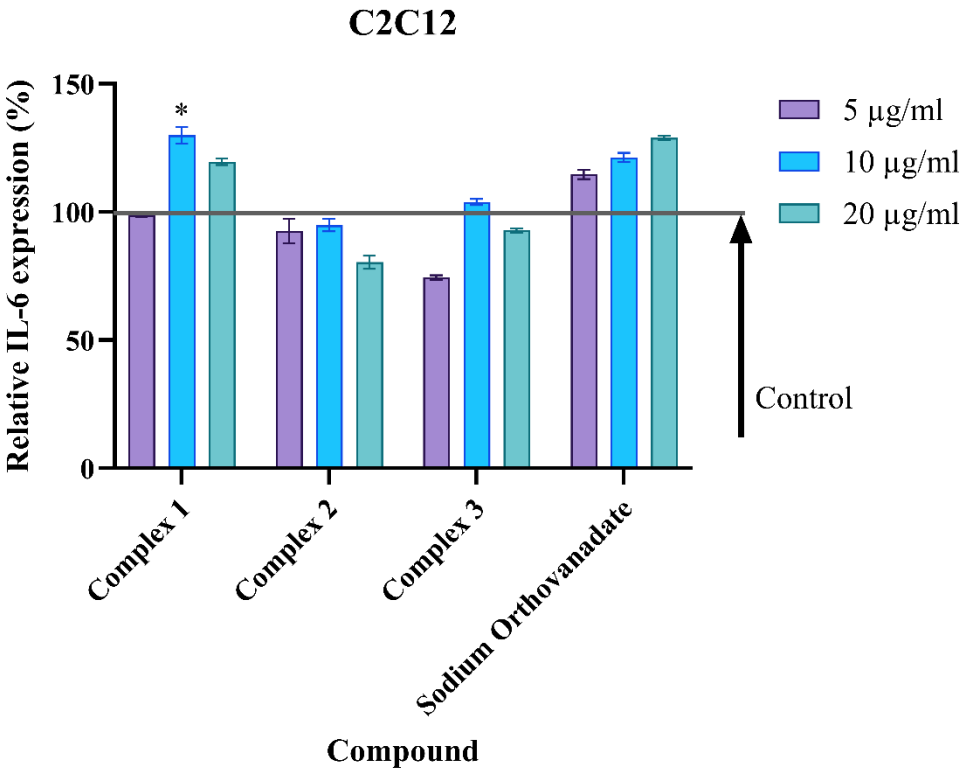


Figure 3.11: Relative IL-6 Expression in C2C12 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$

For the HepG2 cell line, as displayed in Figure 3.12, The Schiff base vanadium compounds (1, 2, and 3) and sodium orthovanadate generally showed a trend towards increased IL-6 expression at the 5 µg/ml concentration compared to the control. However, at higher concentrations (10 and 20 µg/ml), these compounds, including sodium orthovanadate, tended to decrease IL-6 expression.

A one-way ANOVA analysis did not suggest a statistically significant interaction effect among compounds and concentrations ($F = 0.52$, $p = 0.96$) at the $\alpha = 0.05$ level. Dunnett's post hoc revealed a significant decrease in IL-6 expression only with Shiff base vanadium complex 1 at 20 µg/ml compared to the control.

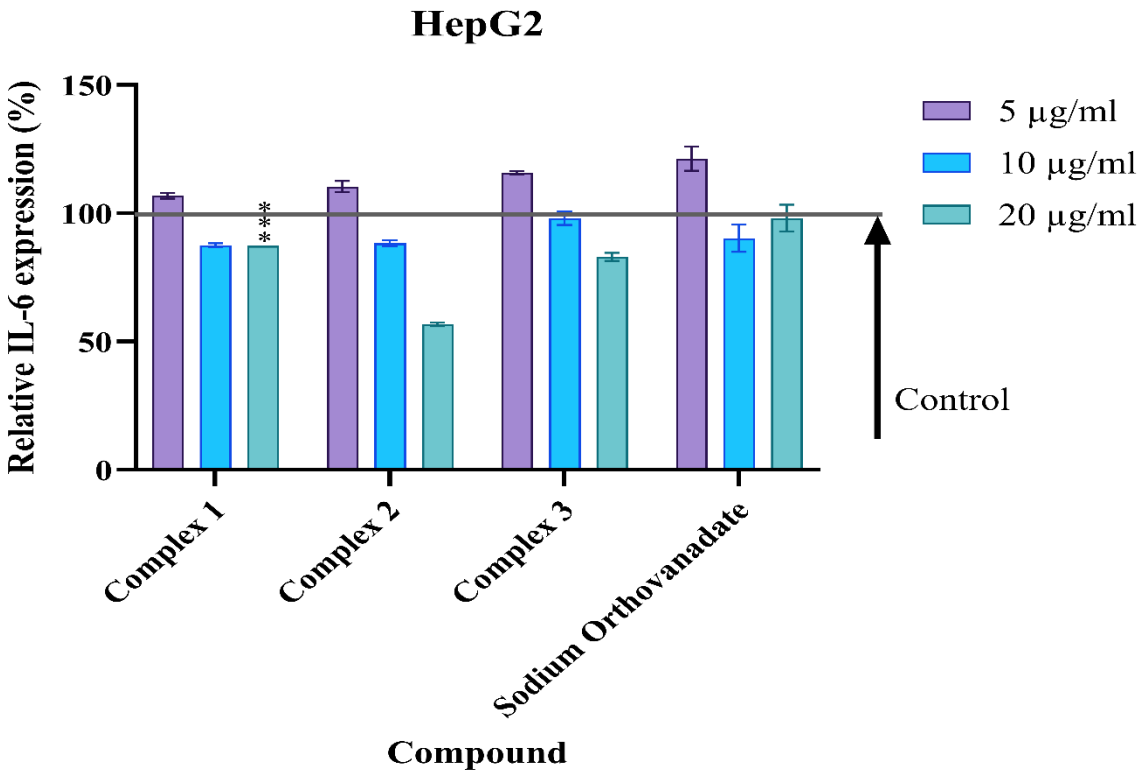


Figure 3.12: Relative IL-6 Expression in HepG2 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk (***) represents the statistical difference between the test compounds and the control at $p < 0.001$

3.1.4.5 MMP1 Expression

The effect of the Schiff base vanadium complexes with, sodium orthovanadate serving as a positive control, on relative MMP1 expression was evaluated in C2C12 and HepG2 cells at

concentrations of 5, 10, and 20 µg/ml. Relative expression is expressed as a percentage compared to the untreated control.

For the C2C12 cell line, as displayed in Figure 3.13, the Schiff base vanadium complexes 1 and 2 generally showed a trend towards reduced MMP1 expression compared to the control with a dose-dependent relationship of increased expression. Schiff base vanadium complex 3 and sodium orthovanadate showed increased MMP1 expression compared to the control, with effects more pronounced at higher concentrations. A one-way ANOVA analysis did not reveal a statistically significant interaction among compounds and concentrations ($F = 0.76$, $p = 0.74$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis revealed statistically significant increases in MMP1 expression compared to the control for sodium orthovanadate at 10 and 20 µg/ml and for Schiff base vanadium compound 3 at 10 µg/ml.

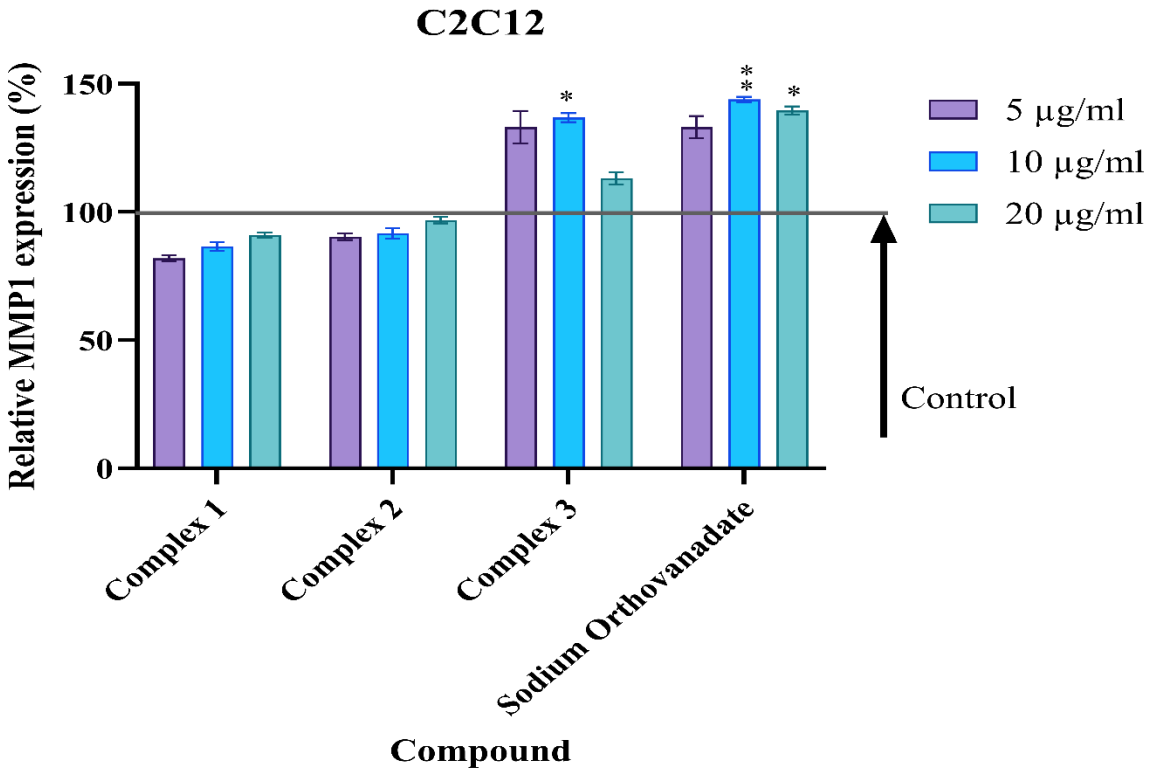


Figure 3.13: Relative MMP1 expression in C2C12 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk

(*) represents the statistical difference between the test compounds and the control at $p < 0.05$, (**) $p < 0.01$

For the HepG2 cell line, as displayed in Figure 3.14, Schiff base vanadium compound 1 consistently decreased MMP1 expression compared to the control, with this effect becoming more pronounced at higher concentrations. Schiff base vanadium complexes 2 and 3 generally demonstrated a decrease in MMP1 expression to the control. Sodium orthovanadate generally showed a trend towards increased MMP1 expression compared to the control, particularly at the higher concentrations. A one-way ANOVA analysis did not suggest a statistically significant interaction across the compounds and concentrations ($F = 1.60$, $p = 0.12$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis revealed statistically significant decreases in MMP1 expression for Schiff base vanadium compound 1 at 5, 10, and 20 µg/ml, and for Schiff base vanadium compound 2 at 20 µg/ml, as well as statistically significant decreases in MMP1 expression for compound 3 at both 10 and 20 µg/ml compared to the control.

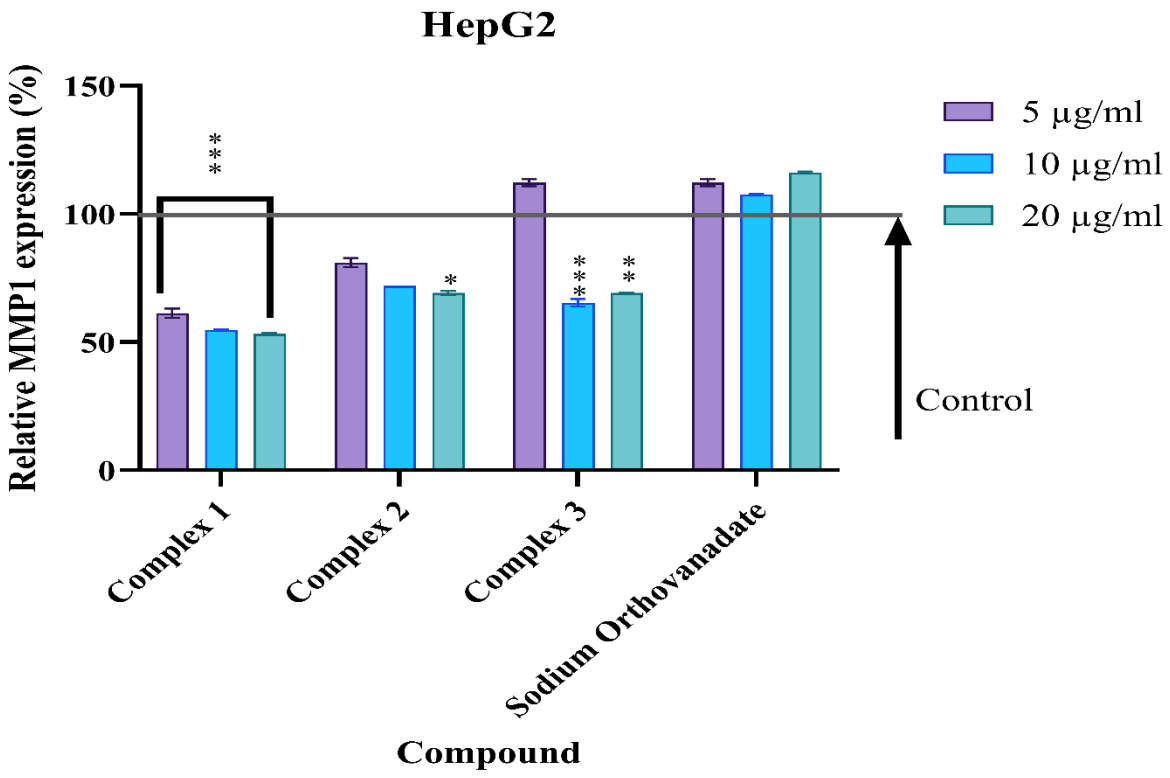


Figure 3.14: Relative MMP1 expression in HepG2 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

3.1.4.6 Relative DPP4 expression

The effect of the Schiff base vanadium complexes with sodium orthovanadate serving as a positive control on relative DPP4 expression was evaluated in C2C12 and HepG2 cells at concentrations of 5, 10, and 20 µg/ml. Relative expression is expressed as a percentage compared to the untreated control.

For the C2C12 cell line, as displayed in Figure 3.15, Schiff Base Vanadium Compound 1 consistently decreased DPP4 expression compared to the control, which became more pronounced at higher concentrations. Compounds 2 and 3, as well as sodium orthovanadate, generally showed a trend towards increased DPP4 expression, particularly at the highest concentration (20 µg/ml). A one-way ANOVA analysis revealed a significant interaction effect among compound and concentration ($F = 1.90$, $p = 0.048$) at the $\alpha = 0.05$ level. Dunnett's post hoc test confirmed statistically significant reductions in DPP4 expression compared to the control for Schiff Base Vanadium Compound 1 at 5, 10, and 20 µg/ml, and for Compound 2 at 10 and 20 µg/ml. Compound 3 showed a statistically significant reduction at 10 µg/ml.

