EFFECTS OF SUSTAINED RELEASE SOMATOSTATIN RECEPTOR TYPE 2 ANTAGONISM ON GLUCAGON COUNTERREGULATION AND GLYCEMIA IN A RAT MODEL OF TYPE 2 DIABETES

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ABSTRACT

Introduction: Effective glucose management in insulin-requiring diabetes is hindered by the challenge of delivering the correct therapeutic amount of insulin, daily, that effectively treats high blood sugar (hyperglycemia) without causing low blood sugar (hypoglycemia). Medications for hyperglycemia treatment (i.e., incretins, insulin sensitizers and insulin secretagogues) are regularly used by individuals with type 2 diabetes (T2D) but no medication is currently available for the prevention of hypoglycemia. One of the reasons why hypoglycemia exists in diabetes is because the main inhibitory hormone, somatostatin (SST), is elevated in diabetes and this elevation results in a blunting in glucagon, the main glucose counterregulatory hormone that can fend off hypoglycemia. This thesis examined if the sustained delivery of a novel SST receptor 2 antagonist (SSTR2a), ZT-01, enhances endogenous glucagon secretion and protects against hypoglycemia, without resulting in a worsening of overall hyperglycemia, in a male rat model of T2D.

Objective: The primary goal was to evaluate the efficacy of this novel SSTR2a, ZT-01, on increasing glucagon levels during insulin-induced hypoglycemia, thereby reducing hypoglycemia exposure, in a rat model of insulin-requiring T2D. The secondary objective was to characterize whole-body insulin resistance, oral glucose tolerance and glucose counterregulatory function during insulin-induced hypoglycemia in response to sustained exposure to ZT-01 as compared to vehicle (i.e., placebo) treatment.

Methods: Male Sprague Dawley rats (n=4/5 per group; Pilot study, n=8 per group; Study 1) were placed on a high-fat diet for ~3 weeks and then injected with low-dose streptozotocin (35 mg/kg) to induce T2D, characterized by high blood glucose levels in the fed state (>12 mmol/L)

but with some residual beta cell function. Therapeutic long-acting/basal (glargine) and short-acting (regular) insulins were administered daily as needed for 7 days (2-3 units/day) to maintain fed-state glycemia targeting 12-25 mmol/L, after which the rats were treated chronically with ZT-01 (0.18 mg/day [low dose, Pilot study]; 0.3 mg/day [moderate dose, Study 1]; or 1.2 mg/day [high dose, Study 2]; Zucara Therapeutics) or vehicle using surgically implanted mini-osmotic pumps (Alzet Osmotic Pumps, DURECT Corporation). An oral glucose tolerance test (OGT) via gavage (2g/kg) followed by a 2–3-day washout period preceded two insulin-induced hypoglycemia challenges (using 12U regular insulin).

Results: ZT-01 treated and vehicle groups displayed similar glycemic levels in the fasted and post-oral glucose gavage state but ZT-01 treated rats tended to display increased baseline glucagon levels and in some cases an increase the glucagon response to hypoglycemia caused by insulin overdose. In most cases, the effect sizes for increased glucagon responses were small and only tended to border on statistical significance. ZT-01 did not seem to alter glycemia in response to oral glucose gavage to assess OGT and the rats glycemic index was not significantly altered in response to glucagon secretion.

Conclusion: Sustained exposure to SSTR2a at a low, moderate, or high therapeutic dose does not appear to affect basal glucose significantly nor affect the response to OGT but modestly, or only marginally, improves the glucose and glucagon response to insulin-induced hypoglycemia in this model of T2D.

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LIST OF ABBREVIATIONS

AUC: Area under the curve

BG: Blood glucose

HFF: High-Fat-Fed

HOMA-IR: Homeostatic model of insulin resistance

ITT: Insulin tolerance test

OGTT: Oral glucose tolerance test

Sapha: Saphenous blood

SST: Somatostatin

SSTR2a: Somatostatin receptor type 2 antagonist

STZ: Streptozotocin

T1D: Type 1 diabetes

T2D: Type 2 diabetes

HBA1C: Hemoglobin A1C (glycated hemoglobin)

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1.0 INTRODUCTION

Defects in glucagon counter-regulation to insulin-induced hypoglycemia is one of the clinical hallmarks of intensive insulin therapy in insulin-requiring diabetes [1]. Hypoglycemia is often the major limiting factor in the glycemic management of type 1 diabetes (T1D) and when insulin or insulin secretagogues are used for the glycemic management of individuals with type 2 diabetes (T2D). For both main forms of diabetes, and for gestational diabetes where insulin may be prescribed, level 1 hypoglycemia is defined as a glucose concentration between 3.0 mmol/L. and 3.9 mmol/L; level 2 hypoglycemia is defined as a glucose < 3.0 mmol/L, while level 3 hypoglycemia is defined as a severe hypoglycemic event characterized by altered mental and/or physical functioning that requires assistance from another person for recovery, irrespective of the glucose concentration [2]. Symptoms of hypoglycemia include but are not limited to, shakiness, irritability, confusion, tachycardia, sweating, and hunger [3]. However, because many people with diabetes demonstrate impaired counterregulatory responses to hypoglycemia and/or experience impaired hypoglycemia awareness, a measured glucose level <3.9 mmol/L is considered “clinically important”, regardless of whether symptoms exist [2]. Both severe (level 3) and non-severe (levels 1 and 2) hypoglycemic events are common in most forms of diabetes, with a majority of insulin-treated patients with diabetes (i.e., all T1D and ~20-30% of all T2D) being exposed to hypoglycemia on a regular basis [4]. Canadian survey studies that included both T1D and T2D who were taking insulin and/or sulfonylureas suggest that ~3.8 severe hypoglycemic events occur per patient per year [5]. Overall, hypoglycemia results in

significant patient burden and healthcare costs in Canada, with the average annual cost reaching $125,932 per patient/year for direct healthcare and indirect costs [6].

Somatostatin (SST) is a pancreatic hormone that can play a role in the defective glucagon counter-regulation in T1D. SST plays an essential role in glycemic control in diabetes by having the capacity to inhibit both insulin and glucagon secretion [7] and high levels of SST in diabetes have been implicated in blunting the glucagon response to the development of hypoglycemia in T1D [8]. However, the impact of this inhibitory hormone is yet to be fully explored in type 2 diabetes.

T2D can be classified into 5 clinical stages [9]. Stage 1 is pre-diabetes. The stages are defined by certain factors such as fasting plasma glucose (FPG), 2-hour plasma glucose (PG) level during an oral glucose tolerance test (OGTT), and hemoglobin A1C (HbA1c).

During Stage 1(Prediabetes), the patients have the following conditions: FPG of 5.6-6.9 mM, 2-h PG level on OGTT of 7.8-11.0 mM, HbA1c of 5.7-6.4%. Stage 2 (diabetes with no complications) is characterized by patients having FPG ≥7.0, 2-h PG level on OGTT of ≥11.1 mM, and a Hemoglobin A1C level of ≥6.5% [9].

Stage 3 of T2D is associated with mild complications. The patients may have hyperglycemia and higher or standard levels of fasting and 2-hour plasma insulin or c-peptide on OGTT.

Stage 4 of T2D is characterized by absolute insulin deficiency. Based on clinical and or laboratory evidence, patients at this stage may experience hyperglycemia and absolute insulin deficiency [9, 10]. At this point, most patients are initiated on basal-only insulin therapy, but these individuals then often progress to basal-bolus insulin therapy and thus the risks for iatrogenic hypoglycemia (i.e., unintentional hypoglycemia as a result of insulin therapy) increase markedly

Stage 5 (T2D with serious complications) is where patients experience complications such as hyperglycemic crises as well as microvascular and macrovascular complications. With hyperglycemia, they may as well have higher, lower, or normal levels of fasting plasma insulin or c-peptide [1, 2]. Somewhat paradoxically, the risk for hypoglycemia is markedly elevated in stage 5, with hypoglycemic events predicting early morbidity and mortality [11]. Microvascular and macrovascular complications of T2D are major causes of blindness, heart attack, kidney failures and lower extremity amputations [12]. To reduce the risk of complications in T2D, diabetes management has made lowering of blood glucose a priority. While diabetes treatments help combat hyperglycemic crises, hypoglycemia becomes the most common adverse effect of diabetes therapy [11]. Based on recent epidemiologic studies, the incidence of hypoglycemia in T2D has been largely underestimated, largely because of infrequent glucose monitoring in T2D relative to T1D, and because symptom recognition may be impaired [4].

Hypoglycemia can be described as a self-perpetuating condition, where each episode of hypoglycemia is accompanied by an attenuation of glycemic threshold for autonomic activation of counterregulatory responses thereby increasing the risk for a subsequent hypoglycemic event [13]. After repeated episodes of hypoglycemia (i.e., 2-3 episodes per week over weeks), most patients with T1D and insulin-requiring T2D develop hypoglycemia-associated autonomic failure (HAAF), which inhibits or delays the onset of neurogenic warning signs associated with each subsequent hypoglycemic [13,14]. In other words, hypoglycemia begets hypoglycemia in diabetes [15]. With impaired hypoglycemia awareness and reduced ability of symptom recognition, patients often experience diminished quality of life as well as a shorter window for restorative intervention (exogenous glucagon rescue treatment, i.e. glucose, etc.) [5 ,6].

As noted above, insulin therapy is frequently initiated in long-standing T2D [16], with the number of insulin users with T2D increasing steadily since 2002, even with the advent of several newer non-insulin therapies in recent years [17]. Although the percentage of T2D insulin users in Canada is unclear, based on US data ~20% of Canadians with T2D are likely on insulin therapy [18]. Intensive insulin therapy (defined as multiple daily insulin injections or the use of an insulin pump) dramatically increases the risk of recurrent hypoglycemia, leading to a need for less strict glycemic targets so that hypoglycemia exposure and recognition can be at least partially restored [11]. Unfortunately, this can result in higher levels of HbA1C and an increased risk of micro- and macrovascular complications over time [19]. This trade-off between hypoglycemia avoidance and glycemic control targets emphasizes the importance of finding preventative measures that allow for more aggressive treatment of T2D without the associated risks [20].

SST type 2 receptor antagonists (SSTR2a's) have been shown to stimulate glucagon release in pre-clinical models of diabetes [21] and ZT-01, a novel SSTR2a has shown promising early results in humans with T1D for the restoration of glucagon counterregulation during insulin-induced hypoglycemia via a hypoglycemic clamp technique [22]. To further explore the therapeutic potentials of this novel SSTR2a, in this study, we will be investigating the effect of sustained (chronic) ZT-01 treatment on glucagon counterregulation, insulin sensitivity, oral glucose tolerance and overall glycemia in a male rat model of insulin-requiring T2D.

2. LITERATURE REVIEW

2.1. NORMAL GLUCOSE REGULATION IN HEALTHY, NON-DIABETIC INDIVIDUALS

2.1.1. PANCREATIC ISLET CELLS

The pancreas, a vital organ in maintaining metabolic homeostasis, consists of exocrine ductal and acinar cells that produce and deliver digestive enzymes into the gut [23]. Islets of Langerhans, which are placed within the exocrine regions are comprised of about 5 endocrine cell types [24].

Here, the focus will be on the three most researched types of pancreatic islet endocrine cells. Alpha (α)-cells secrete glucagon, a glucose-raising hormone; beta (ß)-cells secrete insulin, a glucose-lowering hormone; delta (δ)-cells produce SST an inhibitory hormone for both glucagon and insulin secretion [23]. Additionally, a less common cluster of pancreatic cells, called pancreatic polypeptide (PP) cells (~2% of pancreatic cells), are responsible for the production of pancreatic polypeptides which are also involved in metabolism, albeit to a lesser extent [25]. Over time, the relative expression of these and other islet cells changes, particularly in the various stages of T2D, where ultimately ß-cell mass eventually declines, and α-cell mass eventually expands [26].

2.1.2. GLUCAGON (ALPHA CELLS)

Glucagon is a counterregulatory hormone that acts in opposition to insulin [11]. Glucagon is a peptide made up of 29 amino acids. It is secreted at low levels from pancreatic α-cells in the basal (i.e., non-fasted) state and the hormone normally declines in circulation after feeding [27]. However, glucagon secretion increases during exercise and in the fasted state, particularly when hypoglycemia ensues [28]. Hypoglycemia physiologically is the strongest stimulus for glucagon secretion [29], but strong glucagon secretion also occurs with protein feeding and with stimulation with certain amino acids such as alanine, arginine, cysteine, and proline exsanguination.

2.1.3. INSULIN (BETA CELLS)

Insulin, a hormone secreted by pancreatic ß-cells, is a master regulator of glucose, protein, and lipid metabolism. After ingestion of a meal, or an oral glucose load, there is normally a small rise in plasma glucose concentration and that stimulates insulin secretion by the ß-cells. The insulin response to the rise in circulating glucose is enhanced by the concomitant action of incretins, other nutrient and hormone signalling and the neural responses to nutrient ingestion [30]. The increase in plasma insulin at mealtime, along with the rise in plasma glucose concentration, normally leads to an inhibition in glucagon secretion after the consumption of a meal [31].