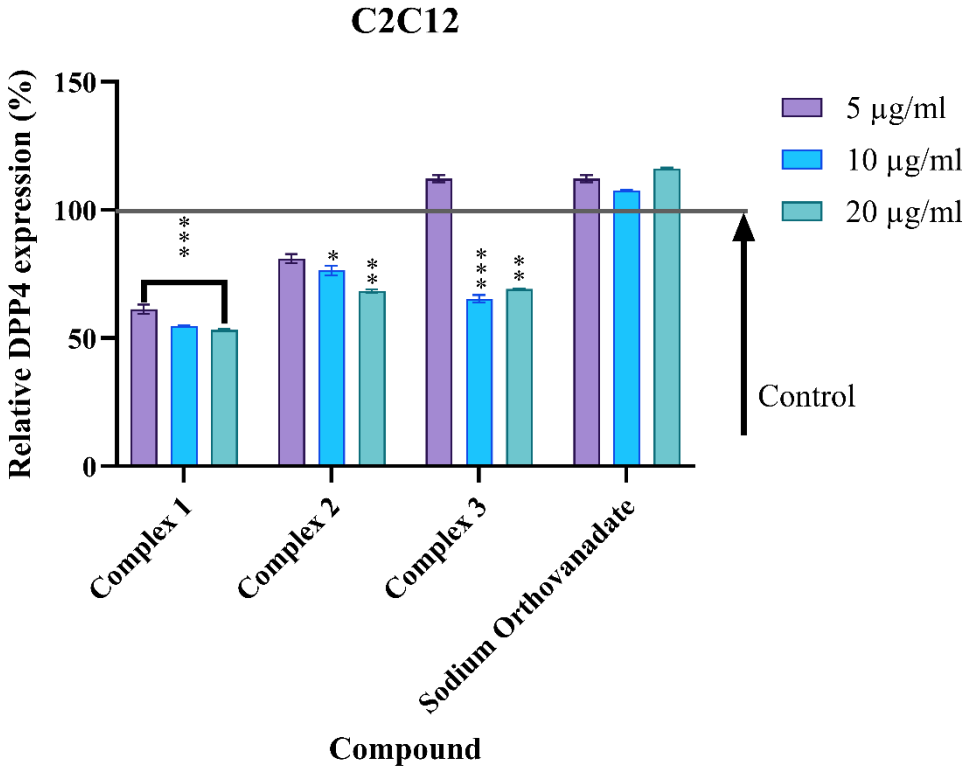


Figure 3.15: Relative DPP4 expression in C2C12 cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line, and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

For the HepG2 cell line, as displayed in Figure 3.16, insulin showed a substantial decrease in DPP4 expression. The Schiff base vanadium compounds (1, 2, and 3) and sodium orthovanadate generally showed decreased DPP4 expression compared to the control, with the reduction in expression becoming more pronounced with increasing concentrations (5, 10, and 20 µg/ml). The one-way ANOVA analysis indicated there was not a statistically significant interaction effect among compound and concentration ($F = 0.33$, $p = 0.99$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis revealed statistically significant decreases in DPP4 expression compared to the control for insulin at 0.05 µg/ml, and all vanadium compounds and sodium orthovanadate at 5, 10, and 20 µg/ml, except for compound 3 at 5 µg/ml.

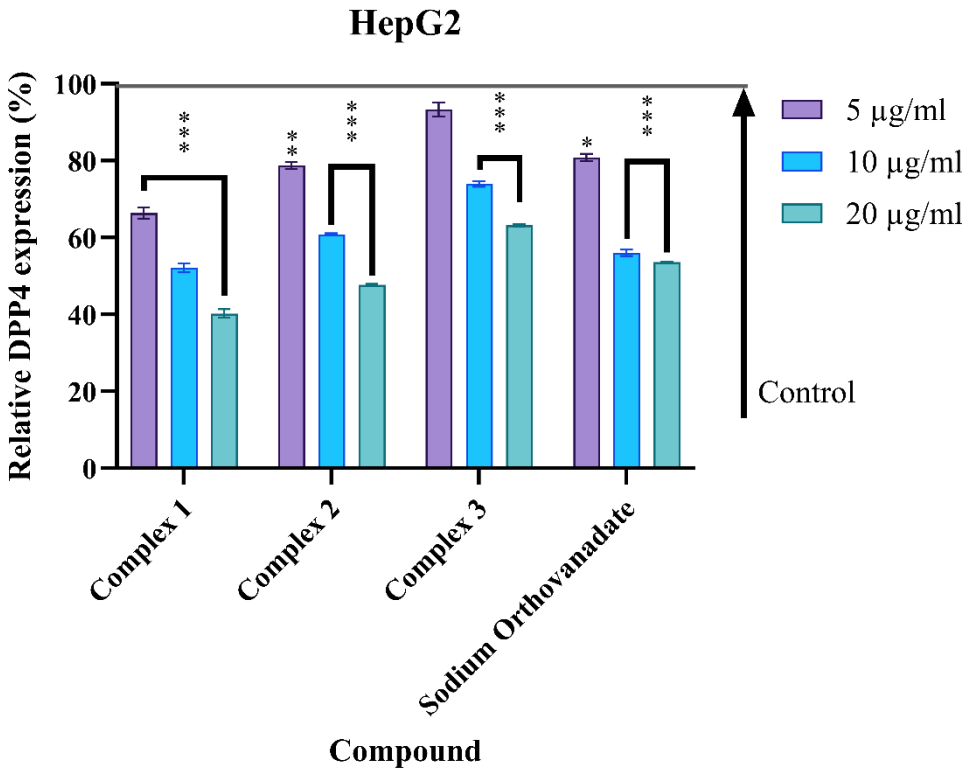


Figure 3.16: Relative DPP4 Expression in HepG2 Cells exposed to vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal line and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

3.1.5 Media ELISA assays

3.1.5.1 Relative IL-6 concentration

The effect of the Schiff base vanadium complexes and sodium orthovanadate, which served as a positive control, on relative IL-6 concentration was evaluated in C2C12 and HepG2 cell media at concentrations of 5, 10, and 20 µg/Relative concentration is expressed as a percentage compared to the untreated control.

For C2C12 cell media, as displayed in Figure 3.17, the Schiff base vanadium compounds (1, 2, and 3) generally showed no significant change in IL-6 concentration compared to the control,

while at the highest concentration (20 µg/ml), complex 2 and 3 showed an increase in IL-6 concentration compared to the control. Sodium orthovanadate significantly increased IL-6 concentration compared to the control. A one-way ANOVA analysis did not reveal a statistically significant interaction among compounds and concentrations ($F = 0.89$, $p = 0.59$). Dunnett's post hoc analysis confirmed the significant increases in IL-6 concentration with complex 2 and sodium orthovanadate at 20 µg/ml compared to the control.

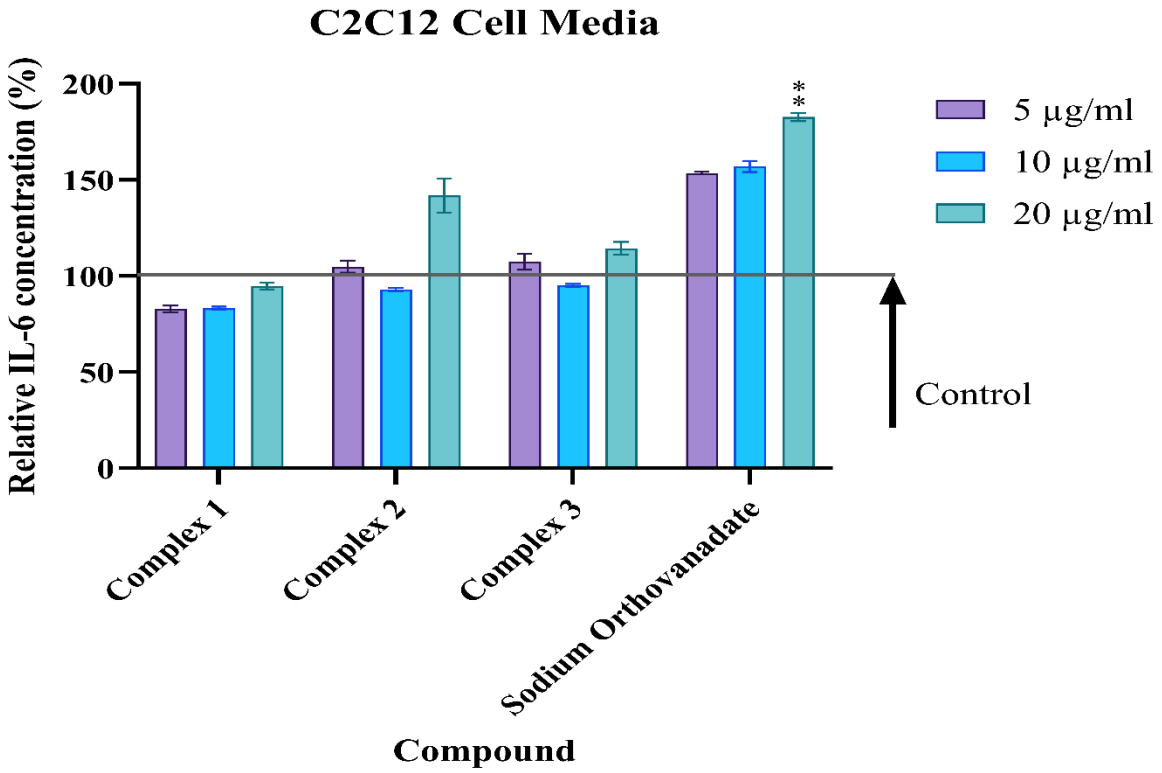


Figure 3.17: Relative IL-6 concentration in C2C12 cell media after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control, and the asterisk (**) represents the statistical difference between the test compounds and the control at $p < 0.01$

For the HepG2 cell media, as displayed in Figure 3.18, the Schiff base vanadium complexes (1, 2, and 3) and sodium orthovanadate exhibited varied effects on IL-6 concentration compared to the control. Schiff base vanadium complex 1 consistently showed a trend towards decreased IL-6 concentration. Schiff base vanadium complexes 2 and 3, as well as sodium orthovanadate,

generally showed a trend towards increased IL-6 concentration, particularly at the higher concentrations (10 and 20 µg/ml). The one-way ANOVA analysis did not reveal a statistically significant interaction among compounds and concentrations ($F = 1.66$, $p = 0.11$). Dunnett's post hoc analysis against the control did not reveal any statistically significant differences in IL-6 concentration

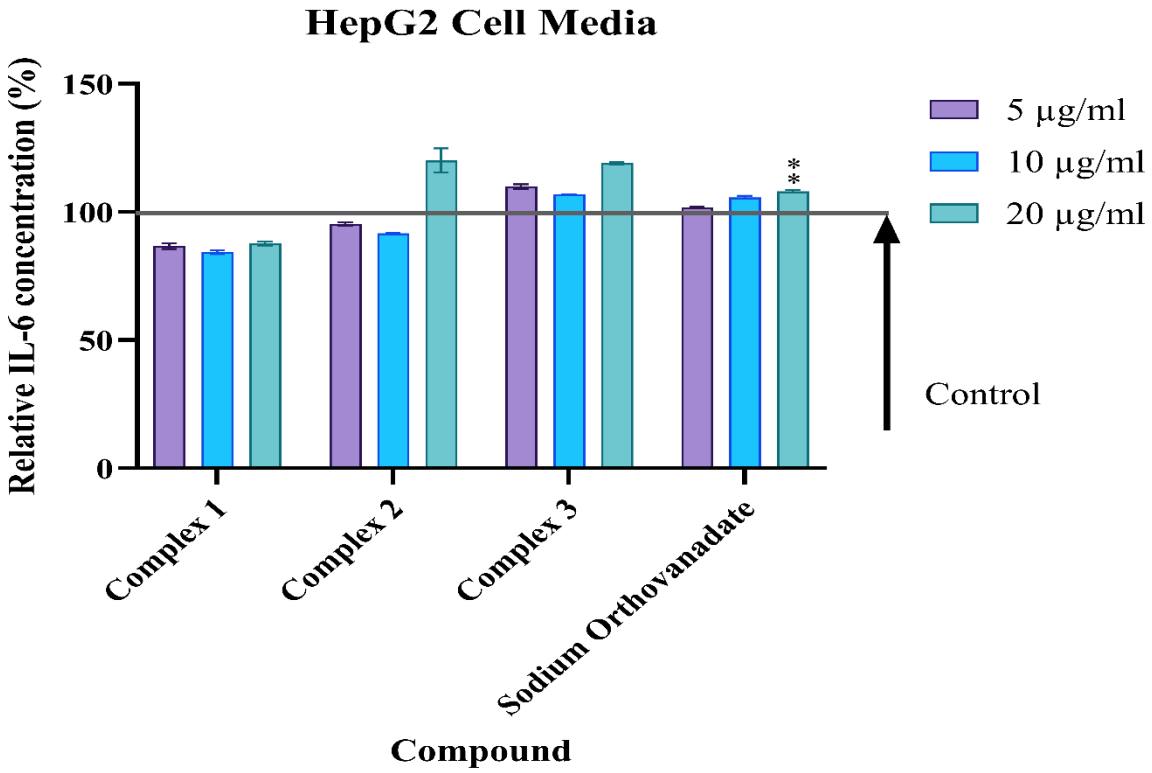


Figure 3.18: Relative IL-6 concentration in HepG2 cell media after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control, and the asterisk (**) represents the statistical difference between the test compounds and the control at $p < 0.01$

3.1.5.2 Relative MMP1 concentration

The effect of the Schiff base vanadium complexes and sodium orthovanadate, which served as a positive control, on relative MMP1 concentration was evaluated in C2C12 and HepG2 cell media

at concentrations of 5, 10, and 20 µg/ml. Relative concentration is expressed as a percentage compared to the untreated control.

For C2C12 cell media, as displayed in Figure 3.19, the Schiff base vanadium complex 1 significantly increased MMP1 concentration compared to the control at 5 and 10 µg/ml. However, at 20 µg/ml, its effect was similar to the control. Schiff base vanadium complexes (2 and 3), as well as sodium orthovanadate, generally showed no significant change in MMP1 concentration compared to the control across the tested concentrations, although some showed minor increases or decreases. A one-way ANOVA analysis suggested a possible interaction effect among compounds and concentrations ($F = 1.91$, $p = 0.057$), approaching significance at the $\alpha = 0.05$ level. Dunnett's post hoc analysis confirmed the significant increase in MMP1 concentration with Schiff base vanadium compound 1 at concentrations of 5 and 10 µg/ml compared to the control.

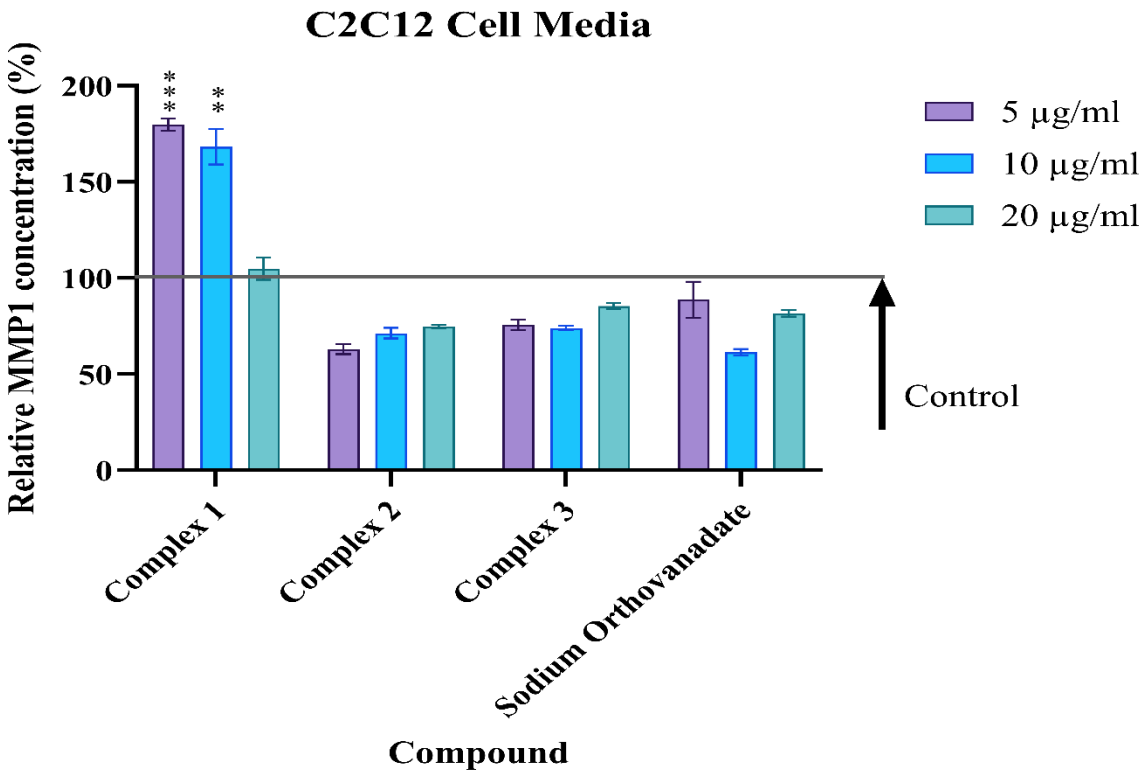


Figure 3.19: Relative MMP1 concentration in C2C12 cell media after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control, and

the asterisk (**) represents the statistical difference between the test compounds and the control at $p < 0.01$, (***) $p < 0.001$

For HepG2 cell media, as displayed in Figure 3.20, the Schiff base vanadium complexes (2 and 3) generally showed a trend towards increased MMP1 concentration compared to the control, while Schiff base vanadium complex 1 showed concentration much closer to the control. Sodium orthovanadate consistently demonstrated the largest increase in MMP1 concentration across all concentrations. A one-way ANOVA analysis did not suggest an interaction effect among compounds and concentrations ($F = 0.26$, $p = 0.99$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis revealed statistically significant increases in MMP1 concentration for Schiff base vanadium complex 2 at 5, 10, and 20 µg/ml and for Schiff base vanadium complex 2 and sodium orthovanadate at 5, 10, and 20 µg/ml compared to the control. Additionally, Schiff base vanadium complex 3 showed a statistically significant increase at 20 µg/ml.

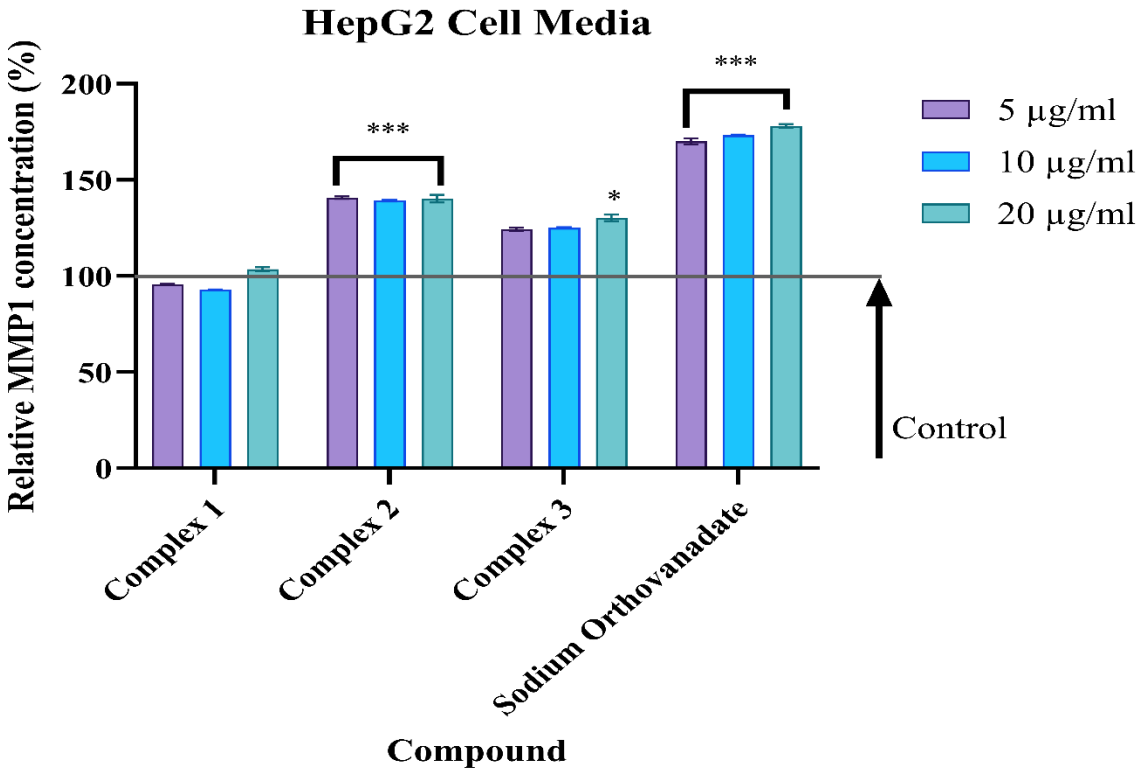


Figure 3.20: Relative MMP1 concentration in HepG2 cell media after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The untreated control is shown by the labelled horizontal

line, and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$, (***) $p < 0.001$

3.1.5.3 Relative DPP4 concentration

The effect of the Schiff base vanadium complexes, with sodium orthovanadate, which served as a positive control, on relative DPP4 concentration was evaluated in C2C12 cell media at concentrations of 5, 10, and 20 μg/ml. The relative concentration is expressed as a percentage compared to the untreated control.

In the C2C12 cell media, as displayed in Figure 3.21, The Schiff base vanadium compounds (1, 2, and 3) and sodium orthovanadate generally showed a trend towards decreased DPP4 concentration compared to the control. This effect was generally more pronounced at lower concentrations. The one-way ANOVA analysis did not suggest a statistically significant interaction effect among compounds and concentrations ($F = 1.22$, $p = 0.29$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the control did not reveal any statistically significant differences in DPP4 concentration.

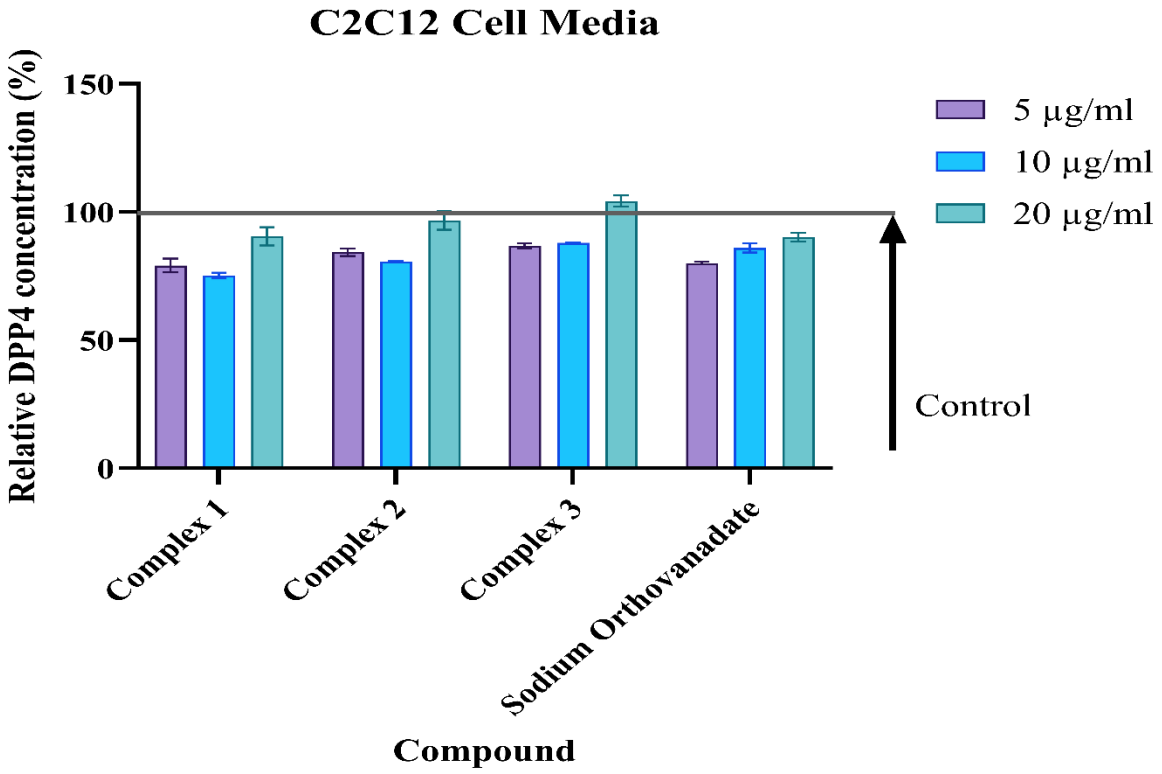


Figure 3.21: Relative DPP4 concentration in C2C12 cell media after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control

For HepG2 cell media, as displayed in Figure 3.22, the Schiff base vanadium compounds (1, 2, and 3) and sodium orthovanadate generally showed no major changes in DPP4 concentration compared to the control across the tested concentrations (5, 10, and 20 µg/ml). However, Schiff base vanadium compound 3 at 20 µg/ml showed a noticeable decrease in DPP4 concentration. A one-way ANOVA analysis did not reveal a statistically significant interaction among compounds and concentrations ($F = 1.12$, $p = 0.36$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the control did not reveal any statistically significant differences in DPP4 concentration.

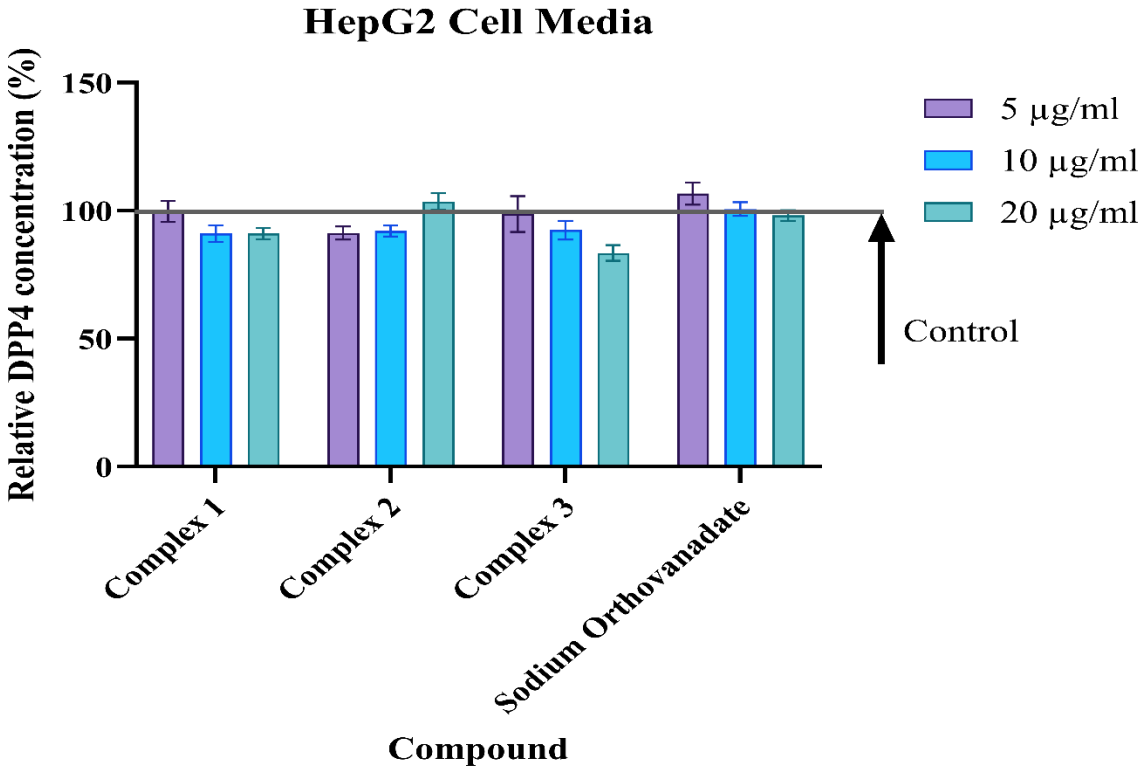


Figure 3.22: Relative DPP4 concentration in HepG2 media after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control

3.1.6 Citrate synthase activity

The effect of the Schiff base vanadium complexes and sodium orthovanadate, which served as a positive control, on citrate synthase activity was evaluated in C2C12 and HepG2 cell media at concentrations of 5, 10, and 20 µg/ml. Relative activity is expressed as a percentage compared to the untreated control.