2.1.4. SST (DELTA CELLS)

Generally, the release and fluctuation in levels of insulin and glucagon are reciprocal, with insulin rising and glucagon dropping at mealtime, with the reverse of these responses occurring during exercise and fasting [32]. The insulin-to-glucagon ratio largely dictates the balance between metabolism and catabolism [33]. Most of the research over the past 100 years has been on insulin and glucagon due to their obvious regulatory effect on metabolism and their role in metabolic syndromes like diabetes mellitus [27,34,35]. However, δ-cells in the islets of Langerhans release SST, which can inhibit both insulin and glucagon secretion and some evidence suggests that this hormone is also dysregulated in diabetes [35]. More specifically as it relates to this thesis, findings from the past ~10-20 years suggest that increased SST signaling in poorly controlled diabetes could play a role in defective glucagon counter-regulation during insulin-induced hypoglycemia [36,37].

2.2. ISLET PHYSIOLOGY IN T2D

The insulin-to-glucagon ratio is the primary regulator of hepatic glucose production, while insulin alone is the main endocrine regulator of glucose disposal into skeletal muscle, liver and adipose tissue [33]. In T2D, there are alterations in the insulin-to-glucagon ratio that may be influenced by several factors including islet mass, cell type and function [26],[38]. Changes observed in T2D include reduced ß-cell mass, altered alpha-beta cell ratios, increased islet fibrosis and amyloid depositions. Evidence suggests that elevated levels of SST may also be a contributing factor to the altered insulin-to-glucagon ratio [39]. SST plays an inhibitory role in both insulin and glucagon secretion. Increased SST levels in diabetes could result in the downregulation of both

insulin and glucagon secretion [40], albeit the clinical implications of elevated SST in diabetes remain unclear [35]. The inhibitory action of SST may lead to further deterioration of islet function and contribute to the overall pathogenesis of T2D [7]. Global ablation of SSTR2 in mice leads to hyperglucagonemia and is associated with impaired glucose control, resulting from decreased hepatic glucose storage, increased glycogen breakdown, and reduced lipid accumulation [41]. On the other hand, the use of pharmacological inhibition (i.e., antagonism) of the SSTR2 on glucagon counterregulation in rodents with insulin-treated T2D remains unclear, which is the primary focus of this thesis.

2.3. T2D COMPLICATIONS AND TREATMENTS

The complications of T2D range from acute to chronic [42]. The acute complications of T2D are diabetic ketoacidosis and coma, hyperglycemia, and hypoglycemia [43].

Hypoglycemia, one of the major complications of diabetes treatment occurs when blood sugar is very low [19]. It may be caused by delivery of an incorrect dose of insulin, reduced food intake (mainly carbohydrate) and intense exercise. The symptoms of hypoglycemia include sweating, headache, dizziness, irritability, absent-mindedness, loss of consciousness and in severe cases death [44].

Hyperglycemia occurs when blood sugar levels are elevated [45]. Hyperglycemia requires treatment as it is the major cause of serious and life-threatening long-term complications in diabetes [46]. Hyperglycemia may be caused by the absence of insulin. Hyperglycemia can happen when insulin treatment is not intensive enough or when the patient misses a dose of their

medication. Other causes of hyperglycemia may be eating habits with improper insulin-to-carb ratio administration [47].

2.4. EPIDEMIOLOGY OF TREATMENT-INDUCED HYPOGLYCEMIA

Treatment-induced hypoglycemia is a pervasive clinical complication of intensive insulin treatment with insulin analogues (T1D/T2D) and/or secretagogues (T2D) [48]. Annual rates of hypoglycemia are three to four times higher in adults with T1D than T2D, because of the ubiquitous need for basal bolus insulin therapy in T1D, which increases hypoglycemia risk [11,49]. However, due to the prevalence of T2D, hypoglycemic events in T2D are now the most diabetes-related hypoglycemic events, including the cases that lead to hospitalization [50]. There have been notable advancements in glycemic control due to the introduction of new basal and bolus insulin secretagogues. While these treatments effectively target hyperglycemia, they do not adequately address hypoglycemia, leading to a rise in the incidence of hypoglycemic episodes [51, 52].

2.5. GLUCAGON COUNTERREGULATORY FAILURE IN DIABETES

Glucose counterregulation during hypoglycemia is mediated by a hierarchy of neuro-hormonal responses in healthy individuals [53]. Initially, a drop in blood glucose within the euglycemic range causes insulin secretion to shut off at around a glucose threshold of 4.4-4.7 mmol/L [54].The ratio of insulin to glucagon in the hepatic portal vein dictates glucose mobilization from the liver [55]. Therefore, lower portal insulin levels favour higher glucose output, as well as lower glucose uptake by insulin-sensitive tissue (excluding the brain).

Once blood glucose drops below the euglycemic range (≤3.9mmol/L, classified as level 1 hypoglycemia [56]), that activates the release of the chief counterregulatory hormone glucagon,

from islet alpha cells at a glucose threshold of 3.6-3.9 mmol/L [54] followed by the emergence of cognitive impairment [57].

2.6. SST RECEPTOR 2 ANTAGONISM AND HYPOGLYCEMIA PREVENTION IN DIABETES

Somatostatin receptor type 2 antagonists (SSTR2a) are a class of drugs designed to modulate hormone secretion in the pancreas, primarily targeting the regulation of glucagon. These drugs act by binding to somatostatin receptor type 2, which is predominantly expressed on the alpha cells of the pancreatic islets, particularly in rats. By antagonizing these receptors, SSTR2a effectively removes the inhibitory influence of somatostatin on glucagon secretion, thereby augmenting its release. This mechanism can be visualized in Figure 1, which illustrates the interplay between different cell types in the pancreatic islet and the action of SSTR2a.

While the primary tissue of interest for SSTR2a is the alpha cells of the pancreas, it is important to note that somatostatin receptors are also expressed in other tissues throughout the body, including the central nervous system, gastrointestinal tract, and certain endocrine glands[7,35,58]. However, the specificity of SSTR2a for the type 2 receptor helps to target its effects more precisely on pancreatic function. The antagonism of somatostatin signalling by SSTR2a not only affects glucagon secretion but can also influence other pancreatic hormones. In addition to augmenting glucagon release, SSTR2a may enhance insulin secretion, by countering the known inhibitory actions of somatostatin on insulin secretion [2] This dual action on both glucagon and insulin highlights the complex interplay of hormones within the pancreatic islets and

underscores the potential of SSTR2a as a therapeutic agent in managing glucose homeostasis, especially in conditions like diabetes where hormone regulation is impaired.

Over the past years, there have been various studies on the metabolic impact of pharmacological SST receptor 2 antagonism (SSTR2a) on different models of diabetes, recurrent hypoglycemia, and pre-diabetes [21]. Of note, different formulations of a SSTR2a, with a range of binding selectivity, were used in these earlier studies, and the development of more potent compounds that had higher selectivity to SSTR2 have been developed by our research laboratory in partnership with Zucara Therapeutics and used in various pre-clinical models and in humans with T1D [59,60,22].

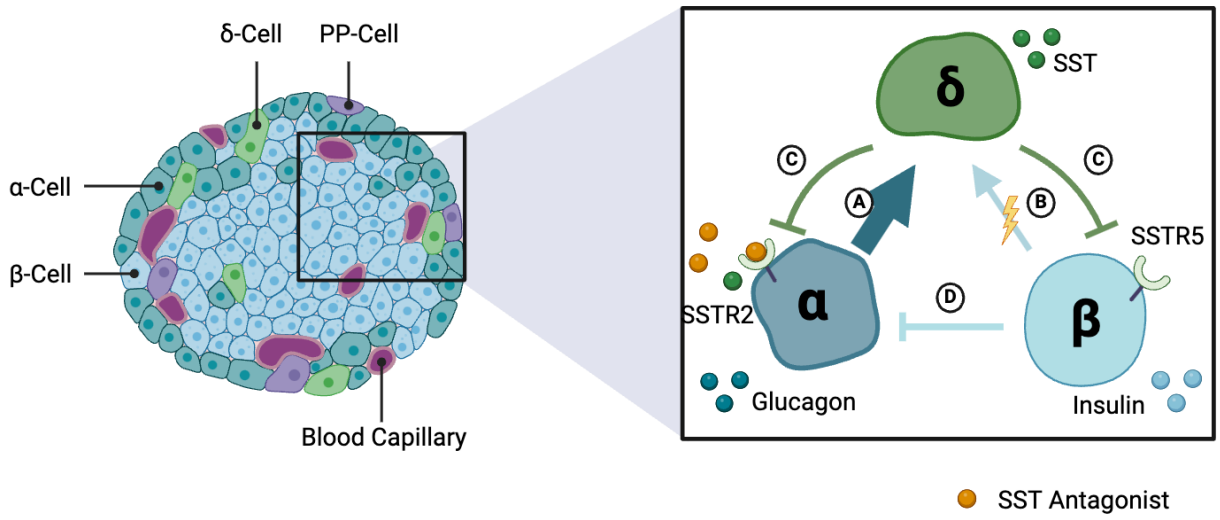


FIGURE 1. INTERRELATIONSHIP AMONG PANCREATIC ALPHA (α), BETA (β) AND DELTA (δ) CELLS.
(A) Glucagon and (B) Insulin stimulate SST secretion. (C) SST inhibits glucagon and insulin release via SSTR2 and SSTR5 respectively. (D) Insulin inhibits glucagon secretion. Yellow dots represent SST2 antagonists, blocking the inhibitory effect of SST on α-cells. SST: somatostatin; SSTR2/5: somatostatin receptor 2/5. (Modified from [Hoffman,2023])

2.6.1. SSTR2A TREATMENT IN T1D: PRECLINICAL STUDIES

Studies on T2D have shown that SST inhibits glucagon and insulin secretion through SST receptor 2 (SSTR2) and SST receptor 5 (SSTR5) [61,62,37]. Data suggests that SSTR2 antagonism can augment glucagon release in response to acute and recurrent hypoglycemia in rodent models of streptozotocin-induced diabetes [37],[14].

2.6.2. SSTR2A TREATMENT IN T1D: CLINICAL STUDIES

In human trials of T1D, ZT-01 has completed Phase 1 clinical trials [22] and the compound has already advanced to Phase 2b clinical trial for the prevention of nocturnal hypoglycemia in T1D (ClinicalTrials.gov ID NCT05762107). As noted above, the short-term use of SSTR2a to restore glucagon counterregulation during insulin-induced hypoglycemia in pre-clinical models of T2D has shown promising results [63], but the efficacy of sustained SSTR2a in animal models of diabetes remains to be demonstrated.

2.6.3. SSTR2A TREATMENT IN T2D: PRECLINICAL STUDIES

Treatment with PRL-2903 and ZT-01 increased glucagon responses in a HFF and low-dose streptozotocin-induced rat model of T2D and resulted in less hypoglycemia exposure after insulin bolus overdose [63]. The effect of ZT-01 seemed to be dose-dependent and the level of glucagon counterregulatory failure appears to be less in this model of diabetes as compared to earlier studies conducted on rats with T1D who had little to no beta cell function [64]. In addition, the administration of ZT-01 (0.3 mg/kg) also increased glucagon and glucose levels transiently even in the euglycemic and mildly hyperglycemic state, thereby suggesting that the drug might deteriorate glucose control at least at initial dosing [59]. However, to date, the effect of chronic or sustained dosing of ZT-01 at mealtime, or soon after meals, and during iatrogenic hypoglycemia in rodents with T2D is unknown.

3.0 RATIONALE

3.1. RATIONALE

As noted above, the newly developed SSTR2a ZT-01 by Zucara Therapeutics, in partnership with the Riddell laboratory at York University, has shown promising results for the amelioration of iatrogenic hypoglycemia in rodents and humans with T1D. However, this drug's therapeutic potential and possible off-target effects on metabolism in T2D have yet to be explored. Specifically in this thesis, I examined the effect of sustained SSTR2a exposure on glucagon counterregulation during recurrent iatrogenic hypoglycemia and oral glucose tolerance in a rat model of late-stage (advanced) T2D, where insulin therapy is required to limit hyperglycemia. In this study, the novel approach to administrating this antagonist is using implantable mini-osmotic pumps in a sustained drug release format. The sustained release with these pumps is aimed at mimicking the possible therapeutic delivery of ZT-01 using either long-acting formulation or constant infusion (pump) therapy.

3.2. OBJECTIVES

The primary purpose of these studies was to characterize insulin resistance, oral glucose tolerance and glucagon counterregulatory function during insulin-induced hypoglycemia in a rat model of T2D treated, or not treated, chronically with SSTR2a using implantable mini osmotic pumps. Overall, the primary goal was to evaluate the efficacy of chronic ZT-01 treatment on the attenuation of insulin-induced hypoglycemia in a T2D model, while a secondary goal was to

determine if this drug results in sustained elevations in circulating glucagon levels (i.e., hyperglucagonemia) and a deterioration of oral glucose tolerance.

I hypothesized that sustained release of ZT-01 using mini osmotic pumps would restore glucagon counterregulation but not alter the glucagon response to oral glucose, nor glycemia, as the glucagon stimulatory effects of SSTR2a may be largely glucose-dependent [37,65].

3.3. STUDY ENDPOINTS

The co-primary endpoints for the hypoglycemic induction protocols are the glucagon area under the curve (AUC) and peak glucagon levels as measured during insulin-induced hypoglycemia. Secondary endpoints include c-peptide levels during hypoglycemia and during oral glucose tolerance tests and liver glycogen levels after the hypoglycemic challenge. The co-primary endpoints during the oral glucose tolerance test were glucagon AUC and glycemia.

4.0 METHODS AND EXPERIMENTAL DESIGN

This study is carried out by the recommendation of the Canadian Council for Animal Care guidelines and has been approved by the York University Animal Care Committee (Protocol # 2017-7).

For this protocol, we used a total of 39 (n=9 pilot, n=22 follow-up study, n=8 higher dose study), healthy (n=6) and T2D (33) male Sprague-Dawley rats (Charles River Laboratories, approximately 300 grams post-weaned) aged 9-10 weeks old. Rats were housed in groups of 3-4, with a 12-hour light/dark cycle. The rats were on high-fat feeding (HFF) for 6 weeks prior to study onset to help induce insulin resistance with ad libitum access to water. Animals were habituated to the vivarium for seven days before any animal handling.

The selection of experimental parameters was carefully considered, drawing from a combination of sponsor recommendations, previous work conducted in our laboratory, and collaborative research from partner labs. The insulin dosage of 12U/kg was specifically chosen based on its demonstrated efficacy in inducing at least level 1 hypoglycemia in rats, as observed in our preliminary studies, and corroborated by earlier research in our lab. This dose strikes a balance between reliably lowering blood glucose levels to the desired range for studying hypoglycemic responses while minimizing the risk of severe hypoglycemia.

The period of insulin maintenance, set at one week before the insertion of mini osmotic pumps delivering the SSTR2a, was crucial for two reasons: firstly, to mimic a model of insulin-treated humans with diabetes, and secondly, to establish and maintain blood glucose levels within a targeted range of 15-25 mmol/L across all groups. This week-long insulin treatment ensured

that the rats were in a stable, insulin-treated state before introducing the experimental drug, allowing for a more accurate assessment of SSTR2a's effects in the context of ongoing insulin therapy. Similarly, the dosages of SSTR2a were selected based on a combination of pharmacokinetic data from previous studies and dose-response relationships observed in preliminary experiments. These carefully chosen parameters were designed to optimize the study's ability to detect the effects of SSTR2a on glucagon secretion and glucose homeostasis while maintaining consistency with established protocols in this field.

4.1. T2D INDUCTION

To induce overt insulin-requiring T2D, the HFF rats were injected with a low-dose (35 mg/kg) streptozotocin (STZ) via the intraperitoneal route, as previously described [63]. Before STZ injection, the rats underwent an overnight fast and diabetes was confirmed the next day with a blood glucose meter and tail nick sample (Figure 2).

4.2. INSULIN MAINTENANCE PERIOD

After induction of diabetes, the rats were placed on a basal-bolus insulin maintenance regimen to keep their basal glucose concentration within the range of 10-25 mmol/L [63]. Primarily, insulin glargine (Lantus, Sanofi-Aventis, Bridgewater, New Jersey), a long-acting basal insulin, was used daily to maintain glucose control in the animals (~3-4 U/kg/day, as needed) based on their glucose levels to maintain a basal glucose level of 10—25 mmol/L. To help reduce the risk of treatment-related hypoglycemia on non-study days, bolus insulin (Regular insulin, Humalog) was only used on non-experimental days if profound hyperglycemia was observed (>33

mmol/L) or if significant ketones were present (i.e., whole blood beta-hydroxybutyrate>1.5 mmol/L). The rats were then randomly placed in drug (STTR2a; ZT-01, Zucara Therapeutics) or vehicle-treated groups and the mini-osmotic pumps (Alzet, Durect Corporation, Cupertino, CA) were implanted which delivered the drug at two possible doses (0.18 mg/day pilot study, 0.3 mg/day follow-up study) or vehicle (Figure 2). The vehicle was composed of the same components as the drug (solvent, stabilizers, etc.), except without the active drug component (also supplied by Zucara Therapeutics).

4.3. MINI OSMOTIC PUMPS

In this study, the drug delivery mode was through mini osmotic pumps (see https://www.alzet.com/products/alzet_pumps/). The pumps selected for this thesis were 2ML2 and 2ML1, releasing the drug at the constant rate of 5.0 µL/h and 10 µL/h respectively (Appendix A).

The formulation of the drug remained consistent throughout the different studies. However, the stock solution carried different concentrations of the drug to allow for a dose-ranging assessment of the various study outcomes. During model development, a very low dose of the drug was chosen which amounted to 0.18 mg/day. After optimizing the protocol and based on the initial results, higher dosages were selected for subsequent Study 1 (0.3 mg/day) and Study 2 (1.2 mg/day).

4.3.1. Pump Priming and Implantation

All the steps prior to and during the implantation surgery were performed under sterile conditions. Before implanting the pumps, they were filled with either the drug or vehicle (placebo). To prepare the pump, 2ml of drug, or vehicle, is injected into the pump using a blunt needle provided in the packaging. The pumps were weighed before and after drug injection to ensure consistency within the injected volume. Following loading the pump with the intended volume (~2ml), the caps were placed on the pump, the capped pumps were placed in a falcon tube that was filled with saline to initiate the release of the drug/vehicle through osmosis. Using inhalable isoflurane, the rats were placed under anesthesia. After two minutes of inhaling isoflurane, the animal was removed from the box and placed on the sterile surgery bench. The animals were placed in a prone position with their face placed in a mask to ensure constant delivery of inhalable isoflurane throughout the surgery.

Before proceeding to make an incision, the rat's reflexes were checked to ensure the required level of anesthesia had been reached (i.e., Level 3; deep sedation). The rats were then injected with local anesthetic (Xylocaine, Aspen Pharmacare, Oakville, Canada) on the incision site for additional local pain management, particularly during the postoperative period. Then using sterile forceps and scissors, a 1-2 cm incision was made in the skin, on the back of the rat, between the scapulae. After the incision, we used a sterile hemostat to spread the subcutaneous connective tissue apart to create a deep enough subcutaneous pocket to accommodate the pump (See Appendix A for the pump). The pump was then inserted into the pocket with the flow moderator facing inside the body and away from the incision site. The skin

incision was then closed using Vicryl suture thread (Ethicon, Sommerville, New Jersey). The surgery took approximately 5-7 minutes. After incision closure, the animal was removed from the surgical bench and placed in their cage with clean bedding.

The animals were monitored for ~2 hours post-surgery to ensure that they regained consciousness and resumed activity post-anesthesia. If the animal was showing signs of pain and distress, it was administered a single dose of Metacam 20mg/ml (Boehringer Ingelheim, lot number: L21013A-33). If animals showed no more signs of discomfort, they were returned to the vivaria and the evening checks (Insulin maintenance) proceeded as scheduled.

4.4. ORAL GLUCOSE TOLERANCE TEST

We performed an oral glucose tolerance test (OGTT; 2 g/kg D-glucose) ~48-72 hours post-surgery on the animals to receive further confirmation of the T2D model. Additionally, an OGTT was performed to help establish whether the drug affects oral glucose tolerance and that both groups (drug and vehicle/Placebo) will behave similarly in response to glucose exposure (See Appendix B for the OGTT timeline).

For the OGTT, rats were given 2g/kg D-glucose (90-100% solution in water) through oral gavage and were monitored for two hours following that. Whole blood glucose concentrations from a tail nick were measured using a glucometer every 15 minutes (in duplicate) and saphenous blood was collected at 0-, 30-, 60- and 120-minutes for hormone (glucagon, c-peptide) analyses (see below).

4.5. INSULIN TOLERANCE TEST (ITT)/HYPOGLYCEMIC CHALLENGE

To investigate the effect of chronic ZT-01 treatment, two hypoglycemic challenges (insulin tolerance tests) were performed (See Appendix. C for the hypoglycemic challenge timeline).

For this procedure, rats were partially fasted by receiving 15 g chow the night before (controlled fed), with any remaining food removed in the morning (9 AM). At approximately 11 AM, rats were injected with a bolus dose (12U/kg) of rapid-acting insulin (NovoRapid, Novo Nordisk, Mississauga, Canada). Whole blood samples were taken from the saphenous vein at t=0-, 30-, 60-,90- and 120 minutes post-insulin administration. The blood was centrifuged for the isolation of plasma and samples were then placed on ice (see hormone analyses below). Whole blood glucose concentrations were monitored from a tail nick or saphenous bleeds every 15 minutes post insulin administration.

4.6. PLASMA AND TISSUE COLLECTION AND PROCESSING

Whole blood samples were collected during the OGT test and an insulin tolerance test (ITT: hypoglycemic challenge). The tubes used for saphenous blood collections were coated with EDTA to prevent coagulation. The whole blood was then centrifuged at 12,000 revolutions per minute (RPM) for ~5 minutes. On the second ITT day, after the ITT, the animals were anesthetized using isoflurane gas. Once anesthetized, portal blood was collected using a 25-gauge needle and an EDTA-coated 1 ml syringe, the needle was then removed (to prevent the blood from being hemolyzed) and the blood was transferred to an EDTA-coated tube and was centrifuged at 12000 RPM for 5 minutes. The animals were euthanized by exsanguination and

the following tissues were collected and flash frozen in liquid nitrogen: heart, liver, pancreas, tibialis anterior muscle, soleus muscle and gastrocnemius. The tissues were then transferred to a -80^{o}C freezer for future histology and/or analyses.

4.7. HORMONAL ANALYSES

Plasma glucagon concentrations were quantified using ELISA (Mercodia, Uppsala, Sweden) and plasma c-peptide concentrations were quantified using ELISA (CrystalChem, Illinois, USA).

4.8. LIVER GLYCOGEN

Liver glycogen content was determined using a modified version of the Carr and Neff method [66]. In brief, frozen tissue samples were digested in 0.5 mL of 1 M KOH at 70°C for 1 hour. The pH of the resulting digest was adjusted to 4.8 before adding acetate buffer and 0.5 mg/mL amyloglucosidase. The glycogen was then hydrolyzed at 40°C for 2 hours. Glucose concentrations were measured enzymatically, with absorbance readings taken at 340 nm using a spectrophotometer (Ultraspec 2100 pro; Biochrom Ltd, Cambridge, UK).

4.9. STATISTICAL ANALYSES

All data are presented as means ± standard deviation (SD), unless otherwise stated. For the two hypoglycemic challenge days, we calculated the “net” glucagon area under the curve (AUC) to assess the possible impact of drug and vehicle on glucagon responsiveness to hypoglycemia (i.e., change for baseline glucagon levels). For these analyses, each animal’s glucagon concentration prior to insulin administration served as baseline and any rise (or drop) from

baseline was included in the analyses (GraphPad Prism Software, San Diego, CA). Statistical analyses were performed with a significance threshold of $P < 0.05$ using Prism software (GraphPad). All data were analyzed using either a t-test or one-way or two-way independent sample analysis of variance (ANOVA) as appropriate unless otherwise stated. Multiple comparisons were done using Tukey's post hoc test.

5.0 RESULTS

5.1. CHARACTERISTICS OF THE RODENT MODEL

In this rodent model of T2D, there was no obvious effect of chronic ZT-01 treatment on average daily blood glucose levels as measured in the morning (fed) or evening, albeit the glucose values were numerically higher, by ~3 mmol/L, on average, with the 0.18 mg/day and 1.2 mg/day groups relative to the vehicle-treated rats with T2D (Table 1). Notably, the moderate dose of ZT-01 (0.3 mg/day) resulted in an average blood glucose level of 19.5 mmol/L, which was slightly lower than the vehicle group. As shown in Table 1, the highest dose (1.2 mg/day) of ZT-01 resulted in an average morning blood glucose value of 23.4±1.8 mmol/L, about 10% higher than the vehicle group. However, there was no significant difference in the average morning glucose values across all treatment groups (vehicle vs 0.18 mg/day, p=0.3093; vehicle vs 0.3 mg/day, p=0.9796; vehicle vs 1.2 mg/day, p=0.6820). A similar pattern of glycemia was consistent in evening glucose levels, where no obvious (or statistical) drug effect on glycemia was observed (vehicle vs 0.18 mg/day, p=0.9781; vehicle vs 0.3 mg/day, p=0.9377; vehicle vs 1.2 mg/day, p=0.9997).

As expected, the nondiabetic (healthy) HFF vehicle controls had significantly lower morning blood glucose levels than the T2D vehicle. Healthy HFF rats treated with vehicle and ZT-01 (0.3 mg/day) had similar morning glucose levels averaging 6.5±0.8 and 6.9±0.6, respectively, with no apparent drug effect observed (p=0.5265). Additionally, there was no significant difference in evening glucose levels in healthy HFF rats treated with vehicle and ZT-01 (6.6±0.1 vs 6.9±0.6, p=0.4789)

As detailed in Table 1, the rats exhibited similar average body weights, for the most part, throughout the studies. However, there was a significant difference between the average body weight of T2D high (1.2 mg/day) ZT-01 treated rats and T2D low (0.18 mg/day) dose ZT-01 treated rats, whereby the higher drug-dosed animals weigh less than the lower drug dosed group (460.9±37.88 vs 401.1±29.82 grams, p=0.0065).

TABLE 1. CHARACTERISTICS OF THE RODENT MODEL

Conditions	N	Average Morning Blood Glucose (mmol/L)	Average Evening Blood Glucose (mmol/L)	Body Weight (Grams)
T2D Vehicle	8	21.3±4.8	22.8±2.1	449.1±38.89
T2D ZT-01 (0.18 mg/day)	5	25.0±2.9	22.8±2.4	460.9±37.88
T2D ZT-01 (0.3 mg/day)	8	20.5±4.5	21.5±3.6	425.3±37.57
T2D ZT-01 (1.2 mg/day)	7	23.4±1.8	21.6±3.3	401.1±29.82*
Healthy HFF Vehicle	3	6.3±1.1#	6.6±1.0#	474.8±23.99
Healthy HFF ZT-01 (0.3 mg/day)	3	6.5±0.8#	6.9±0.6#	459.9±8.935

The results indicate that sustained exposure to ZT-01 did not significantly influence morning or evening glucose levels. *= significantly different from T2D ZT-01 (0.18 mg/day) group (p=0.0402) and Healthy HFF Vehicle group (p=0.0065). $^{\#=}$ significantly different from all T2D groups.