For C2C12 cells, as displayed in Figure 3.23, treatment with the vanadium complexes (1, 2, and 3) showed varying effects on the activity of citrate synthase compared to the control. Complex 1 at 5 µg/ml showed an increase in activity, but activity decreased at higher concentrations (10 and 20 µg/ml). Complexes 2 showed relatively minor fluctuations compared to the control with no clear dose-dependent trend. Complex 3 showed a minor increase in activity at the concentrations

of 5 µg/ml and 20 µg/ml and a significant increase in activity at 10 µg/ml. Sodium orthovanadate consistently increased citrate synthase activity across all concentrations tested (5, 10, and 20 µM). A one-way ANOVA indicated a statistically significant interaction among compounds and concentrations ($F = 2.28$, $p = 0.022$) at the $\alpha = 0.05$ level. Dunnett's post hoc test revealed statistically significant increases in citrate synthase activity for complex 1 at the lowest concentration (5 µg/ml) and for complex 2 at 10 µg/ml. Sodium orthovanadate exhibited statistically significant increases in citrate synthase activity across all tested concentrations, and insulin similarly demonstrated statistically significant increases.

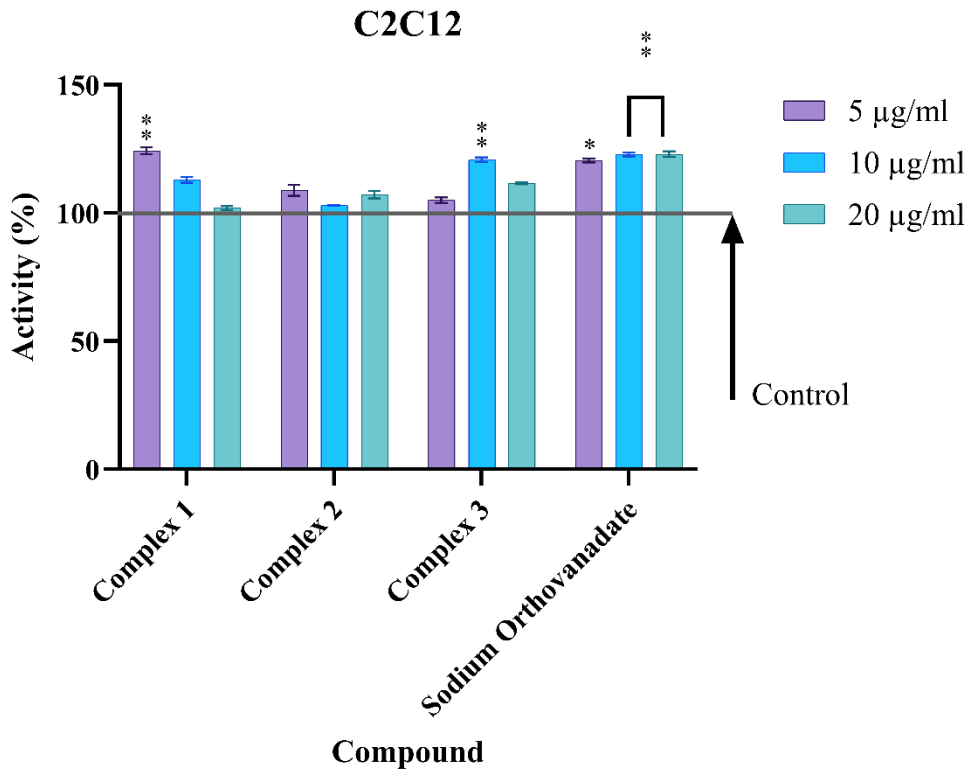


Figure 3.23: Citrate synthase activity in C2C12 cells after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control, and the asterisk (*) represents the statistical difference between the test compounds and the control at $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

For the HepG2 cell line, as displayed in Figure 3.24, the Schiff base vanadium complexes (1, 2, and 3) generally showed a trend towards decreased citrate synthase activity compared to the control, with this effect becoming more pronounced at higher concentrations (10 and 20 µg/ml). On the other hand, sodium orthovanadate showed varied effects, slightly decreasing activity at 5 µg/ml but increasing activity at 10 µg/ml and a slight increase in activity at 20 µg/ml. The one-way ANOVA analysis did not indicate a statistically significant interaction among the groups on citrate synthase activity ($F = 0.87$, $p = 0.62$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the control did not reveal any statistically significant differences in citrate synthase activity.

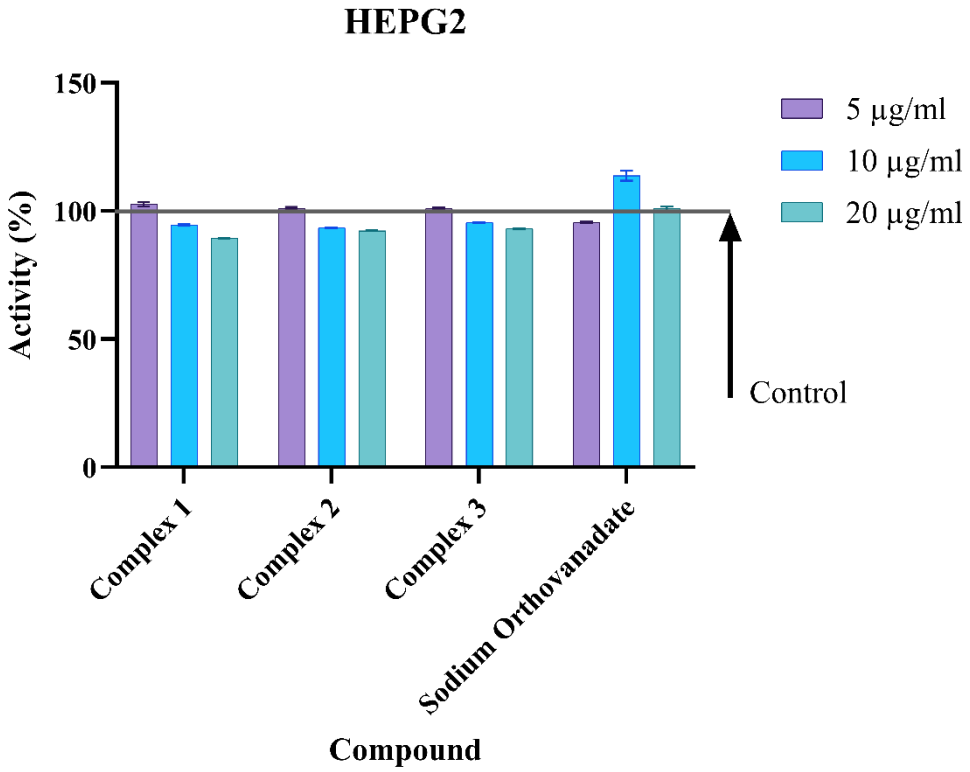


Figure 3.24: Citrate synthase activity in HepG2 cells after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control

3.1.7 Cell media pH

The effect of the Schiff base vanadium complexes, with sodium orthovanadate serving as a positive control, on cell media pH was evaluated in C2C12 and HepG2 cell media at concentrations of 5, 10, and 20 µg/ml.

For the C2C12 cell line, as displayed in Figure 3.25, the analysis revealed minimal changes in media pH for all the compounds compared to the control. One-way ANOVA analysis did not indicate a statistically significant interaction effect among compound and concentration ($F = 0.80$, $p = 0.71$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the control did not reveal any statistically significant differences in media pH.

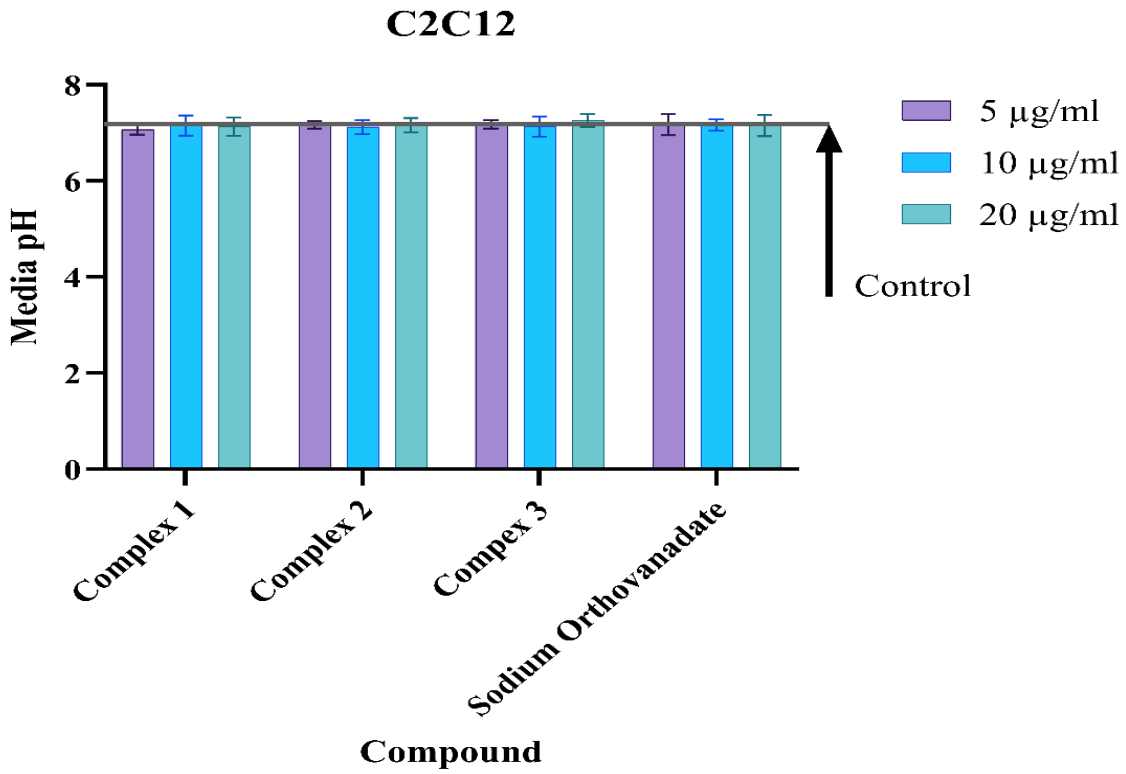


Figure 3.25: Media pH in C2C12 cells after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control

For HepG2 cells, as displayed in Figure 3.26, the analysis revealed slight increases in pH across all compounds and concentrations, except for Complex 1 at 10 µg/ml and Complex 2 at 5 µg/ml, which exhibited pH values closer to the control. The one-way ANOVA analysis did not indicate

a statistically significant interaction effect between compound and concentration ($F = 0.85$, $p = 0.63$) at the $\alpha = 0.05$ level. Dunnett's post hoc analysis against the control did not reveal any statistically significant differences in media pH.

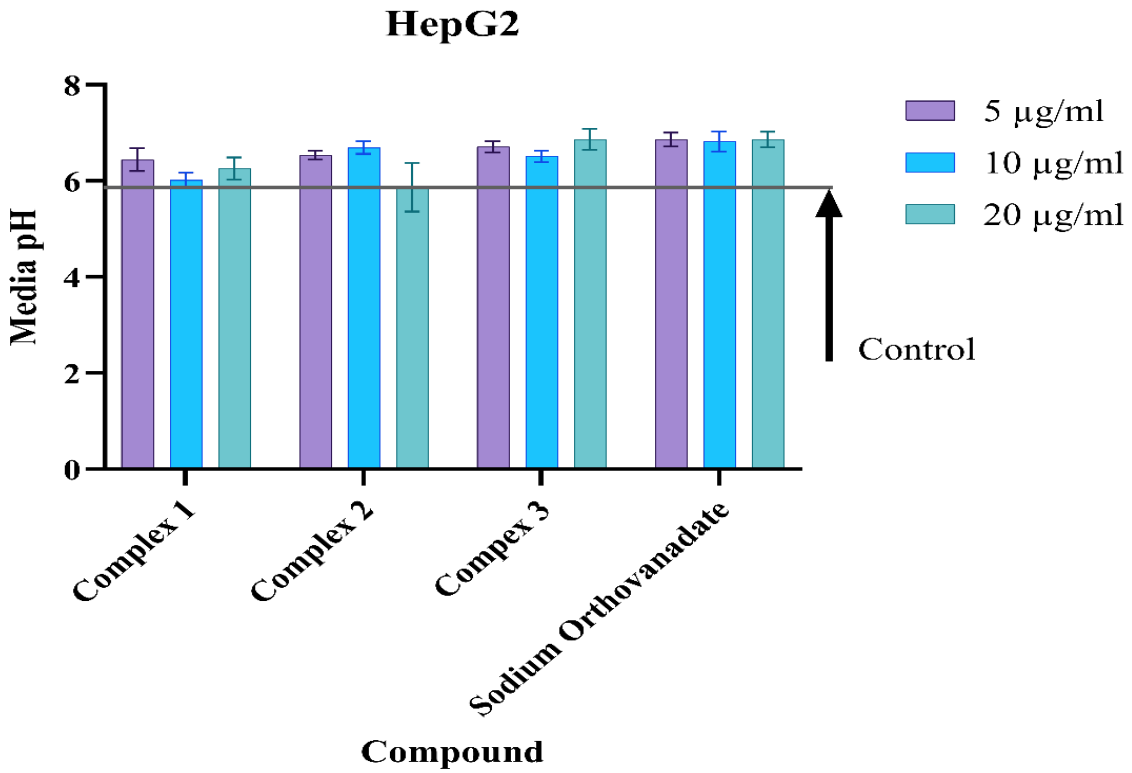


Figure 3.26: Media pH in HepG2 cells after treatment with vanadium compounds at concentrations of 5, 10, and 20 µg/ml for 24 hours. The data are presented as mean ± SD, with error bars (n = 3). The labelled horizontal line shows the untreated control

3.2 Cell-free assays

3.2.1 Pharmacokinetic properties of vanadium complexes

The drug-likeness of the vanadium complexes was assessed using the Lipinski parameters via the online Swiss ADME prediction tool, with the results shown in Table 2 below. Complexes 1 and 2 produced identical output data due to their identical canonical SMILES structures, resulting in

similar physicochemical properties. Both complexes violated one of Lipinski's parameters because their molecular weights exceeded the recommended 500 g/mol threshold. Complex 3, however, showed two violations: a molecular weight above 500 g/mol and a higher-than-acceptable number of hydrogen bond acceptors (13, exceeding the limit of 10). Despite these violations, all three complexes fall within the acceptable drug-likeness threshold, as compounds can typically have up to two violations and still be considered drug-like.