5.2. HYPOGLYCEMIC CHALLENGES

5.2.1. IMPACT OF SUSTAINED SSTR2A ON GLUCAGON AUC AND PEAK GLUCAGON LEVELS DURING IATROGENIC HYPOGLYCEMIA

The influence of low (0.18 mg/day), moderate (0.3 mg/day) and high (1.2 mg/day) dose ZT-01 treatment on baseline glucagon concentrations (pre-hypoglycemia induction) is shown in Figure 3, while the glucagon AUCs during ITT/hypoglycemia in these same groups are shown in Figure 4. Of note, peak glucagon levels during ITT/hypoglycemia are also shown in Supplementary Figures 3B and 3E.

During the first ITT/hypoglycemic challenge, as displayed in Figure 3A, Supplementary Figure 5 and Supplementary Figure 6, baseline glucagon concentrations were significantly elevated in the high (1.2 mg/day) dose ZT-01 treated group as compared to the vehicle-treated group (61.82±45.56 vs 12.94±9.540, p=0.0075). The low (0.18 mg/day) and moderate (0.3 mg/day) doses of ZT-01 had similar baseline glucagon concentrations as the HFF T2D rats and vehicle-treated T2D rats (p=0.7533 and p=0.9843 respectively).

As shown in Figure 3B, during the second hypoglycemic challenge, there was a significant difference in baseline glucagon levels between the low dose ZT-01 group (0.18 mg/day) and the vehicle group (26.48±5.10 vs 6.41±6.46, p=0.0015). The moderate and high dose ZT-01 groups

did not demonstrate differences in baseline glucagon concentrations as compared to the vehicle-treated group (p=0.6367 and p=0.1483).

As depicted in Figure 4, there was no clear dose-dependent effect of chronic ZT-01 treatment on glucagon AUC to insulin-induced hypoglycemia, with the moderate dose (0.3 mg/day) resulting in higher net glucagon AUC during hypoglycemia, while the highest dose resulted in a net reduction in glucagon AUC. There was a statistically significant difference in net glucagon AUC between the high dose ZT-01 (1.2 mg/day) rats and the vehicle-treated rats (-3968±3400 vs 1998±1384, p=0.0176). The moderate dose ZT-01-treated rats (0.3 mg/day) exhibited a higher net glucagon AUC as compared to high dose ZT-01-treated rats (1.2 mg/day) (4190±5547 vs - 3968±3400, p=0.0010). The relationship between baseline glucagon concentration and net glucagon AUC for each of the treatment groups on both hypoglycemic challenge days is shown in Supplementary Figure 5. There was no clear association between baseline glucagon concentrations and net glucagon AUC in the vehicle-treated and low-dose drug-treated groups. However, the medium dose (0.3 mg/day) tended to result in a positive association between baseline glucagon concentration and net glucagon AUC while the highest dose (1.2 mg/day) resulted in a negative association.

5.2.2. IMPACT OF SUSTAINED SSTR2A ON HYPOGLYCEMIA RESISTANCE

The incidence rate of hypoglycemia during the two hypoglycemia induction challenges is shown in Figure 5. During the first hypoglycemic challenge (Figure 5A), the highest dose of ZT-01 (1.2 mg/day) was the most effective at attenuating hypoglycemia, with only 1 of 7 animals having a

glucose concentration <3.9 mmol/L during the 2-hour challenge. This attenuation may be more related to the elevated baseline glycemia than the net glucagon response, however.

Surprisingly, there was no clear ZT-01 dose-response effect on the incidence rate of hypoglycemia in challenge 1 or 2. Specifically, the moderate dose did not significantly affect the hypoglycemia incidence rate as compared to the vehicle-treated rats, while the lowest dose (0.18 mg/day) showed an apparent attenuation of hypoglycemia by ~40%.

On the second hypoglycemic challenge (Figure 5B), the highest dose (1.2 mg/day) was moderately effective in preventing hypoglycemia, with a ~40 % attenuation in the incidence of hypoglycemia as compared to vehicles. Similarly, during the second hypoglycemic challenge, the lowest dose (0.18 mg/day), resulted in ~40 % attenuation in hypoglycemia while the moderate dose was not at all effective at attenuating hypoglycemia with 8 out of 8 animals having a glucose concentration <3.9 by the end of the challenge (at 120 minutes).

5.2.3. IMPACT OF SUSTAINED SSTR2A ON LIVER GLYCOGEN AT THE END OF HYPOGLYCEMIC CHALLENGE

Liver glycogen content as measured at the end of hypoglycemia challenge 2 was highly variable among the animals and not statistically different across the four T2D treatment groups (Figure 6). Thus, there was no apparent effect of ZT-01 on hepatic glycogen content.

5.3. IMPACT OF SUSTAINED SSTR2A ON OGTT

5.3.1. IMPACT OF SUSTAINED SSTR2A ON HOMA-IR AT THE TIME OF OGTT

Markers of whole-body insulin resistance in ZT-01-treated and non-drug-treated rats were assessed from HOMA-IR in select healthy and T2D rats (Figure 7). HOMA-IR tended to be higher overall in the rats with T2D as compared to healthy rats, but the differences did not reach statistical significance. In addition, ZT-01 did not appear to influence HOMA-IR in healthy or T2D rats, albeit the values were numerically higher in the 0.3 mg/day and 1.2 mg/day ZT-01 groups relative to all other groups.

5.3.2. IMPACT OF SUSTAINED SSTR2A ON GLYCEMIA, GLUCAGON AND C-PEPTIDE LEVELS DURING OGTT

Glucose, glucagon, and c-peptide response to OGTT in rats with and without ZT-1 are shown in Supplementary Figures 3 and 4. A trend was noted for ZT-01 to deteriorate glucose tolerance throughout the OGTT, but the differences were again not statistically significant. At baseline, the rats exposed to the lowest dose of ZT-01 (0.18 mg/day), did not appear to experience significantly higher blood glucose levels compared to the vehicle-treated group (13.7±10.2 vs 14.8±5.8, p=0.8824). Additionally, at the same dose (0.18 mg/day), the average blood glucose was not altered in the ZT-01-treated group compared to the vehicle-treated group (25.1±4.0 vs 25.1±5.0 and p>0.999).

During the OGTT, at the moderate (0.3 mg/day) dose, baseline glucose levels (Supplementary Figure 3A), were not significantly different between the T2D ZT-01-treated group and the T2D vehicle-treated group, albeit glucose values were numerically higher in the ZT-01-treated group (11.3±9.0 vs 7.1±5.5, p=0.2862). Furthermore, the average blood glucose throughout OGTT did not appear to be significantly altered by exposure to ZT-01 in T2D rats (17.9±3.1 vs 16.0±3.9 in ZT-01 vs vehicle, p=0.5690).

At the highest dose of ZT-01 used in this study (1.2 mg/day), baseline glucose levels were significantly higher (18.3±4.2 mmol/L) than the vehicle-treated group (7.9±5.4 mmol/L; p=0.0019), as were average glucose levels during the OGTT (Supplementary Figure 4A). By the end of the OGTT, average glucose levels remained higher in the ZT-01-treated rats as compared to the vehicle-treated rats (26.6±2.36 vs 18.9±5.25 mmol/L, p=0.0004). Thus, overall, the highest dose of ZT-01 appeared to result in some deterioration in glycemia at baseline and in response to an OGTT.

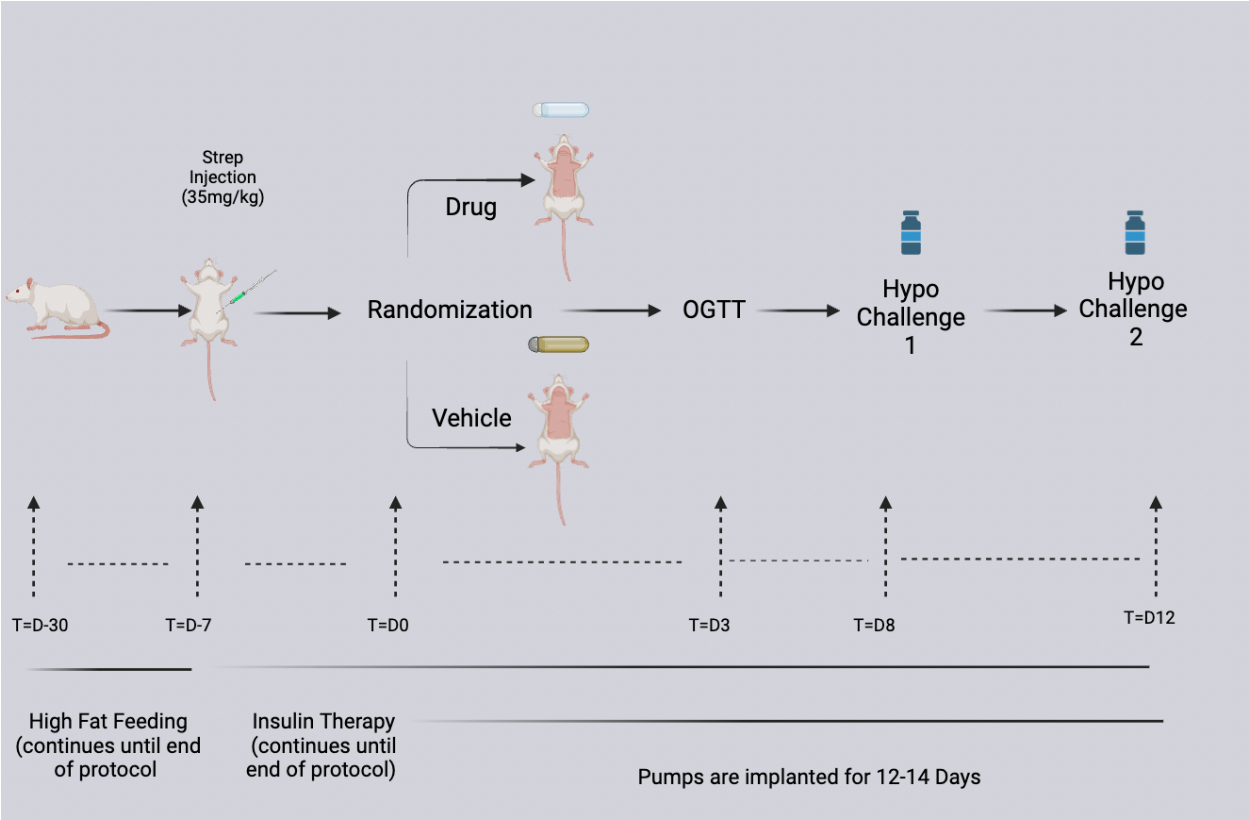
Glucagon levels during OGTT are shown in Supplementary Figures 3B and 4B. Chronic exposure to the lowest dose of ZT-01 (0.18 mg/day) resulted in baseline glucagon levels that were similar to the vehicle-treated rats (p=0.782). Furthermore, sustained exposure to higher doses of ZT-01 (0.3 mg/day, Supplementary Figure 3B and 1.2 mg/day, Supplementary Figure 4B) do not appear to impact glucagon levels significantly during OGTT (p=0.1667 and p=0.1370 respectively). Similarly, baseline glucagon levels were not different from vehicles at the lower dosages of ZT-01 (Supplementary Figure 3).

C-peptide levels during the OGTT are shown in Supplementary Figures 3C and 4C. Baseline c-peptide levels in exposure to the lowest dose of ZT-01 (0.18 mg/day) did not appear to be

significantly different among treatments. That was further translated into average c-peptide values throughout the OGTT in exposure to all three different doses of ZT-01 (0.18 mg/day, 0.3 mg/day and 1.2 mg/day) compared to vehicle treatment (p=0.7430, p=0.9330 and p=2077 respectively). Overall, the results demonstrated that ZT-01 does not significantly alter c-peptide levels in HFF-T2D animals compared to vehicle-treated HFF T2D animals.