Table 2: Lipinski parameters of vanadium compounds

Compound	Molecular Weight (g/mol)	Donor Hydrogen Bond	Acceptor Hydrogen Bond	Lipophilicity Log P o/wa[a]	#rotorb[b]	Rule of 5 violations
Acceptable range	≤ 500	≤ 5	≤ 10	-2.0 ≤ x ≤ 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
BMOV	317.15	0	7	- 0.58	0	0
$NaVO_3$	121.93	0	13	3.62	0	1

The pharmacokinetic properties of the vanadium complexes (Complex 1, Complex 2, and Complex 3) were assessed alongside two reference compounds, BMOV and sodium orthovanadate, focusing on key factors influencing oral bioavailability, which are gastrointestinal (GI) absorption, water solubility, metabolism, and bioavailability as shown in Table X. The pharmacokinetic properties of the tested compounds are summarized in Table 3. Complexes 1, 2, and 3 displayed low gastrointestinal (GI) absorption, poor water solubility, metabolism primarily through CYP2C19, CYP2C9, and CYP3A4, and low bioavailability (0.11). BMOV showed high GI absorption, soluble in water, and had a higher bioavailability (0.56), with P-glycoprotein as an additional factor in its metabolism. NaVO exhibited low GI absorption, poor solubility, metabolism by CYP2C9 and CYP3A4, and low bioavailability (0.11).

Table 3: Summary of pharmacokinetic properties of vanadium compounds

Compound	Gastrointestinal	Water Solubility	Metabolism	Bioavailability

	Absorption			
Complex 1	Low	Poorly Soluble	CYP2C19, CYP2C9, CYP3A4	0.11
Complex 2	Low	Poorly Soluble	CYP2C19, CYP2C9, CYP3A4	0.11
Complex 3	Low	Poorly Soluble	CYP2C9, CYP3A4	0.11
BMOV	High	Soluble	P-gp	0.56
$NaVO_3$	Low	Poorly Soluble	CYP2C9, CYP3A4	0.11

3.2.2 Molecular docking

The docking analysis revealed that the Schiff base vanadium complexes and reference compounds, including BMOV and sodium orthovanadate, demonstrated distinct binding affinities and interactions with the protein PTP1B. Among the Schiff base complexes, Complex 3 exhibited the highest binding affinity (-8.9 kcal/mol), forming key interactions with residues such as Tyr46 and Cys215, while Complex 1 showed the lowest affinity (-7.9 kcal/mol). Sodium orthovanadate, with a binding affinity of -3.5 kcal/mol, formed minimal interactions, primarily hydrophobic. BMOV, with a binding affinity of -8.4 kcal/mol, displayed significant interactions through both hydrogen bonds and hydrophobic contacts, similar to the Schiff base complexes. These results indicate that the Schiff base complexes, particularly Complex 3, exhibit favourable docking characteristics for PTP1B inhibition, with interaction profiles comparable to BMOV.

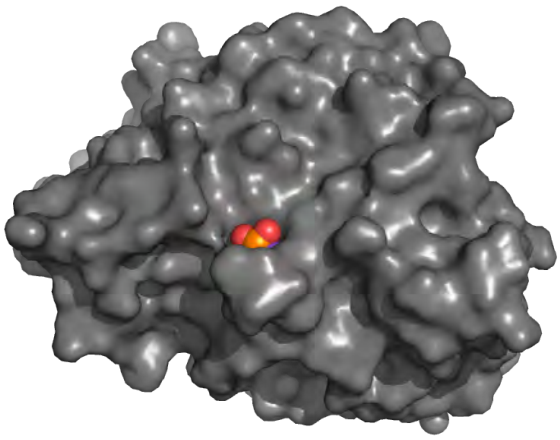


Figure 3.27: 3D render of sodium orthovanadate docked into PTP1B

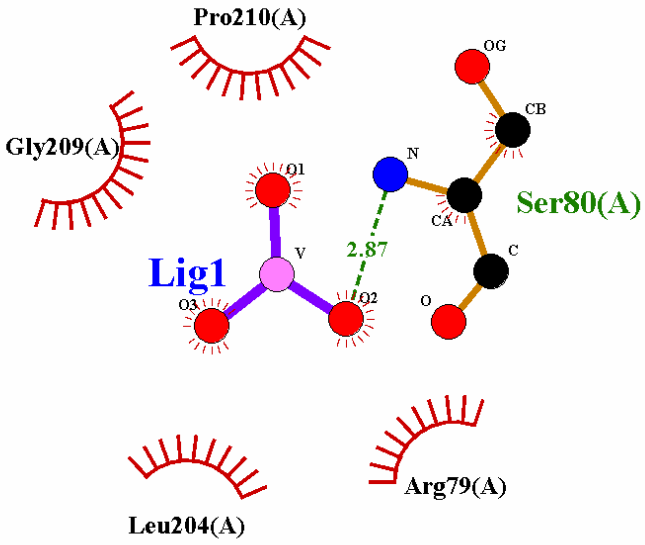


Figure 3.28: 2D Plot of Sodium orthovanadate docked into PTP1B, showing hydrophobic interactions with Pro210, Gly209, Leu204 and Arg79 as well as hydrogen bonds with ser80

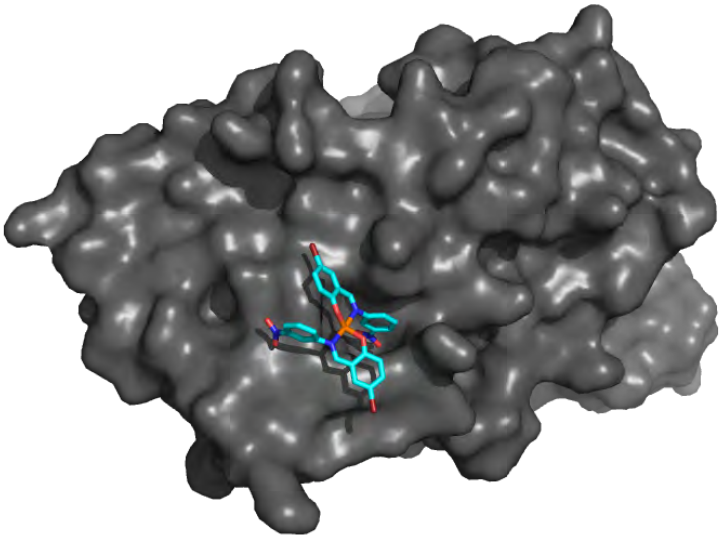


Figure 3.29: 3D render of Schiff base vanadium complex 1 docked into PTP1B

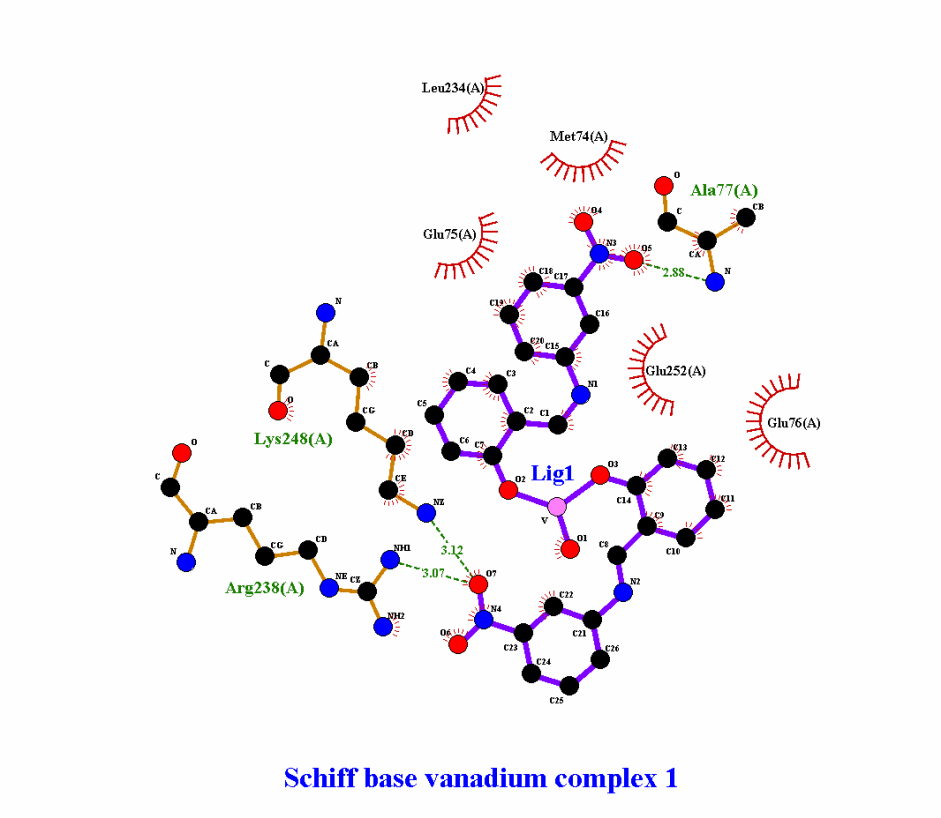


Figure 3.30: 2D plot of Schiff base vanadium complex 1 docked into PTP1B, showing hydrophobic interactions with Leu234, Met74, Glu75, Glu252 and Glu76 as well as hydrogen bonds with Ala77, Arg238 and Lys248

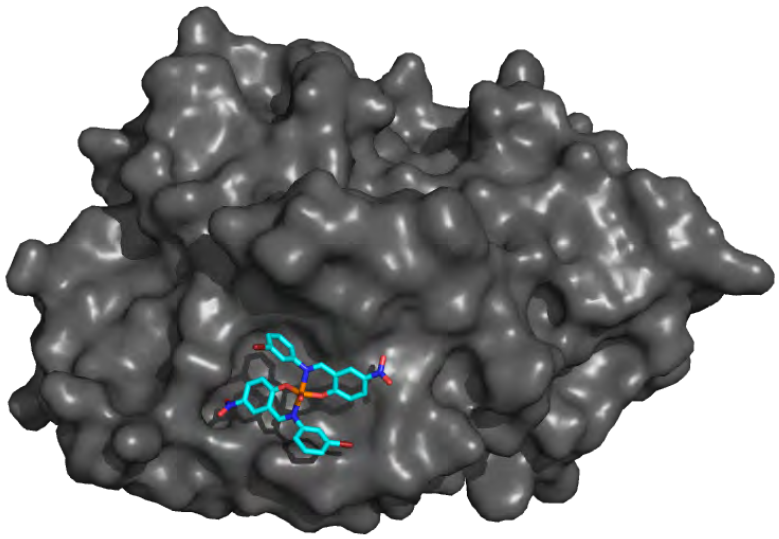


Figure 3.31: 3D render of Schiff base vanadium complex 2 docked into PTP1B

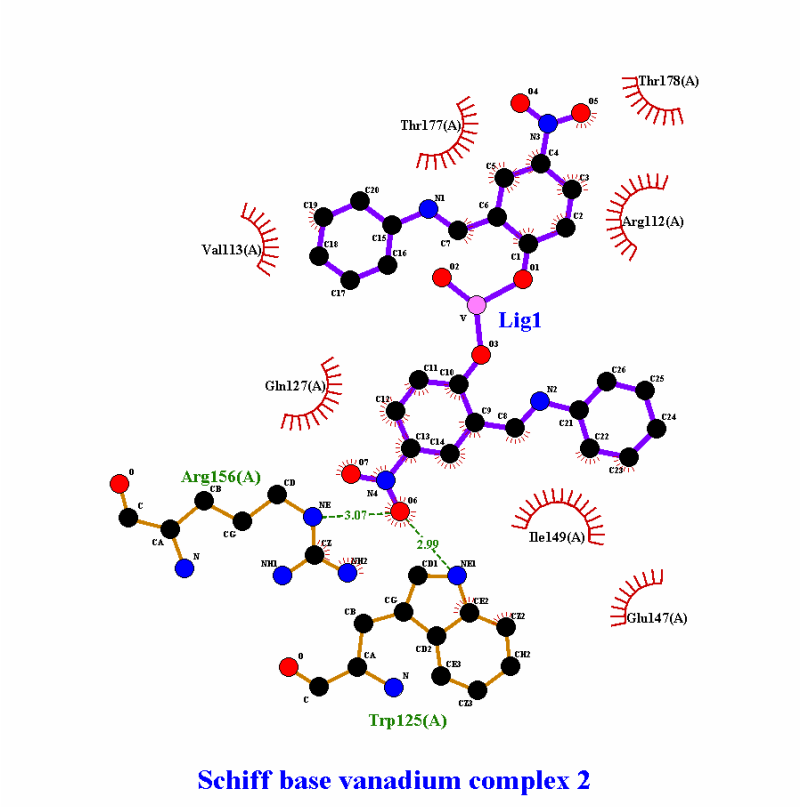


Figure 3.32: 2D plot of Schiff base vanadium complex 2 docked into PTP1B, showing hydrophobic interactions with Thr177, Thr178, Arg112, Val113, Gln127, Ile149, Glu147 as well as hydrogen bonds with Trp125 and Arg156

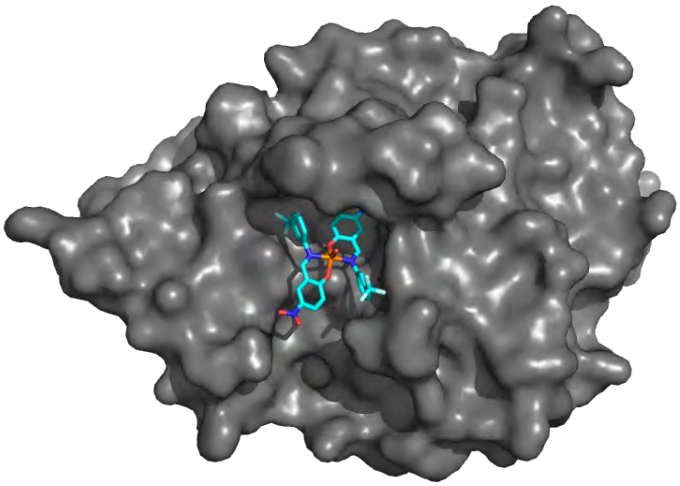
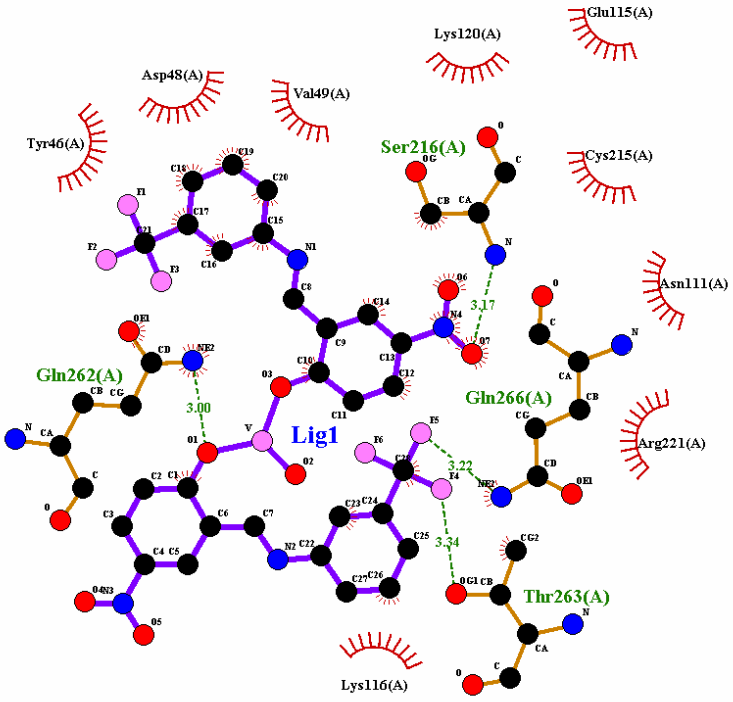