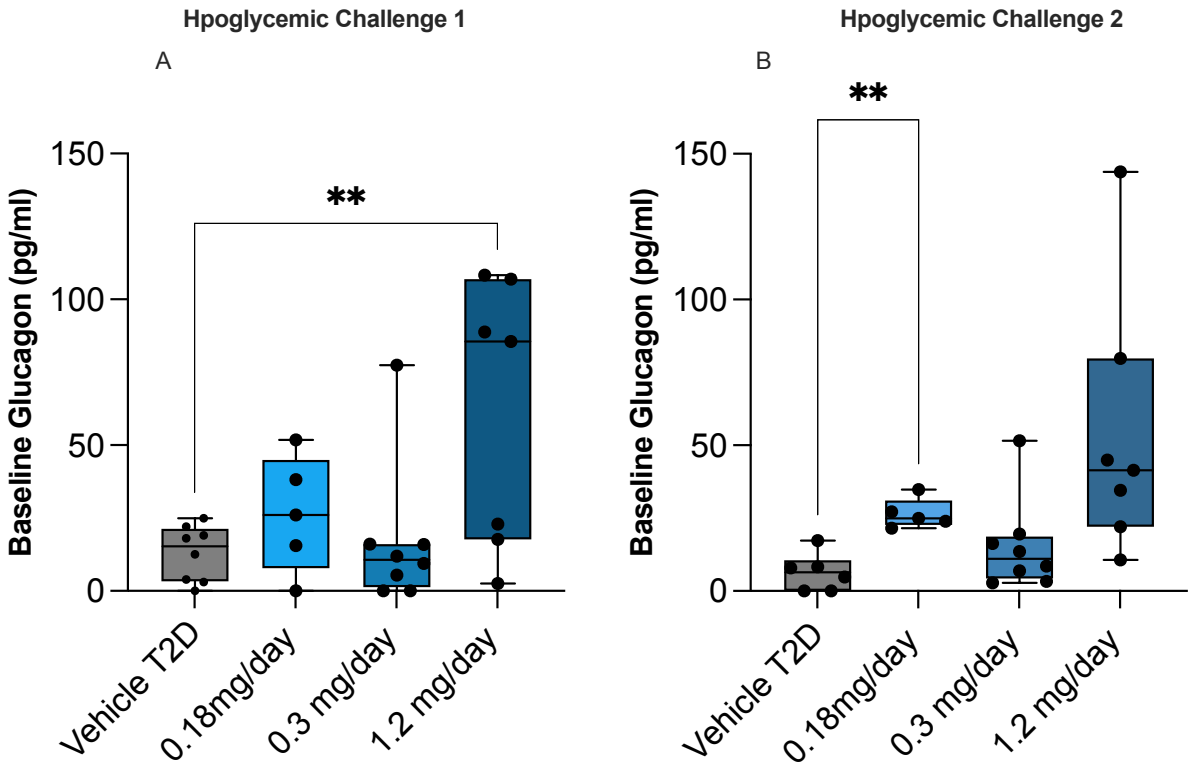
6.0 FIGURES

FIGURE 2. STUDY DESIGN.



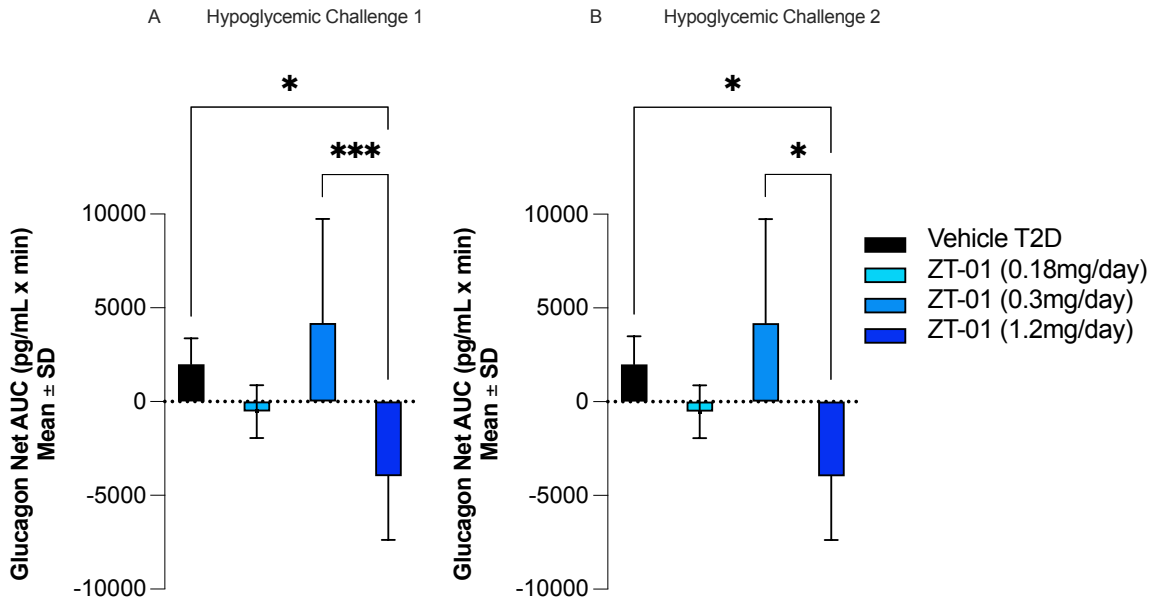
Notes: Animals received on D=-30, started high fat feeding (HFF) upon arrival. Low-dose Streptozotocin injection (35mg/kg) on D=-7. Animals underwent an insulin maintenance period for 1 week before randomization and pump implantation surgery. The maintenance period was followed by an oral glucose tolerance test (OGTT) on D=3. They were exposed to two insulin tolerance tests (ITT) on D=8 and D=12. On each ITT, rats will receive a bolus dose (12U/kg) of Rapid-acting insulin (Novorapid). (For the highest dose study, OGTT took place on D=2, and hypo challenges 1 and 2 took place on D=4 and D=7 respectively).

FIGURE 3. BASELINE GLUCAGON LEVELS (PG/ML) IN RESPONSE TO HYPOGLYCEMIC CHALLENGES IN HFF RATS WITH T2D.



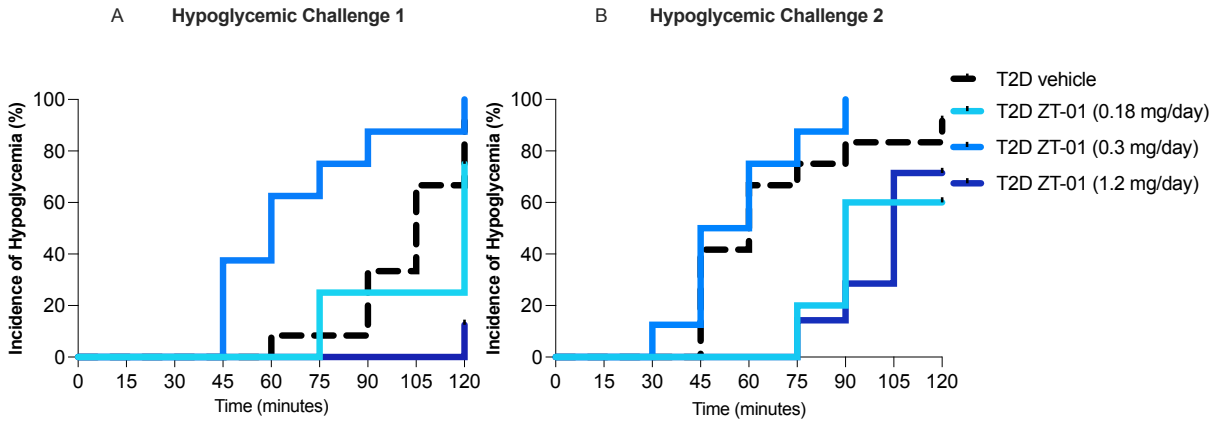
Notes: Panels A and B show baseline glucagon levels (pg/mL) prior to hypoglycemic challenges 1 and 2, respectively. Data are presented as box plots with individual data points, representing means ± SD. A: Baseline glucagon levels before Hypoglycemic Challenge 1. B: Baseline glucagon levels before Hypoglycemic Challenge 2. The treatment groups are depicted as follows: Vehicle T2D (grey), ZT-01 at 0.18 mg/day (light blue), ZT-01 at 0.3 mg/day (blue), and ZT-01 at 1.2 mg/day (dark blue). Statistical significance between groups is indicated by asterisks: **P < 0.01.

FIGURE 4. GLUCAGON AREA UNDER THE CURVE (AUC) DURING HYPOGLYCEMIC CHALLENGES.



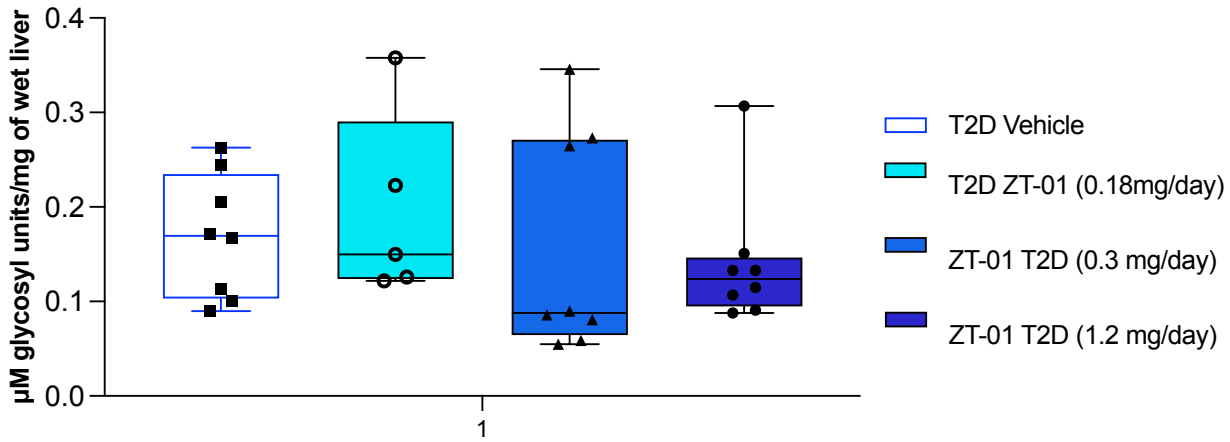
Notes: Panels A and B depict the net area under the curve (AUC) of glucagon levels (pg/mL x min) in response to two hypoglycemic challenges. Data are expressed as means ± SD. A: Hypoglycemic Challenge 1, B: Hypoglycemic Challenge 2. Different treatment groups are represented by distinct bars: Vehicle T2D (black), ZT-01 at 0.18 mg/day (light blue), ZT-01 at 0.3 mg/day (blue), and ZT-01 at 1.2 mg/day (dark blue). Statistical significance is denoted by asterisks: *P < 0.05 and ***P < 0.001.

FIGURE 5. INCIDENCE OF HYPOGLYCEMIA (%) DURING HYPOGLYCEMIC CHALLENGES IN RATS WITH T2D.



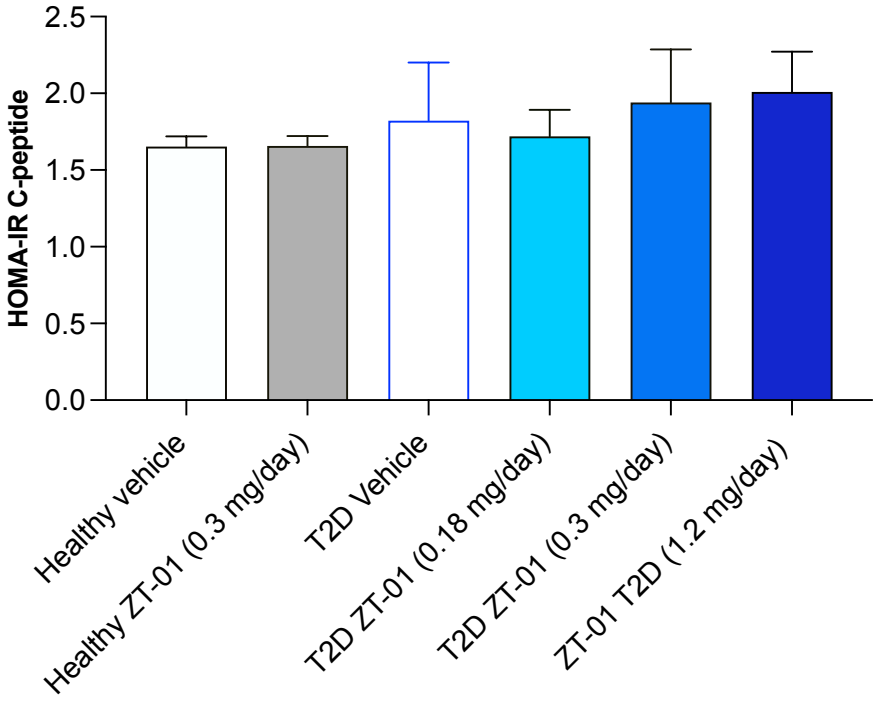
Notes: Panels A and B illustrate the incidence of hypoglycemia over time during two hypoglycemic challenges. The data are expressed as the percentage of rats experiencing hypoglycemia at different time points (minutes). A: Hypoglycemic Challenge 1. B: Hypoglycemic Challenge 2. The treatment groups are represented by different lines: T2D vehicle (black dashed line), T2D ZT-01 at 0.18 mg/day (light blue line), T2D ZT-01 at 0.3 mg/day (blue line), and T2D ZT-01 at 1.2 mg/day (dark blue line). The x-axis represents time in minutes, while the y-axis shows the percentage incidence of hypoglycemia.

FIGURE 6. LIVER GLYCOGEN LEVELS (µM GLYCOSYL UNITS/MG OF WET LIVER) IN RATS WITH T2D WHO UNDERWENT DRUG (ZT-01) AND VEHICLE TREATMENT.



Notes: The box plots illustrate liver glycogen levels for rats under different treatment conditions, with data presented as means ± SD. Treatment groups include ZT-01 at 0.18 mg/day (white bar with blue outline), ZT-01 at 0.3 mg/day (light blue bar), ZT-01 at 1.2 mg/day (blue bar) and vehicle (white bar with blue outlines). The x-axis represents the different groups, while the y-axis shows liver glycogen levels. No significant differences were observed among the groups, indicating that sustained exposure to ZT-01 did not significantly influence liver glycogen levels.

FIGURE 7. HOMEOSTATIC MODEL ASSESSMENT FOR INSULIN RESISTANCE (HOMA-IR) IN HFF HEALTHY AND RATS WITH T2D WHO UNDERWENT DRUG (ZT-01) AND VEHICLE TREATMENT.



Notes: The bar graphs illustrate the HOMA-IR values for rats under different treatment conditions. Data are presented as means ± SD. The treatment groups are represented as follows: White bar: Healthy controls, grey bar: T2D controls, White bar with blue outline: T2D ZT-01 at 0.18 mg/day, Light blue bar: T2D ZT-01 at 0.3 mg/day, blue bar: T2D ZT-01 at 1.2 mg/day
The x-axis represents the different groups, while the y-axis shows the HOMA-IR values. There were no significant differences observed among the groups.

7.0 DISCUSSION

7.1. PRINCIPLE FINDINGS

Iatrogenic hypoglycemia (i.e., accidental hypoglycemia caused by therapeutic insulin) is the main treatment barrier to insulin therapy in T1D and insulin-requiring T2D [67,68]. The failure of glucagon levels to rise during hypoglycemia may be at least partially related to elevations in SST levels in diabetes [65]. The recent development of SSTR2a allows for testing the hypothesis that glucagon counterregulation can be restored with SSTR2a treatment in insulin-requiring diabetes [21], but previous literature had yet to examine the effect of sustained-release SSTR2a on glucagon physiology and in particular glucagon counterregulation to iatrogenic hypoglycemia, in-human or pre-clinical rodent models of insulin-requiring T2D. Thus, in the present study, we aimed to investigate the effect of chronic (i.e., sustained) SSTR2a treatment using mini osmotic infusion pumps on glucagon counterregulation and OGTT in rats with insulin-requiring T2D. While treatment strategies exist for the "rescue" of hypoglycemia in insulin-treated diabetes [69,70], no pharmacological solution exists for the prevention of hypoglycemia for either diabetes subpopulation. While pre-clinical and early-phase human trials for hypoglycemia prevention exist for hypoglycemia in T1D, where beta cell mass is lost and glucagon deficiency is consistently observed [71], few investigations to date have explored the safety and effectiveness of acute or sustained SSTR2a treatment in T2D where glucagon hypersecretion may be observed at mealtime and a blunted glucagon response to hypoglycemia may also exist [72].

This study aimed to assess if sustained release of SSTR2a, at varying dosages using mini osmotic pumps, could augment glucagon counterregulation and attenuate hypoglycemia without increasing basal glucagon secretion and worsening oral glucose tolerance. In support of the literature that SSTR2a can delay hypoglycemia onset following iatrogenic insulin "overdose" in T1D [21,22,63,64], this study is the first to demonstrate that sustained SSTR2a exposure can delay or even prevent hypoglycemia in an animal model of T2D, it can also potentially raise basal glucagon levels and deteriorate glucose tolerance at mealtime.

From these results, it was found that there was no significant effect of chronic drug treatment with ZT-01 on average daily blood glucose levels, albeit the highest dose of the drug given (1.2 mg/day) did appear to be associated with slightly elevated glycemia prior to hypoglycemia induction and it was the most effective in limiting hypoglycemia exposure during the first iatrogenic hypoglycemia challenge. During the second hypoglycemic challenge, the groups treated with 0.18 mg/day and 1.2 mg/day of ZT-01 experienced moderate attenuations in hypoglycemia incidence rate, albeit with no obvious increase in glucagon responsiveness to hypoglycemia, while all the rats in the moderate dose group (0.3 mg/day) experienced at least level 1 hypoglycemia. In summary, the highest dose of ZT-01 significantly reduced the incidence of hypoglycemia during the hypoglycemic challenges, indicating the drug's effectiveness in preventing hypoglycemia. Interestingly, this came with elevated baseline glycemia and basal glucagon levels, suggesting that the drug's hypoglycemia-preventing effects may operate through mechanisms beyond acute glucagon responsiveness. This observation points to a complex interplay between ZT-01's effects on basal hormone levels and its ability to prevent hypoglycemia, potentially offering protection at the cost of altered baseline glycemic control.

Several earlier studies have focused on examining the effects of acute SSTR2a treatment on glucagon counterregulation, glycemia, c-peptide levels, and liver glycogen in various rodent models of diabetes or counterregulatory failure. In one study by Farhat et al., the effectiveness of PRL-2903, a preliminary SSTR2a formulation, as well as the new formulation, ZT-01, were evaluated in a repeat drug dosing protocol (not chronic administration) in male T1D rats subjected to two episodes of hypoglycemia [64]. The results and others [60,22,65], clearly demonstrate that SSTR2a effectively increases the glucagon response to iatrogenic hypoglycemia in a rodent model of T1D. Similarly, in previous studies by us and others, rats exposed to a bolus overdose of insulin showed improved glucagon counterregulation with SST antagonism [37,60,64,73].

The most recent studies, conducted by D'Souza et al. focused on male T2D rats treated with ZT-01 [74,63]. These studies included male rat models subjected to high-fat feeding to induce insulin resistance, followed by partial beta cell knockout using low-dose streptozotocin. The rats were then treated with insulin to maintain basal blood glucose levels between 15-25 mmol/L before exposure to hypoglycemic challenges. Similar to our study, ZT-01 significantly increased glucagon levels within 60 minutes of acute dosing, and these levels remained elevated during the ITT/hypoglycemia challenge. In that study, peak glucagon concentration (Cmax) was 156 ± 50 pg/mL in the ZT-01 group compared to 77 ± 46 pg/mL in the control group ($p < 0.01$). In terms of hypoglycemia prevention, ZT-01 reduced the incidence of hypoglycemia by about 40% (63% vs. 100% in controls) and delayed the onset of hypoglycemia in those rats that did develop hypoglycemia (103.1 ± 24.6 minutes vs. 66.1 ± 23.6 minutes, $p < 0.05$). During the OGTT, ZT-01 increased glucagon response without exacerbating hyperglycemia, likely due to a corresponding

increase in c-peptide levels (6251 ± 5463 vs. 14008 ± 5495, p = 0.013). The authors concluded that ZT-01 effectively enhances glucagon responses and reduces hypoglycemia exposure in T2D rats but more research was needed to determine the optimal dosing schedules for chronic SSTR2 antagonism in T2D [63]. Interestingly, in our study, it was also found that while the moderate dose did not significantly affect hypoglycemia incidence, the highest dose was effective in reducing hypoglycemia during the first challenge but the glucagon responsiveness to hypoglycemia was clearly dysfunctional, thereby demonstrating a paradoxical effect of high dose ZT-01 treatment on glucose counterregulation. Future studies are required to determine if high dose ZT-01 blunts glucagon responsiveness to hypoglycemia, perhaps because it increases baseline glucagon secretion, which may be detrimental to the normal functioning of the pancreatic α-cell [75].

As noted above, the inability to observe a boost in glucagon levels with higher doses of ZT-01 (under sustained-release conditions) may be attributed to the drug-induced elevation in baseline glucagon levels. A study by Farhy et al., aimed to explore the relationship between basal hyperglucagonemia and impaired glucagon counterregulation in T1D [1]. Using a combination of *in vivo* and *in silico* studies, it was demonstrated that higher basal glucagon levels were linked to a reduced glucagon counterregulation response to hypoglycemia. This was evidenced by a negative correlation between basal glucagon and cumulative glucagon levels during hypoglycemia. The study concluded that basal hyperglucagonemia contributes to impaired glucagon counterregulation in T1D, highlighting the importance of managing basal glucagon levels to improve hypoglycemia counterregulation. Even though their study was done on a T1D cohort, we may be able to conclude that elevated baseline glucagon levels in all forms

of diabetes, and when SSTR2a is given at high pharmacological levels chronically, as in this thesis work, might impair glucagon pulsatility to hypoglycemia, thereby limiting the pancreatic α-cells full glucagon secretion potential [1,75].

In our study, we clearly observed that higher doses of ZT-01 under sustained-release conditions led to elevations in glucagon levels even before exposure to a bolus overdose of insulin. Specifically, the group exposed to 1.2 mg/day of ZT-01 showed significantly higher glucagon levels, even before exposure to bolus overdose of insulin, compared to the vehicle group (61.82 ± 45.56 pg/mL vs 12.94 ± 9.540 pg/mL, p = 0.0294). This suggests that sustained-release ZT-01 can elevate basal glucagon levels, potentially impacting its counterregulation to hypoglycemia. This is particularly evident through the glucagon AUC, where under 1.2 mg/day conditions, we observed a negative glucagon AUC (Figure 4, Supplementary Figure 5). A negative glucagon AUC indicates a decrease in glucagon levels over time, suggesting that despite the initial elevation, the overall glucagon response was reduced under sustained-release conditions. However, one might also argue that since the glucose concentration did not reach level 1 hypoglycemia with the highest dose of ZT-01, the need for a rise in glucagon counteregulation may not have been met.

The observed differences between T1D and T2D in response to SSTR2a treatment may be attributed to the differences in underlying pathophysiology between the two conditions. In T1D, where beta cell mass is lost and glucagon deficiency is consistently observed, SSTR2a treatment aims to enhance the deficient glucagon response [64]. In contrast, T2D is characterized by insulin resistance and often an overproduction of glucagon at mealtimes. Therefore, SSTR2a treatment in T2D must balance the enhancement of counterregulation during hypoglycemia

with the risk of exacerbating hyperglucagonemia and hyperglycemia at other times. These findings highlight the complexity of SSTR2a treatment in T2D compared to T1D. While SSTR2a aims to enhance deficient glucagon response in T1D, in T2D, it must balance enhancing counterregulation during hypoglycemia with avoiding hyperglucagonemia and hyperglycemia at other times. This underscores the importance of precise dosing and monitoring in T2D treatment to achieve optimal therapeutic outcomes.

While previous studies have primarily focused on the glucose-glucagon axis [60,64,65], our findings suggest that the effects of SSTR2a may be more complex. The paradoxical effect of high-dose ZT-01 on glucose counterregulation, where glucagon responsiveness to hypoglycemia appeared blunted despite reduced hypoglycemia incidence, hints at alternative mechanisms of action. These could include effects on other hormones regulated by somatostatin, such as growth hormone or insulin, or even direct effects on hepatic glucose production or peripheral glucose utilization.

In conclusion, while our study demonstrates the potential of sustained-release SSTR2a in preventing hypoglycemia in T2D, it also reveals the complexities of long-term somatostatin receptor antagonism. The effects of SSTR2a likely extend beyond simple enhancement of glucagon secretion, potentially involving multiple hormonal and metabolic pathways. Future research should aim to elucidate these alternative mechanisms and explore optimal dosing strategies that balance hypoglycemia prevention with overall glycemic control in T2D. Moreover, investigation into the effects of SSTR2a on other somatostatin-regulated hormones

and metabolic processes could provide a more comprehensive understanding of its therapeutic potential and limitations in diabetes management.

7.2. STUDY STRENGTHS AND LIMITATIONS

This is the first study to examine chronic administration of the drug, ZT-01, through mini-osmotic pumps in an HFF T2D rat model and to explore the differences in glucagon AUC and glycemia during an OGTT and iatrogenic hypoglycemia under sustained release of three dose levels of SSTR2a. Individuals with diabetes often manage their condition with a combination of medications, adding to the complexity of their treatment regimen. Daily injections of any drug, including insulin, are burdensome, requiring regular physician consultations for dose adjustments among other considerations. Therefore, investigating a sustained release format of ZT-01 is both novel and important. Such formulations could potentially offer longer intervals between injections, reducing the burden and cost associated with daily injections, including the need for syringes.

This study presents several strengths that help to highlight its novelty. Firstly, it is the first to test a sustained-release formulation of the drug, which had not been previously explored. Further enhancing the study's uniqueness is the experimentation with multiple ZT-01 dosages, allowing us to assess the effects of varying rates of drug exposure. We also conducted oral glucose tests and examined daily glycemia and ITT designed to simulate insulin treatment-related hypoglycemia. Moreover, daily insulin administration was standardized across all groups, with modifications made only when necessary, based on ketone body measurements (no apparent effect of chronic SSTR2a on ketone levels was observed).

This research also has limitations that should be acknowledged. Firstly, the sample size of rodents allocated to each experimental group was small, particularly in the low-dose ZT-01 group as it was a pilot study. Secondly, a significant limitation lies in the methodology of glucose collection. It is crucial to acknowledge that blood glucose measurements are subject to a ceiling effect, reaching a maximum value of 33.3 mmol/L. Consequently, during oral glucose tolerance tests (OGTT), the resulting error bars tended to be narrow at the higher glucose levels and exhibited reduced data variability at the detectable glucose threshold of the glucose meters used. Another limitation stems from the variability inherent in the glycemic response observed across the rodents that were run in batches rather than all at once. This pronounced variability could potentially be mitigated through the inclusion of more expansive sample sizes and with more staggered ZT-01 dose-level testing.