Figure 3.33: 3D render of Schiff base vanadium complex 3 docked into PTP1B



Schiff base vanadium complex 3

Figure 3.34: 2D plot of Schiff base vanadium complex 3 docked into PTP1B, showing hydrophobic interactions with Tyr46, Asp48, Val49, Lys120, Glu115, Cys215, Asn111, Arg221 and Lys116 as well as hydrogen bonds with Gln262, Gln266, Ser216 and Thr263

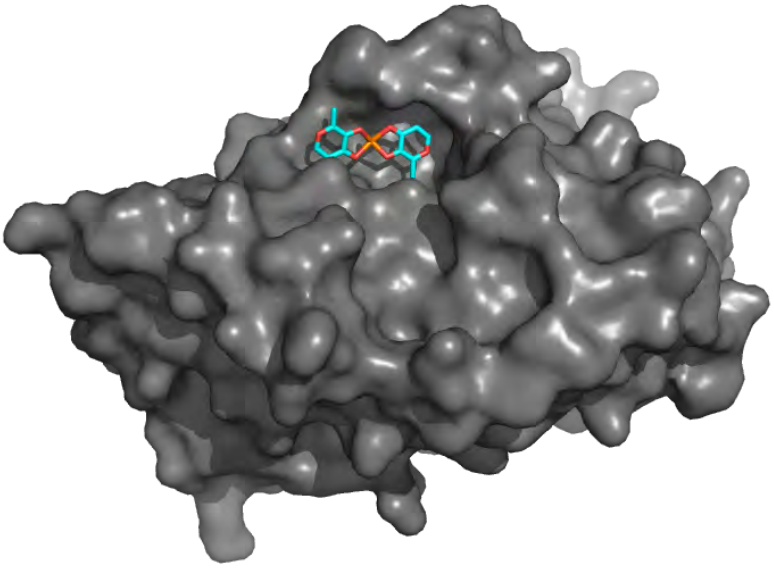
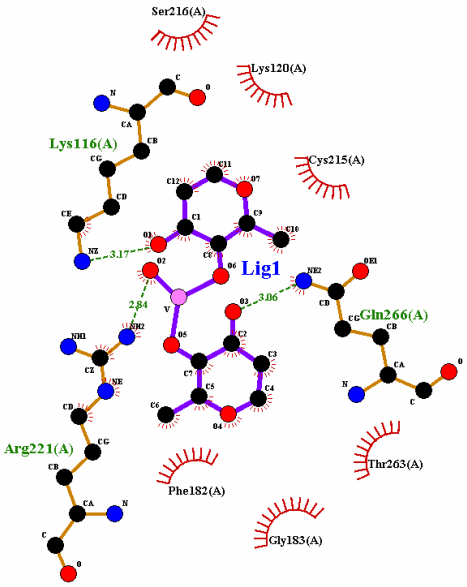


Figure 3.35: 3D render of BMOV docked into PTP1B



Bis(maltolato)oxovanadium(IV)

Figure 3.36: : 2D plot of BMOV docked into PTP1B, showing hydrophobic interactions with Ser216, Lys120, Cys215, Thr263, Gly183 and Phe182 as well as hydrogen bonds with Arg221, Lys116 and Gln266

Table 4: Summary of protein docking results

	Sodium orthovanadate	Schiff base vanadium complex 1	Schiff base vanadium complex 2	Schiff base vanadium complex 3	Bis(maltolato)oxovanadium(IV)
Hydrophobic Interactions	Pro210, Gly209, Leu204, Arg79	Leu234, Met74, Glu75, Glu252, Glu76	Thr177, Thr178, Arg112, Val113, Gln127, Ile149, Glu147	Tyr46, Asp48, Val49, Lys120, Glu115, Cys215, Asn111, Arg221, Lys116	Ser216, Lys120, Cys215, Thr263, Gly183, Phe182
Hydrogen Bonds	Ser80	Ala77, Arg238, Lys248	Trp125, Arg156	Gln262, Gln266, Ser216, Thr263	Arg221, Lys116, Gln266
Affinity (kcal/mol)	-3.5	-8.6	-7.9	-8.9	-8.4

Chapter 4 : Discussion and Conclusion

4.1 Discussion

Insulin therapy for managing DM has several drawbacks, including high costs and potential side effects, which can negatively influence treatment adherence of people living with the condition. These factors significantly impact patient quality of life, particularly in resource-limited settings where socioeconomic barriers and infrastructural challenges restrict access to insulin. As a result, there has been a continuous search for alternative therapies that are more accessible, cost-effective, and user-friendly, with an improved safety and efficacy profile. Vanadium complexes have emerged as a promising option, as earlier research with vanadium salts showed encouraging results highlighting their insulin-mimetic properties. These vanadium compounds enhanced glucose uptake, stimulated insulin signalling pathways, and reduced hyperglycaemia in diabetic animal models. However, the development of vanadium-based therapies was hindered by issues related to toxicity associated with the chronic administration of vanadium salts (188). Later research focused on reducing the toxicity of vanadium compounds by improving on the poor oral bioavailability of vanadium salts by chelating them with organic ligands, leading to much more promising outcomes by positively influencing processes of absorption, transport and stability of the complexes, therefore, overall improving their antidiabetic activity. This research envisaged investigating the potential enhancement of the insulin-mimetic activity of vanadium compounds in cell line models by chelating them with Schiff base ligands, aiming to overcome the main challenges of toxicity and bioavailability that have hindered vanadium clinical applications. Therefore, this research ultimately seeks to contribute to the development of safer and more effective antidiabetic therapies.

Our study investigated cytotoxicity by assaying Schiff base vanadium complexes in an MTT cell viability assay in C2C12 and HepG2 cell lines. Sodium orthovanadate was used as a reference form of vanadium compound. The results demonstrated that although viability decreased with increased concentrations of treatment with the vanadium complexes, it did not compromise the overall viability of both cell lines since it was higher than the recommended cytotoxicity threshold of 80% according to the International Organization of Standardisation (ISO) where these values of more than 80% viability are classified under slight cytotoxicity (189). These findings are consistent with the study by Kowalski et al., which demonstrated the non-cytotoxic profile of a Schiff base vanadium complex derived from phenanthroline at concentrations similar to those in our study in a pancreatic ductal adenocarcinoma cell line (PANC-1). Notably, they observed viability values exceeding 80% at concentrations corresponding to the highest concentration in our research (20 µg/ml). Additionally, their study reported a dose-dependent decrease in cell viability, mirroring our observations (190). This is further supported by a study conducted by Romanowski et al., who performed an MTT assay on Schiff base vanadium complexes derived from chiral 3-amino-1,2-propanediol enantiomers in the Ht-22 neuronal cell line. They observed viability values exceeding 80% at concentrations equivalent to the highest concentration in our study (20 µg/ml). Similar to our findings, their study demonstrated a dose-dependent decrease in cell viability with increasing concentrations (191).

The non-cytotoxic profile of Schiff base vanadium complexes represents a promising advancement for the clinical application of vanadium in DM therapy. One of the major limitations of vanadium therapy has been gastrointestinal (GI) upset, which is directly linked to the cytotoxicity of vanadium compounds. This cytotoxicity manifests through multiple mechanisms, including direct mucosal irritation of intestinal epithelial cells via oxidative stress and disruption of ion transport channels, particularly Na^+/K^+-ATPase activity, which disturbs normal fluid homeostasis in the gut (192). Therefore, the reduced cytotoxicity observed in these Schiff base vanadium complexes suggests potential mitigation of these adverse GI effects (193).

Interestingly, at lower concentrations, some of the viability values for the Schiff base vanadium complexes in our study were more than 100%. This viability above the untreated control is a promising result in terms of the glucose uptake-enhancing properties of these compounds, as the

MTT cell viability assay measures the reduction of MTT into formazan by succinate dehydrogenase, which is found in metabolically active cells and is crucial for cellular energy production through its roles in cellular ATP generation and overall cellular metabolism. Therefore, this finding may indicate an increase in the metabolic activity of the cells (194). Turtoi et al., also observed this in an MTT cell viability assay on HepG2 cells using Schiff base vanadium complexes derived from L-/D- valine, where they observed cell viability values of more than 100% of the control (195). However, it is important to acknowledge that increased mitochondrial activity can also come from cellular stress (196). The possible increase in metabolic activity observed in our MTT cell viability assay aligns well with the increase in glucose uptake observed in the glucose uptake assays in our study.

To further understand the effect of our compounds on cytotoxicity, we explored mitochondrial health by examining cellular citrate synthase activity. The Schiff base vanadium complexes showed a minimal yet noteworthy trend in reducing citrate synthase activity, particularly at higher concentrations, which could indicate a subtle potential for cytotoxicity, as observed in the MTT assay. Importantly, the assay specifically targeted cytoplasmic citrate synthase, which is not typically present in healthy cells. The presence of citrate synthase in the cytoplasm suggests mitochondrial leakage, a sign of mitochondrial damage. Citrate synthase, a key enzyme in the TCA cycle, is critical for cellular energy production, and its leakage into the cytoplasm may reflect early signs of mitochondrial stress or compromised metabolic function (197). This observation aligns with the idea that mitochondrial damage can lead to the release of citrate synthase into the cytoplasm, further supporting the potential cytotoxic effects of the complexes. However, the absence of statistically significant changes across the treatments, as determined by post hoc analysis, suggests that these effects must be more pronounced to indicate a severe impact on mitochondrial function conclusively. While not definitive, these findings raise the possibility of subtle cytotoxic effects associated with these compounds, particularly at higher concentrations. Vanadium compounds have been shown to generate reactive oxygen species (ROS), which can lead to oxidative stress, mitochondrial membrane depolarization and cytotoxicity. This aligns with findings from a review by Aureliano et al., which highlights the role of lipid peroxidation (LPO) in vanadium-induced oxidative stress. LPO, driven by ROS, can disrupt cellular membrane integrity and impair mitochondrial function. The study further

emphasizes the importance of vanadium speciation, as the diverse forms of vanadium under physiological conditions can exhibit distinct effects, both protective and harmful (198,199).

The tendency of vanadium to undergo speciation changes, alternating between its +4 oxidation state as (oxovanadium(IV), VO^{2}) and +5 oxidation state as (Orthovanadate, VO_4^{3-}) in physiological environments is a significant limitation for vanadium therapy, as these compounds alter their structure when exposed to acidic and reductive conditions within the gastrointestinal tract and bloodstream, therefore diminishing their effectiveness or even being harmful. Therefore, the therapeutic form of vanadium should be preserved long enough to reach the target cells to achieve optimal therapeutic outcomes for patients (200,201).

The media pH assay showed that the vanadium complexes did not significantly alter the pH of the culture medium. Therefore, reducing the risk of speciation changes at the target site. This also supports the citrate synthase results, which suggests that mitochondrial function was not compromised after treatment with the vanadium complexes. Media pH is closely related to mitochondrial function because changes in extracellular pH can influence intracellular pH, which in turn affects the mitochondria, which are highly sensitive to pH changes, as this can disrupt the proton gradient across the mitochondrial membrane (202). This gradient is critical for ATP production; therefore, the stable pH observed in the media pH assays suggests that the vanadium complexes did not induce conditions that might disrupt the proton gradient and, therefore, impair mitochondrial function .

The findings that these vanadium complexes may not severely compromise mitochondrial function are important since mitochondrial dysfunction is a major side effect of some antidiabetic drugs, such as thiazolidinediones. A study by Hu et al. showed that troglitazone can induce mitochondrial respiratory dysfunction, oxidative stress, and structural changes in Human hepatoma cells (HepaRG), which, therefore, can lead to cell death through decreased oxygen consumption and ATP levels (203). Therefore, this presents a significant advantage of the Schiff base vanadium complexes for DM therapy, as they appear to maintain mitochondrial integrity while potentially offering therapeutic benefits.

Cytotoxicity often leads to secondary inflammatory responses, as damage caused to the cells triggers the release of cytokines such as MMP1, DPP4 and IL-6. This is particularly relevant in the context of DM, where chronic inflammation exacerbates the progression of the disease and insulin resistance (55). To explore the impact of vanadium complexes on inflammation, we investigated their effects on MMP1 expression, which revealed distinct, compound and cell-type-specific trends. In C2C12 cells, the reduction in MMP1 expression observed with Schiff base vanadium compounds 1 and 2, even at the highest tested concentrations, suggests their potential to modulate extracellular matrix remodelling in skeletal muscle cells. This effect could be advantageous in therapeutic contexts where reduced MMP1 activity is desirable, such as in controlling tissue damage or inflammation, which is particularly important in a DM context where chronic inflammation leads to further complications related to DM. However, the dose-dependent increase in expression within this lower range highlights a concentration-dependent balance that warrants further exploration. Conversely, the pronounced increase in MMP1 expression observed with Compound 3 at higher concentrations suggests a potential pro-inflammatory effect, particularly in the C2C12 context. While this may indicate off-target effects or stress-induced signalling pathways, the significance of these increases only at higher doses highlights a threshold-dependent response that could limit the therapeutic window of these compounds. In HepG2 cells, the consistent suppression of MMP1 expression by Schiff base vanadium compound 1 across all concentrations underscores its potential as a regulator of hepatic extracellular matrix dynamics. The mixed effects observed with Compounds 2 and 3 and the overall trend toward increased expression with sodium orthovanadate reflect the complexity of MMP1 regulation in liver cells, which is influenced by diverse signalling pathways related to liver function and metabolism. The significant suppression of MMP1 expression by Compound 1 in HepG2 cells further supports its specificity and potential as a therapeutic candidate.