7.3. THESIS SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

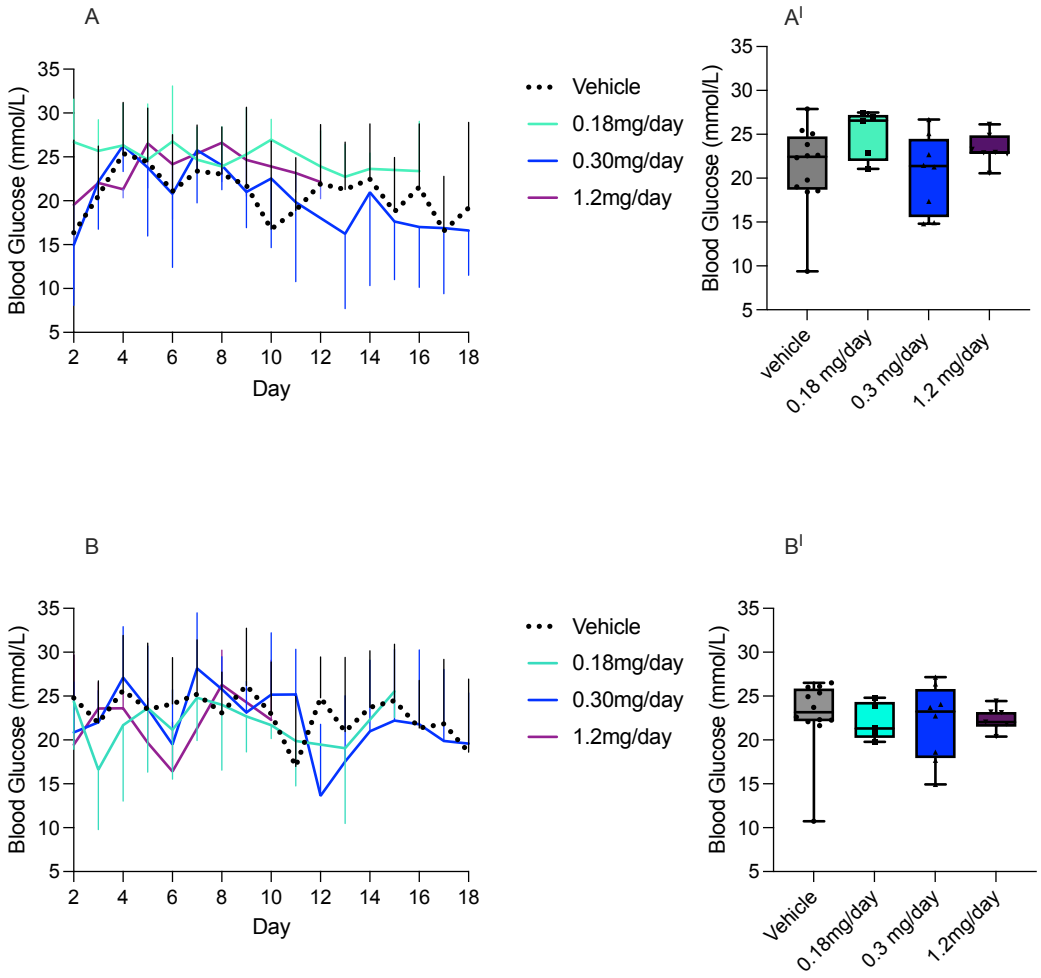
Overall, these findings align with previous literature suggesting that SSTR2a can impact glucagon responsiveness to hypoglycemia in diabetes and potentially reduce hypoglycemia exposure. This work also highlights the potential implications of drug-induced alterations in baseline glucagon levels when evaluating the efficacy of ZT-01 under hypoglycemic conditions. This study supports the potential of sustained-release SSTR2a may be a viable strategy for hypoglycemia prevention in T2D, albeit the drug dosing amount for sustained drug treatment remains unclear. Nonetheless, these results provide a foundation for further research into optimizing dosing regimens and exploring long-term outcomes of ZT-01 treatment in diabetic populations.

Future directions for this study include exploring different doses of the drug to identify therapeutic doses of ZT-01. Additionally, long-term formulations will be investigated, such as once-weekly and once-monthly injections. These formulations will be tested to ensure that, after injection, the drug solidifies in the body and then slowly releases. Pharmacokinetic (PK) studies will be performed to confirm drug concentration. These long-term formulations will initially be tested in preclinical settings and, after completing the appropriate levels of testing, will proceed to clinical trials.

In conclusion, this study demonstrated that ZT-01 can attenuate the incidence of hypoglycemia. However, sustained release of ZT-01 through mini-osmotic pumps could introduce the risk of baseline hyperglucagonemia in certain animals and possibly a diminished glucagon counterregulatory response to iatrogenic hypoglycemia caused by insulin overdose.

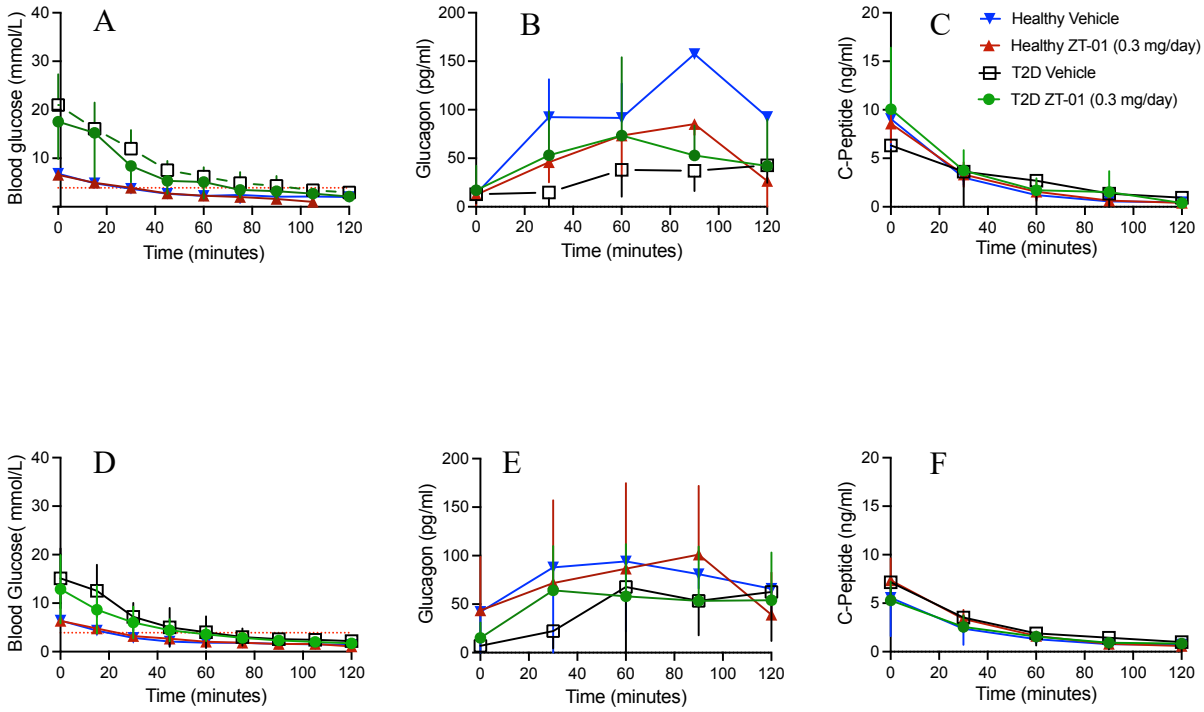
8.0 SUPPLEMENTARY FIGURES AND TABLES

SUPPLEMENTARY FIGURE 1. MORNING AND EVENING GLUCOSE LEVELS ACROSS THE STUDY DAYS.



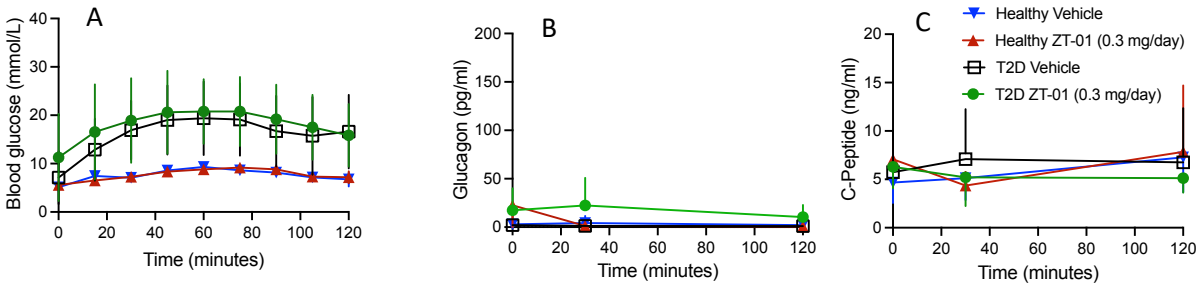
Notes: A) Time course of morning glucose levels in animals over the study days under different treatment conditions. The treatments include Vehicle T2D (black), ZT-01 at 0.18 mg/day (light blue), ZT-01 at 0.3 mg/day (blue), and ZT-01 at 1.2 mg/day (dark blue). Data points represent means ± SD. A') Corresponding average values for glucose levels measured over the same period and under the same treatment conditions as in Panel A. The bar graph presents the average blood glucose values (mean ± SD) for each treatment group, with statistical significance indicated by asterisks where applicable. B) Time-course of evening blood glucose levels of animals over the study days, using the same treatment groups. Data points represent means ± SD. B') Corresponding average values for glucose levels measured over the same period and under the same treatment conditions as in Panel A. The bar graph presents the average blood glucose values (mean ± SD) for each treatment group. There is no statistical difference denoted in this figure.

SUPPLEMENTARY FIGURE 2. TIME-COURSE ANALYSIS OF BLOOD GLUCOSE (MMOL/L), GLUCAGON (PG/ML) AND C-PEPTIDE (NG/ML) LEVELS DURING TWO HYPOGLYCEMIC CHALLENGES IN RESPONSE TO ZT-01 (0.3 MG/DAY) AND VEHICLE IN HFF T2D AND HFF HEALTHY RAT MODELS.



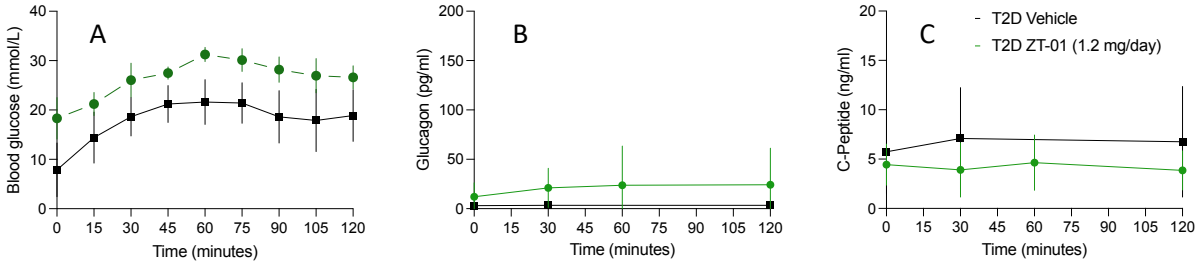
Notes: This figure shows the time course of blood glucose, glucagon, and c-peptide levels during two hypoglycemic challenges in response to ZT-01 and vehicle treatments in T2D and healthy rat models over 120 minutes. In Hypoglycemic Challenge 1 (Panels A-C), T2D rats treated with ZT-01 exhibited lower blood glucose levels compared to T2D vehicle, indicating improved glycemic control. Glucagon levels were higher in T2D vehicle compared to healthy controls, with a reduction seen in the ZT-01 treated groups. c-peptide levels showed minimal differences between groups. In Hypoglycemic Challenge 2 (Panels D-F), T2D rats treated with ZT-01 continued to show numerically lower blood glucose levels compared to T2D vehicle but the results were not statistically significant. Glucagon levels remained elevated in T2D vehicle but were reduced in ZT-01 treated groups, like Challenge 1. C-peptide levels were consistent across all groups. Data points represent means ± SD for each group.

SUPPLEMENTARY FIGURE 3. TIME-COURSE ANALYSIS OF BLOOD GLUCOSE (MMOL/L), GLUCAGON (PG/ML), AND C-PEPTIDE (NG/ML) LEVELS DURING AN OGTT IN HFF HEALTHY AND HFF T2D RATS TREATED WITH ZT-01 (0.3 MG/DAY) OR VEHICLE.



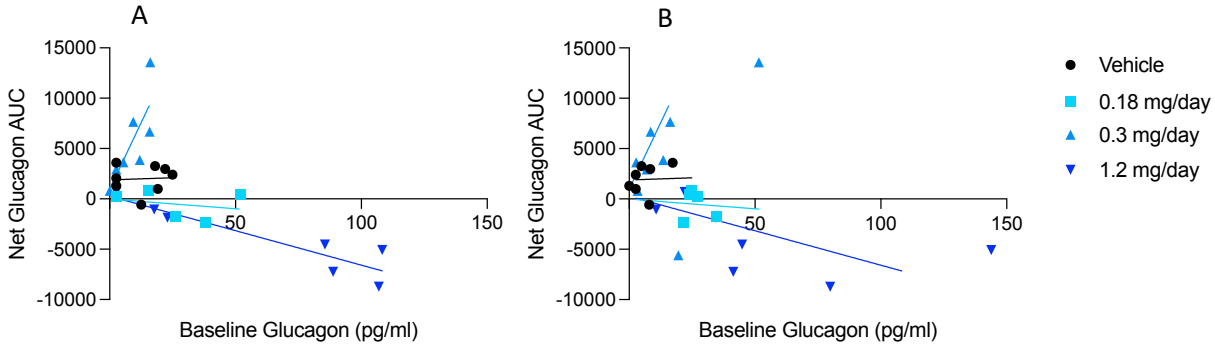
Notes: A) Blood glucose levels (mmol/L) measured over 120 minutes in four treatment groups: Healthy Vehicle (blue), Healthy ZT-01 at 0.3 mg/day (red), T2D Vehicle (black), and T2D ZT-01 at 0.3 mg/day (green). Data points represent means ± SD. B) Glucagon levels (pg/mL) were measured over 120 minutes in the same four treatment groups. Data points represent means ± SD. C) c-peptide levels (ng/mL) were measured over 120 minutes in the same four treatment groups. Data points represent means ± SD. The x-axis in all panels represents time in minutes, while the y-axis represents the respective measured parameter: blood glucose (mmol/L) in Panel A, glucagon (pg/mL) in Panel B, and c-peptide (ng/mL) in Panel C.