The observed effects of Schiff base vanadium complexes on DPP4 expression underscore the complexity of their biological activities and suggest differential mechanisms of action that may be cell-type specific. The reduction in DPP4 expression by Compound 1 in C2C12 cells aligns with its potential therapeutic relevance, as lower DPP4 levels are generally associated with enhanced glucose tolerance and insulin sensitivity (204). This effect, remarkably consistent across all tested concentrations, may indicate a direct inhibitory interaction with DPP4. In

contrast, the trend toward increased DPP4 expression with Compounds 2 and 3 in this cell line at higher concentrations raises questions about their dual roles, as elevated DPP4 levels may indicate possible inflammation, potentially reflecting compensatory mechanisms or off-target effects that emerge at higher doses. In HepG2 cells, the consistent decrease in DPP4 expression across all compounds is notable, suggesting that the metabolic context of the liver may favour the suppression of this enzyme more than in skeletal muscle tissue. DPP4 is a critical regulator of incretin hormones, and its inhibition is a validated strategy for managing T2DM (98). The observed reduction in DPP4 expression highlights the pharmacological potential of these compounds, particularly in hepatic contexts where this effect was consistently observed with the vanadium complexes. These observations echo the findings from the MMP1 expression assay. This correlation can be explained by the literature findings from a study by Röhrborn et al., who reported that MMP1 mediates DPP4 shedding (205). Our findings suggest a mechanistic link between the DPP4 and the MMP1 expression patterns across both cell lines. The correlations between reduced MMP1 expression and decreased DPP4 levels suggest that MMP1-mediated DPP4 shedding may be a key mechanism influenced by these vanadium complexes. Therefore, this relationship provides insights into how these complexes may modulate inflammatory responses through a shared pathway.

To further understand the effect of our compounds on inflammation, the subsequent investigation of IL-6 expression in cells treated with these compounds revealed noteworthy results, particularly for Compound 1 in C2C12 cells. An increase in IL-6 expression was observed in cells treated with these compounds. This finding may explain the results from the glucose uptake assays where Compound 1 did not exhibit the same anti-diabetic effect as Compound 2 and Compound 3, suggesting that cells treated with Compound 1 were under stress, potentially indicating cytotoxicity associated with this compound, which is also correlated with the MTT cell viability assays where compound 1 showed slight toxicity. Similarly, the increase in IL-6 expression observed aligns with existing literature, which has reported elevated IL-6 levels in response to vanadium compound treatment (206). However, it is important to note that IL-6 is a pleiotropic cytokine with both pro-inflammatory and anti-inflammatory roles (207). Therefore, the expression of IL-6 alone does not definitively indicate cytotoxicity. Further functional assays,

such as lactate dehydrogenase (LDH) release (208), would be required to more conclusively assess cytotoxic effects associated with Compound 1 in particular.

The observed effects of the Schiff base vanadium complexes on inflammation can be explained from the literature where they have shown therapeutic potential in modulating inflammation at low doses, as seen in a study by Ki et al,. (174) where the researchers observed the anti-inflammatory potential of their Schiff base vanadium complexes derived from tryptamine and observed these potential therapeutic effects at low concentrations in the nanomolar treatment ranges, therefore evading the issues of toxicity. Hence, the observed anti-inflammatory properties of Schiff base vanadium complexes, as demonstrated by their efficacy at low nanomolar concentrations, highlight their potential as therapeutic agents with minimal toxicity concerns.

Building on this foundation, the glucose uptake assays provided evidence of the potential insulin-mimetic effects of these vanadium complexes. In C2C12 cells, Schiff base vanadium complexes 2 and 3 showed significant increases in glucose uptake at the 10 µg/ml concentration compared to the untreated control. The highest uptake values for most compounds were observed at 10 µg/ml, indicating a therapeutic window for maximizing glucose uptake without affecting cell viability. Schiff base vanadium compound 1 displayed limited glucose uptake enhancement, with values similar to the control at lower concentrations and a decrease at 20 µg/ml.
In HepG2 cells, the response to treatment with the vanadium complexes did not exhibit a clear peak at 10 µg/ml. Both 10 and 20 µg/ml concentrations showed similar levels of glucose uptake for all tested compounds, suggesting a broader range of effective doses. While the insulin-treated control showed slightly increased glucose uptake over the untreated control, the Schiff base vanadium complexes and sodium orthovanadate control generally showed modest increases in glucose uptake, with complex 2 showing the highest uptake among the complexes. The significant interaction effect between compound and concentration in HepG2 cells highlights the importance of dose optimization to enhance glucose uptake effectively.

These findings align with existing literature on the antidiabetic potential of vanadium complexes as seen in a study by Nejo et al., (209) who conducted their research on symmetrical tetradentate Schiff bases derived from substituted salicylaldehyde and aliphatic diamine, through an *in vitro*

glucose uptake assay, which revealed significant increases in glucose uptake by C2C12 cells with glucose uptake values exceeding even those of insulin with negligible cytotoxicity, interestingly the authors observed these findings at concentrations even lower than those in our study. They attributed the increase in glucose utilization to the substitution of the salicylaldehyde, where substitution with negative electron-withdrawing groups increased the glucose utilization. This was observed in our study, particularly in C2C12 cells. Complex 3 differs from the other two Schiff base complexes with a more negative electron-withdrawing trifluoromethyl group substituent instead of bromine, which is on complex 1 and complex 2. Therefore, this may, in part, explain why treatment with complex 3 showed the highest glucose utilization by C2C12 cells. It is important to note that this finding was only true for C2C12 cells, with the results in HepG2 cells showing the opposite, with complex 3 showing the least glucose uptake amongst the Schiff base vanadium complexes, which can be attributed to the difference in the cell lines and possibly a difference in the bioavailability and stability of the complex, further research in these parameters would provide deeper insights into the mechanism of action and differential cellular responses (171).

To further understand the effects of the Schiff base vanadium complexes on glucose metabolism, we conducted a glucose uptake assay using cells pretreated with palmitic acid. Palmitic acid is a saturated fatty acid known to induce insulin resistance by activating protein kinase C (PKC) (210). The activation of PKC inhibits the insulin signalling pathway in peripheral cells, primarily by reducing the tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and impairing the downstream activation of the Akt pathway, ultimately hindering glucose uptake (19). The observed reduction in glucose uptake upon insulin treatment served as a positive control, confirming the successful induction of insulin resistance in these cells. Consistent with this result, our findings suggest that these compounds exert effects similar to those of insulin. Interestingly, the vanadium complexes exhibited relatively lower glucose uptake in comparison to the experiment where cells were not pretreated with palmitic acid. This indicates that the insulin-mimetic effects of these complexes are largely dependent on the functionality of the PI3K/Akt signalling pathway. However, it is worth noting that, compared to insulin treatment, treatment with the Schiff base vanadium complexes resulted in a significant increase in glucose uptake. In a similar study by Kazek et.al, (211) where the researchers induced insulin resistance

by incubating HepG2 cells in oleic acid and observed a reduction in glucose uptake. After incubation with Schiff bases vanadium complexes derived from thioamide, they observed the compounds to reverse the decrease in glucose uptake in hepatocytes in which insulin resistance was induced by oleic acid, with glucose-enhancing effects that were greater than those of 1 mM metformin. This highlights the potential of these compounds as candidates for addressing insulin resistance, which is a critical aspect of conditions such as T2DM (211).

To further understand the mechanism behind the effect of the Schiff base vanadium complexes, we examined their effect on the PI3K/Akt signalling pathway proteins, with a focus on Akt and GLUT 4.

For Akt expression studies, Schiff base vanadium complexes 2 and 3 significantly increased the expression of activated Akt at higher concentrations in C2C12 cells. This is supported by the literature from which phosphorylation of Akt has been implicated as a mechanism of the antidiabetic effects of vanadium compounds (212). Therefore, these results indicate that these compounds may act through the same shared mechanism, which is through the PI3K/Akt pathway. However, in contrast, HepG2 cells showed a marked reduction in Akt expression with increasing concentrations of the same complexes. These results align with observations from the MTT cell viability assay and glucose uptake assay, which indicated potential cytotoxic effects at the highest concentrations, particularly in HepG2 cells. This discrepancy between the two cell lines likely stems from inherent differences in glucose metabolism and signalling pathway regulation between skeletal muscle and hepatic tissue. C2C12 cells, upon differentiation into multinucleated myotubules, exhibit metabolic and signalling dynamics, which are more representative of mature skeletal muscle, where glucose uptake is tightly regulated and enhanced by insulin signalling pathways. In contrast, HepG2 cells, which remain in an undifferentiated hepatic state, may exhibit altered sensitivity to external stimuli, rendering them more susceptible to cytotoxic effects at higher doses and less likely to show increased Akt expression. Additionally, the differentiation state of the C2C12 cells may play a significant role, as the mature-like myotubules are metabolically active and capable of mitigating potential toxic effects more effectively than the undifferentiated HepG2 cells (28). However, this interpretation remains

speculative, and other contributing factors, such as oxidative stress or off-target interactions, may also underlie the observed cytotoxicity.

Furthermore, an ELISA assay examining GLUT4 expression in C2C12 cells revealed increased expression for cells treated with complexes 2 and 3. This finding confirms that these compounds produce insulin-mimetic effects primarily through the Akt pathway, as supported by the results for the expression of Akt since activation of Akt is linked with upregulation of GLUT4 gene expression at the transcription level (213). The subsequent ELISA on GLUT4 translocation showed that complexes 2 and 3 increased GLUT4 translocation. Whilst the GLUT4 expression assay showed the production of GLUT4 in the cell, these translocation results show the movement of the GLUT4 to the cell membrane, where it facilitates glucose uptake (19). However, a reduction in translocation was observed at the highest concentration, consistent with previous assays and suggesting a therapeutic window between the highest concentration (20 μg/ml) and the lowest concentration (5 μg/ml). These results are supported by the literature with a study by Ki et al.., where they synthesised a Schiff base vanadium complex consisting of the tryptamine moiety, and observed this complex to promote GLUT4 translocation (174). These findings suggest that the PI3K/Akt pathway may be involved in the insulin mimetic effects of these complexes.

The main literature-reported mechanism of the antidiabetic action of vanadium is the inhibition of PTP1B (214), which is a negative regulator of the insulin signalling pathway through the dephosphorylation of the insulin receptor, essentially deactivating the insulin receptor and, therefore, the rest of the insulin signalling pathway. The results of molecular docking simulations of the Schiff base vanadium complexes on PTP1B show that the vanadium complexes bind to PTP1B with negative binding energies, suggesting favourable interactions with the protein. Notably, the organic vanadium complexes demonstrated greater affinity for regions outside the Cys215-containing P-loop of the active site, where orthovanadate preferentially binds. This observation aligns with the hypothesis that these vanadium complexes act as prodrugs, delivering the vanadate ion to PTP1B, where it binds, occupying the active site and preventing phosphate binding, thereby hindering the catalytic activity of the enzyme (133). Specifically, binding these complexes to non-active site regions, such as the proline-rich domain (301–400) or regulatory

loops, may facilitate allosteric modulation, which can also contribute to the inhibition of PTP1B. This binding to non-active site regions could also allow targeted delivery of the vanadate ion near the catalytic centre. Such interactions could also stabilise the enzyme in conformations favouring inhibition, although they may also potentially enhance the activity (215). Together, these findings suggest organic vanadium complexes have the potential to provide targeted delivery of the active vanadate species while minimising competition for the catalytic site (216).

The pharmacokinetic profiles of the vanadium complexes investigated in this study reveal critical insights into their potential as therapeutic agents, particularly regarding drug-likeness and bioavailability. Lipinski's rule of five, which offers a simple framework for evaluating the drug-likeness of compounds, was applied to the synthesised vanadium complexes (183). Complexes 1 and 2 each violated only one of Lipinski's rules, specifically the molecular weight threshold of 500 g/mol, which is still within an acceptable range for drug development. In contrast, complex 3 violated two rules: a high molecular weight and an excessive number of hydrogen bond acceptors (13, exceeding the limit of 10). Despite these violations, all complexes fall within the broader drug-like threshold, as compounds are often considered viable even with up to two violations of Lipinski's rules.

It is essential to highlight that the rule of five does not provide a definitive measure of oral absorption, particularly for metal-based complexes like vanadium compounds. This is an important consideration, as Lipinski's criteria alone cannot fully capture the absorption, distribution, metabolism and excretion (ADME) properties of such complexes. The expansion of the 'drug-like' definition over time has incorporated additional parameters such as polar surface area (PSA), rotatable bond count, and molecular flexibility, all of which provide a more comprehensive understanding of the pharmacokinetic properties of a compound and its ability to reach target sites (217). In this context, the Schiff base vanadium complexes investigated in this study display significant flexibility, a beneficial trait in drug discovery. The excellent flexibility of these complexes allows them to potentially access deep-rooted binding sites that more rigid analogues may struggle to reach, making them promising candidates for further optimisation (218).

Looking into the other pharmacokinetic properties, which are aqueous solubility and lipophilicity, the vanadium compounds exhibit moderate lipophilicity, likely due to aromatic structures facilitating hydrophobic interactions. However, this hydrophobicity may contribute to the poor aqueous solubility observed in these compounds (219). Since solubility in aqueous media is crucial for *in vivo* bioavailability, it will enhance their clinical applicability. The gastrointestinal (GI) absorption and blood-brain barrier (BBB) permeability predictions further underline this poor aqueous solubility. All three vanadium complexes demonstrated poor GI absorption and BBB permeability, raising concerns about their therapeutic potential, particularly for systemic administration. Formulation strategies can be employed to address this. For example, in our experiments, we initially dissolved the complexes in DMSO, then diluted the solution in cell culture medium to result in a final DMSO concentration of less than 0.1% a concentration that is widely reported not to adversely affect cell viability or proliferation in various cell types and assays (220,221). Therefore, dissolving in DMSO helped bypass the solubility hurdle and ensured the compounds were available for testing.

Interestingly, BMOV, a reference vanadium compound, exhibited significantly better GI absorption and bioavailability compared to the other complexes, suggesting that molecular modifications could enhance the absorption profile. The higher bioavailability of BMOV may be attributed to its favourable solubility and interaction with P-glycoprotein (P-gp), a transporter protein that facilitates drug absorption and distribution (222). In contrast, NaVO , another reference compound, demonstrated similarly poor bioavailability to the investigated vanadium complexes, highlighting the crucial role of structural differences in BMOV. These differences contribute to improved aqueous solubility and, consequently, enhanced bioavailability.

To improve the pharmacokinetic properties of these compounds, a key focus should be on enhancing solubility and optimising molecular size to reduce violations of Lipinski's parameters. Strategies to improve GI absorption and reduce the compounds' reliance on CYP-mediated metabolism could further enhance their therapeutic potential. Reducing molecular weight and a better balance between lipophilicity and hydrophilicity may help optimise these compounds for clinical applications, especially in targeting diseases like DM.