SUPPLEMENTARY FIGURE 4. TIME-COURSE ANALYSIS OF BLOOD GLUCOSE (MMOL/L), GLUCAGON (PG/ML), AND C-PEPTIDE (NG/ML) LEVELS DURING AN OGTT IN HFF T2D RATS TREATED WITH ZT-01 (1.2 MG/DAY) OR VEHICLE.



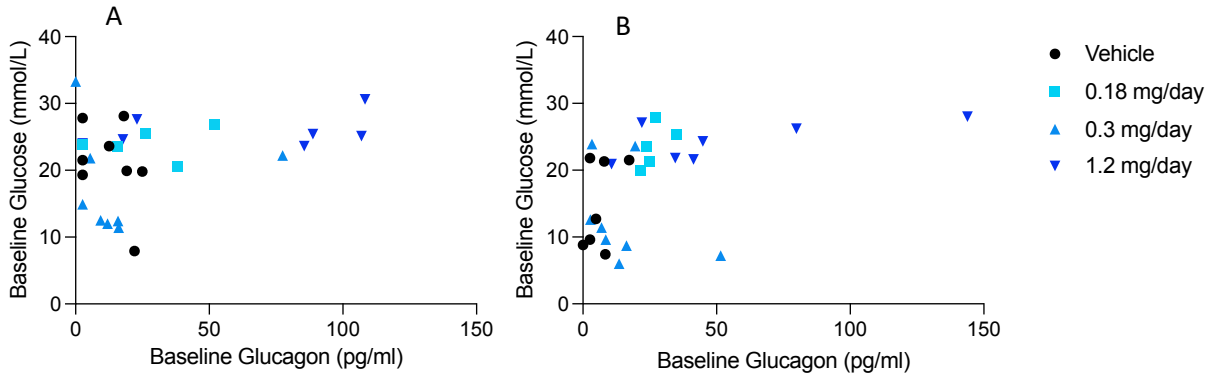
Notes: Panel A shows blood glucose levels over time. At baseline, glucose levels were significantly elevated in the ZT-01 treated group compared to the vehicle group (18.3 ± 4.2 mmol/L vs. 7.9 ± 5.44 mmol/L, p = 0.0019). This elevation persisted throughout the experiment, and the average blood glucose throughout the OGTT appeared to be significantly higher in the ZT-01-treated group compared to the vehicle-treated group (26.6 ± 2.36 mmol/L vs. 18.9 ± 5.25 mmol/L, p=0.0004). Panel B displays glucagon levels over time. Glucagon levels remained relatively stable across both treatment groups, with no significant differences observed between ZT-01 treated and vehicle groups. Panel C illustrates c-peptide levels over time. c-peptide levels also remained stable and showed no significant differences between the ZT-01-treated group and the vehicle group. Data points in all panels represent means ± SD.

SUPPLEMENTARY FIGURE 5. RELATIONSHIP BETWEEN BASELINE GLUCAGON LEVELS AND NET GLUCAGON AUC DURING HYPOGLYCEMIC CHALLENGES



Notes: Scatter plots show net glucagon AUC plotted against baseline glucagon (pg/ml) for different treatment groups. Black circles represent vehicle control. Coloured symbols indicate treatment groups: 0.18 mg/day (light blue squares), 0.3 mg/day (light blue triangles), and 1.2 mg/day (dark blue inverted triangles). Blue lines represent the linear regression for each plot. Panel A shows the relationship for the first hypoglycemic challenge. A negative correlation is observed between baseline glucagon levels and net glucagon AUC across all groups. Panel B displays the same relationship for the second hypoglycemic challenge, with a similar negative trend. In both panels, the 1.2 mg/day group tends to have lower net glucagon AUC values, particularly at higher baseline glucagon levels, suggesting a dose-dependent effect on glucagon response. The vehicle group shows more variability in net glucagon AUC across baseline glucagon levels compared to the treatment groups. Data points represent individual measurements for each animal.

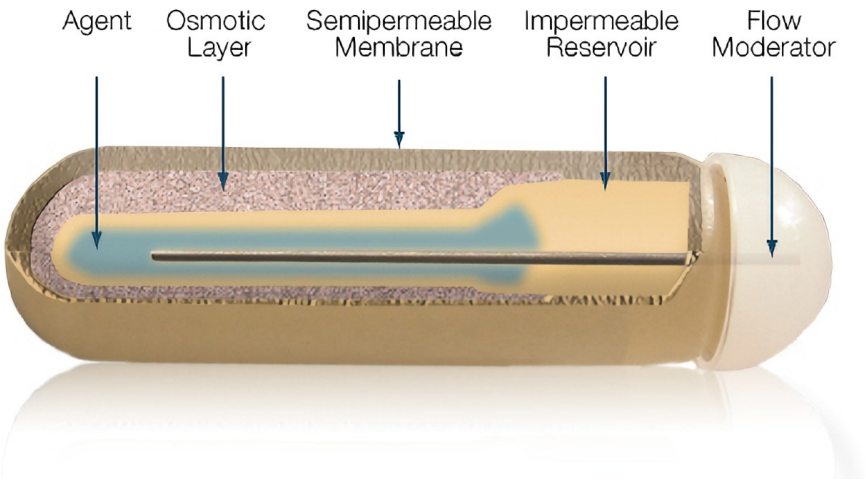
SUPPLEMENTARY FIGURE 6. RELATIONSHIP BETWEEN BASELINE GLUCOSE AND GLUCAGON LEVELS DURING HYPOGLYCEMIC CHALLENGES.



Notes: (A) Hypoglycemic challenge 1 and (B) Hypoglycemic challenge 2. Scatter plots show baseline glucose (mmol/L) plotted against baseline glucagon (pg/ml) for different treatment groups. Black circles represent vehicle control. Coloured symbols indicate treatment groups: 0.18 mg/day (light blue squares), 0.3 mg/day (light blue triangles), and 1.2 mg/day (dark blue inverted triangles). Each point represents an individual measurement. The figure illustrates the distribution of baseline glucose and glucagon levels across treatment groups during two separate hypoglycemic challenges, allowing for comparison of the metabolic profiles between vehicle and drug-treated subjects.

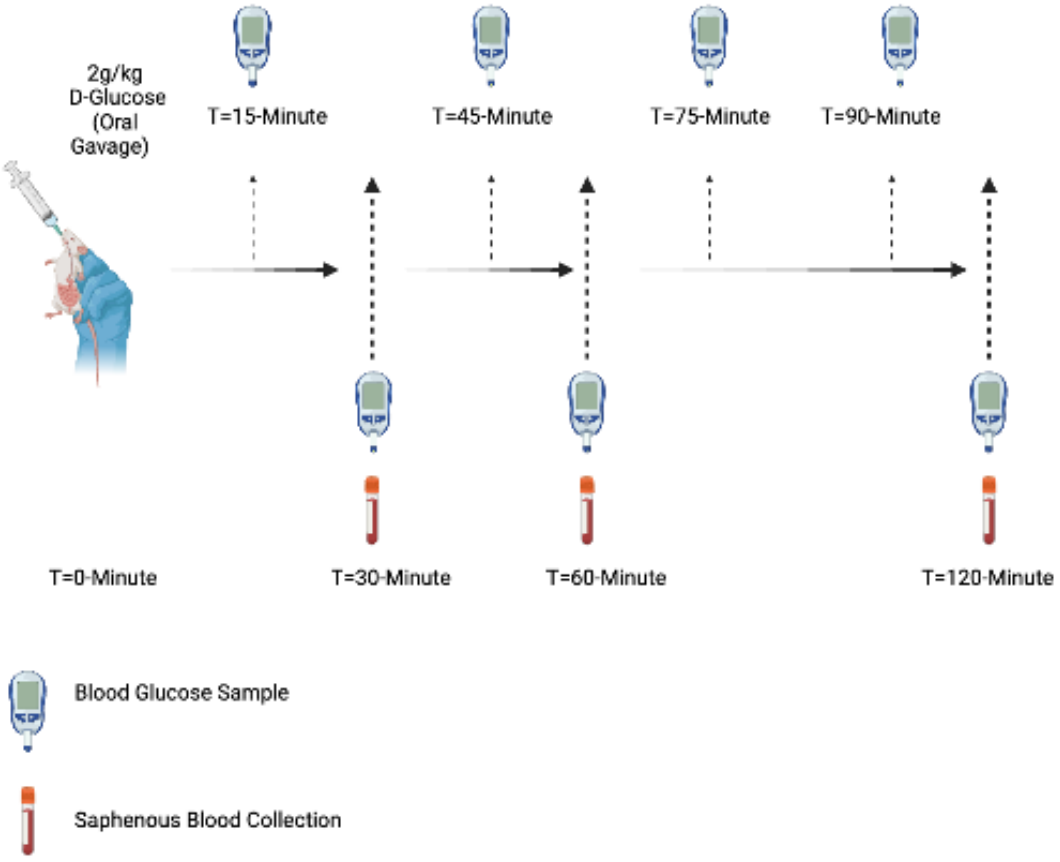
9.0 APPENDICES

APPENDIX A. DIAGRAM OF ALZET OSMOTIC PUMP.



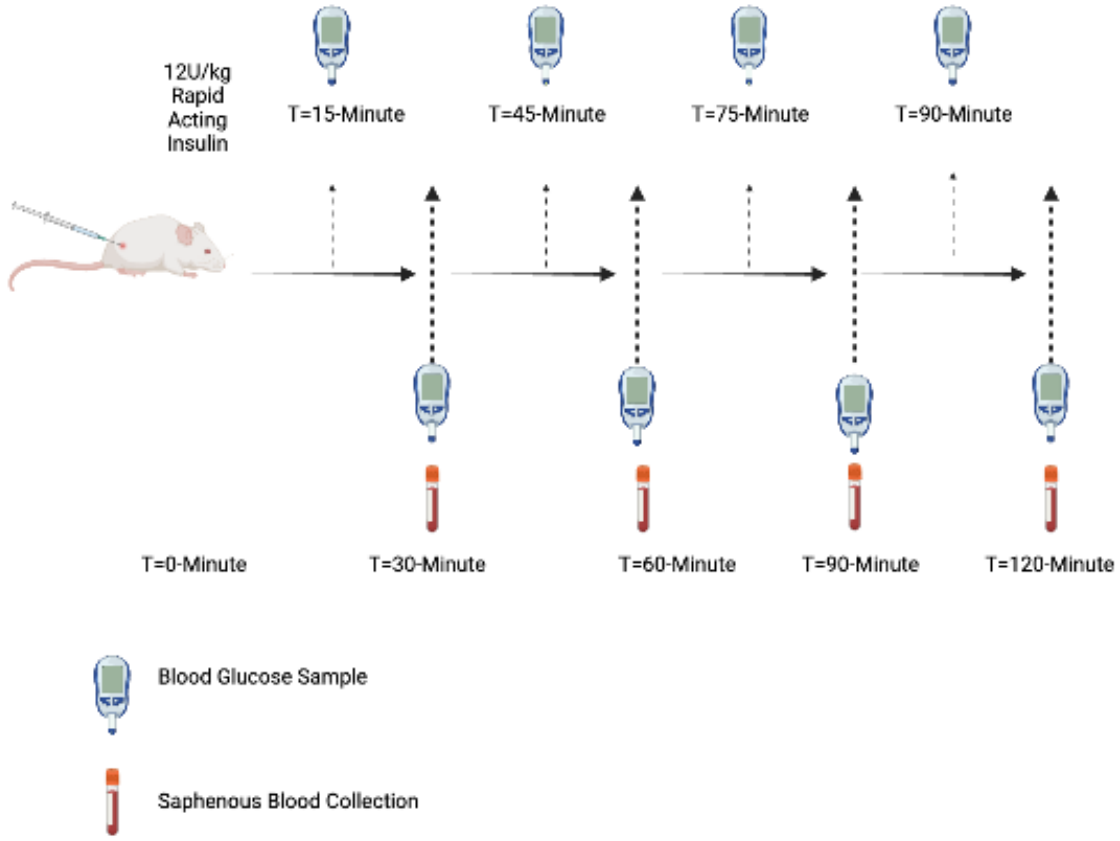
Notes: The pump used for the first two studies was a 2ML2 model with a delivery rate of 5µL/h for 14 days. The pump in the final study was the 2ML1 model which delivered the drug at the constant rate of 10 µL/h for 7 days.

APPENDIX B. TIMELINE OF THE ORAL GLUCOSE TOLERANCE TEST.



Notes: Rats received 2g/kg of D-glucose via oral gavage. They were monitored for 120 minutes. Whole blood glucose concentration (BG) measurements were collected every 15 minutes. Saphenous blood collection followed at 30,60 and 120 minutes. The blood was centrifuged, and the plasma was collected and stored at -80 °C.

APPENDIX C. TIMELINE OF THE HYPOGLYCEMIC CHALLENGE.



Notes: Rats received a bolus dose (12U/Kg) of rapid-acting insulin subcutaneously. They were monitored for 120 minutes. Whole blood glucose concentration (BG) measurements were collected every 15 minutes. Saphenous blood collection followed at 30,60 and 120 minutes. The Saphenous blood was centrifuged, and the plasma will be collected and stored at -80 °C for future analyses.

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