Future studies could explore the potential of encapsulating Schiff base vanadium complexes within nanoparticle carriers, which may offer a promising solution to address stability challenges inherent to these compounds. Due to their prodrug nature and sensitivity to environmental conditions, these complexes often face issues with rapid degradation and speciation changes, which can hinder their bioavailability and therapeutic efficacy (223). Nanoparticle encapsulation could provide a protective matrix stabilising the vanadium complexes, reducing undesired transformations, and improving cellular uptake. Furthermore, nanoparticles could allow for controlled and targeted drug release, enhancing the specificity of these compounds in insulin-target cells and potentially reducing systemic toxicity (224). This strategy could enhance the pharmacokinetic profile of vanadium complexes, ensuring a more consistent therapeutic effect and expanding their clinical viability as insulin-mimetic agents. This approach could facilitate a return to the use of vanadium salts. By encapsulating the vanadium salts with nanoparticles, it is possible to enhance their bioavailability, addressing the stability and degradation issues typically associated with them. Nanoparticle encapsulation could offer a more efficient and controlled delivery mechanism, improving the therapeutic outcomes of vanadium salts while minimising the challenges of toxicity and off-target effects related to their direct administration.

Repurposing approved drugs as ligands for these vanadium complexes is another direction future research can take, as seen in a study by Sousa-Coelho et al., (225) where they studied metformin complexed with decavanadate to form metformin decavanadate complexes, which demonstrated enhanced antiproliferative effects on melanoma cells at concentrations 10 times lower than orthovanadate. Therefore, this can be valuable in DM research since the main issue has been the toxicity of these vanadium compounds. However, if their potency can be increased by complexing with approved drugs, this could lead to the development of safer, more effective vanadium-based DM therapeutics.

Vanadium complexes offer distinct advantages in treating DM, particularly in resource-limited settings, as they can be chemically synthesised, unlike insulin, which typically requires a complex and resource-intensive biotechnological process. Insulin production often involves recombinant DNA technology using genetically engineered microorganisms, followed by purification and formulation under strict conditions (226). In contrast, the chemical synthesis of

vanadium compounds can be scaled more efficiently for higher manufacturing volumes. Additionally, vanadium complexes would likely not require refrigeration, a critical limitation for insulin storage in low-resource environments. This eliminates the cold chain logistics required for insulin distribution, making vanadium-based therapies a more accessible and cost-effective alternative for managing DM in regions with scarce refrigeration infrastructure.

Looking at the application of our findings, our research contributes significantly to the existing literature on vanadium complexes and their potential as DM treatments. Specifically, our complexes demonstrated low cytotoxicity and minimal potential for inflammation, positioning them as promising candidates for further therapeutic development. One crucial avenue for future research is the optimization of ligand structures. Previous studies have highlighted the correlation between ligand structures and compound stability (227). Our findings, which showed varying results across complexes with different chemical structures, support this relationship. Future studies could, therefore, focus on tailoring ligand structures to enhance stability and efficacy, particularly in gastrointestinal environments. This approach may lead to the development of highly efficacious vanadium complexes with broad therapeutic applications.

Additionally, expanding the scope of these complexes beyond DM treatment could open new research directions. Vanadium is known for its cardioprotective, anticancer, and antimicrobial properties (227), while Schiff bases also exhibit significant biological activities such as antimicrobial and antioxidant effects (228). Exploring the potential of these compounds in these contexts could be transformative. Notably, managing hyperglycaemia with current antidiabetic agents often places patients at risk of developing complications such as cardiovascular diseases. Some antidiabetic drugs, such as sulphonylureas and thiazolidinediones, even exacerbate cardiovascular risks (229). Vanadium-based therapies, with their multifaceted therapeutic potential, could provide means to address DM while simultaneously mitigating such complications.

This multi-therapeutic potential also suggests the possibility of reducing polypharmacy in the management of DM and its associated complications, thereby improving patient compliance. Furthermore, investigating the synergistic effects of vanadium complexes in combination

therapies for DM and other health conditions could lead to enhanced therapeutic outcomes and reduced side effects. Such an approach supports a more comprehensive and integrative strategy for managing diverse health conditions (230).

4.2 Conclusion

This study demonstrated the potential of Schiff base vanadium complexes as promising therapeutic agents for DM, with apparent insulin-mimetic effects observed at lower concentrations. Complexes 2 and 3 can enhance glucose uptake, activate insulin signalling pathways, and reduce inflammatory markers such as DPP4. The dose-dependent cytotoxicity observed still necessitates further investigation to optimise therapeutic windows and evaluate long-term safety. Future studies should also explore the bioavailability of these compounds *in vivo* to translate their therapeutic potential into clinical applications.

4.3 Limitations and future studies

A limitation of this study was the unavailability of BMOV for comparison with the synthesised vanadium compounds. BMOV is widely recognised as a standard reference compound in studies of vanadium complexes due to its established bioactivity, particularly in glucose uptake assays and related pharmacological applications (10,152,231). Without BMOV, directly benchmarking the synthesised compounds' efficacy and safety profiles against a well-characterised vanadium agent was impossible, which may affect the generalizability and interpretation of the results. Future studies incorporating BMOV would strengthen comparative analyses and enhance the understanding of these novel compounds' potential as therapeutic agents.

Additionally, according to Dunnett's test, while the study showed promising trends in the bioactivity of the synthesised compounds, some of these results were not statistically significant. Further assays would be needed to confirm and build upon these initial findings, but time and resource constraints limited the extent of additional experiments.

Although three concentrations were tested for each compound, a more comprehensive dose-response analysis with additional concentration points would have provided greater insight into the vanadium compounds' pharmacological profiles. Dose-response curves would have been advantageous as they would allow for visualization of concentration ranges associated with different biological activities. Therefore, incorporating dose-response profiling in future studies would provide a more precise pharmacological characterization of the compounds and assist in identifying optimal therapeutic windows.

Moreover, stability studies in different pH conditions were not conducted, which would have provided further insights into the stability of the vanadium compounds under varying physiological conditions. The study also lacked the inclusion of an adipocyte cell line, which is critical when studying insulin target cells. While assays were conducted on skeletal (C2C12) and liver (HepG2) cell lines, an adipocyte cell line would have added a more comprehensive understanding of the compounds' effects on insulin sensitivity. Furthermore, if included in the ELISA assays, other proteins, such as IRS, PI3K, LDH, CRP, and TNF-α, could have provided valuable results. Their inclusion could help better elucidate the mechanisms of action of the compounds in relation to glucose uptake and related metabolic pathways.

Furthermore, future studies should prioritise evaluating the long-term cytotoxic effects of the synthesised compounds in more physiologically relevant models, including in vivo systems. This is particularly important for assessing potential gastrointestinal (GI) toxicity, a known concern with vanadium-based therapies. Although this study highlighted the possibility of GI side effects, these were speculative in the absence of direct assessments. Incorporating animal models would provide a more accurate reflection of clinical relevance. Additionally, analysing oxidative stress markers and inflammatory mediators in these models could offer deeper mechanistic insights into the safety profile of these compounds.

It is crucial to recognise that the complex behaviour of vanadium in biological systems and its lack of target specificity and challenges in controlling speciation have hindered interest from major pharmaceutical companies. While academic research and small business initiatives have expanded our understanding of the therapeutic potential of vanadium, significant barriers such as

toxicity, bioavailability, regulatory concerns, and off-target effects persist. These challenges, along with the rise of alternative non-vanadium-based therapies, have contributed to the reluctance of "Big Pharma" to invest in vanadium-based drugs despite the ongoing need for novel antidiabetic treatments (122).

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4.4 Appendices

4.4.1 56th Annual Conference of the South African Society for Basic and Clinical Pharmacology Certificate of Participation

South African Society for Basic and Clinical Pharmacology

Certificate of Participation

This is to confirm that

Mr Munyaradzi Chiwundura

Has attended the 2023 Conference
of
The South African Society for Basic and Clinical Pharmacology
at Rhodes University
on the 24th - 27th August 2023

(accredited for 18 CPD Points)

56th ANNUAL CONFERENCE

"Pharmacology celebrating resilience: Current and Future strategies"

Prof Mamza Mothibe
Chairperson: 2023 Organising Committee

Ms Tanusha Dukhan
Secretary: 2023 Organising Committee

4.4.2 Rhodes University Faculty of Pharmacy Research Day Abstract

Munyaradzi Chiwundura

THE EFFECT OF SCHIFF BASE VANADIUM COMPLEXES ON GLUCOSE UPTAKE IN INSULIN TARGET CELLS

Authors:

Chiwundura, M; Sibiya, N

Affiliations:

Faculty of Pharmacy, Rhodes University, Grahamstown, South Africa

Introduction/Background:

Diabetes mellitus has been declared by the world health organisation as one of the leading causes of death worldwide. Hence, there is a need to investigate potential therapeutic agents that have fewer side effects and high patient compliance. Vanadium complexes have been reported to improve glucose homeostasis in various diabetes models. Hence, this study was aimed at exploring the potential application of schiff base vanadium complexes in promoting glucose uptake in insulin target cells.

Methods:

In this study, MTT cell viability and glucose uptake assays were conducted on HeLa cells and skeletal cells. The MTT Assay evaluated the cytotoxicity of the synthesized vanadium compounds which have been named compound 1, 2 and 3 compounds, while the glucose uptake assay assessed their ability to enhance glucose uptake. The assays were performed at various concentrations of the vanadium complexes, with a focus on concentrations below 60 µg/ml.

Results:

The MTT cell viability assay demonstrated that the compounds exhibited low levels of toxicity on both HeLa cells and skeletal cells at concentrations below 60 µg/ml. This finding suggests that the synthesized vanadium complexes have potential as safe candidates for further investigation. Regarding glucose uptake, compound 1 exhibited insulin mimetic activity at a concentration of 60 µg/ml, as determined by the glucose uptake assay. However, this activity decreased with decreasing concentrations of compound 1. Conversely, compounds 2 and 3 did not show a similar insulin mimetic effect, indicating that their glucose uptake enhancement capabilities may be limited or absent.

Conclusion:

In conclusion, the study findings suggest that the Schiff base vanadium complex, compound 1, holds promise as an insulin mimetic agent to enhance glucose uptake in insulin target cells. This compound exhibited insulin-like activity at a concentration of 60 µg/ml, indicating its potential therapeutic value. Further research is needed to elucidate the underlying mechanisms and explore the possibilities of optimizing compound 1 for potential clinical applications.

Keywords:

Schiff base vanadium complexes, glucose uptake, insulin mimetic, MTT assay, HeLa cells, skeletal cells

4.4.3 57th Annual Conference of the South African Society for Basic and Clinical Pharmacology acceptance letter

57TH ANNUAL SASBCP CONFERENCE 2024

15-17 September 2024 Irene Country Lodge, Centurion, South Africa

South African Society for Basic and Clinical Pharmacology
www.sapharmacol.co.za

Department of Pharmacology & Therapeutics
Email: conference@sasbcp2024.co.za
www.sasbcp2024.co.za

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School of Medicine Telephone: 083 407 8621 | Fax: 012 521 4121
PO BOX 235, MEDUNSA, 0204
South Africa
E mail: conference@sasbcp2024.co.za

Dear M. Chiwundura,

Re: SASBCP Annual Conference 2024

The 57th SASBCP Annual Conference organizing committee is delighted to advise that your abstract, "Investigation of schiff base vanadium complexes: effects on glucose uptake, cytotoxicity, and inflammation in insulin target cells" has been accepted for oral presentation.

Please complete your registration for conference attendance at http://sasbcp2024.co.za/ to secure your space.

Looking forward to seeing you in September 2024.

Sincerely,

Prof E Osuch
HOD & Conference Chairperson
Department of Pharmacology and Therapeutics
Sefako Makgatho Health Sciences University
South Africa

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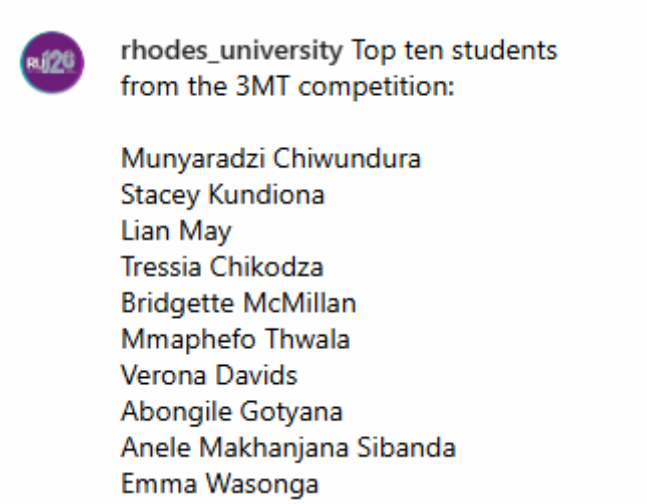
Organizing committee: Prof E Osuch (Chairperson) | Cell: +27 82 770 0629 | Prof PH van Wyk | Cell: +27 83 407 8621

4.4.4 Rhodes University Centre for Postgraduate Studies 3-minute thesis competition finalists

Link



4.4.5 Rhodes University Faculty of Pharmacy Research Symposium abstract

Investigation of Glucose Modulation and Cytotoxicity of Insulin Target Cells by Schiff Base Vanadium Complexes

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Background:
Diabetes mellitus is a major global health challenge, with significant morbidity and mortality. Current therapies often have limitations, necessitating new treatments with improved efficacy and patient compliance. Vanadium complexes have shown potential in modulating glucose metabolism. This study investigates the effects of Schiff base vanadium complexes on glucose uptake and cytotoxicity in insulin-target cells, aiming to assess their therapeutic potential [1].

Purpose:
To evaluate the glucose uptake and cytotoxicity of three Schiff base vanadium complexes in C2C12 and HepG2 cells, with sodium orthovanadate as a reference, focusing on GLUT4 and AKT expression and inflammatory response markers.

Methods:
Three Schiff base vanadium compounds (Compounds 1, 2, and 3) were synthesised. MTT assays determined cytotoxicity across concentrations (5, 10, and 20 µg/ml). Glucose uptake was assessed in C2C12 and HepG2 cells, with additional GLUT4 and AKT ELISA assays performed on C2C12 cells. Media IL-6 levels were measured to evaluate potential inflammatory effects.

Results:
The MTT assay showed low cytotoxicity for all compounds except Compound 1 at 20 µg/ml. All compounds enhanced glucose uptake, with the highest uptake seen in non-toxic concentrations. ELISA assays indicated increased GLUT4 expression and AKT activation in C2C12 cells following treatment. No significant increase in IL-6 was observed, suggesting an absence of inflammatory response.

Conclusion:
Schiff base vanadium complexes, especially at lower concentrations, may function as insulin mimetics, enhancing glucose uptake with minimal cytotoxicity and no inflammatory response. These findings support further investigation into the clinical potential of these compounds.

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