

GLX10, A Novel Immunometabolism Modulator, Improves Glycemic Control and Modulates Inflammatory Readouts in a High Fat Diet and Streptozotocin Induced Rat Model of Type 2 Diabetes: An In Vivo and In Vitro Study

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Abstract

Type 2 diabetes mellitus (T2DM) is a progressive metabolic disease characterized by chronic hyperglycemia, insulin resistance, progressive β -cell dysfunction, and persistent low-grade inflammation. Although current therapeutic approaches, particularly metformin, remain central to disease management, sustained metabolic control is still not consistently achieved in many patients. This highlights the need for novel treatment strategies that address not only glucose dysregulation but also the broader immunometabolic disturbances underlying disease progression. In the present study, GLX10 was evaluated in a high-fat diet/low-dose streptozotocin (HFD/STZ) induced rat model of T2DM over a 91-day period. In vivo efficacy was assessed through serial random blood glucose measurements and oral glucose tolerance testing (OGTT), with glucose exposure quantified using the area under the curve from 0 to 120 minutes (AUC₀₋₁₂₀). To ensure a rigorous safety assessment, histopathological and hematological evaluations were conducted in a separate cohort of six normal rats that received GLX10 administration at the start of the study for 20 days. Hematological parameters were subsequently monitored at 10-day intervals throughout the 91-day study period to capture potential time-dependent systemic effects. At study termination, animals were sacrificed, and comprehensive histopathological examination was performed. This approach enabled longitudinal evaluation of hematological changes alongside end-point tissue assessment, allowing for the characterization of potential GLX10-related toxicities independently of confounding effects associated with HFD and streptozotocin (STZ). Complementary in vitro studies in hepatic and macrophage-based systems were conducted to assess cytocompatibility, nitric oxide production, inflammatory cytokine release, and selected pathway-related signaling changes. GLX10 treatment was associated with a significant reduction in random blood glucose levels and a marked improvement in glucose tolerance compared with diabetic controls. Under the same experimental conditions, GLX10 also demonstrated greater improvement in OGTT AUC than metformin. In vitro, GLX10 preserved macrophage viability across functionally relevant concentrations while reducing nitric oxide production and suppressing IL-6 and TNF- α release. Exploratory signaling analyses further suggested modulation of NF- κ B- and mTOR-related pathways.

Taken together, these findings support GLX10 as a promising investigational immunometabolic modulator with antidiabetic potential in a preclinical setting. Nevertheless, further mechanistic, pharmacological, and translational studies are required to confirm its therapeutic relevance.

Keywords: Type 2 diabetes mellitus, GLX10, Immunometabolic modulation, Inflammation, Macrophages, Glucose tolerance, Streptozotocin, High-fat diet, Preclinical therapeutics

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic and multifactorial metabolic disorder characterized by persistent hyperglycemia resulting from the combined effects of insulin resistance and progressive pancreatic β -cell dysfunction [1,2]. Over recent decades, the global burden of T2DM has increased substantially, driven by lifestyle, demographic, and epidemiological transitions worldwide. This expanding prevalence has made T2DM one of the most pressing public-health challenges worldwide. Persistent hyperglycemia is strongly associated with both microvascular and macrovascular complications, including nephropathy, neuropathy, retinopathy, and cardiovascular disease [3].

Current therapeutic strategies are primarily directed toward improving glycemic control and enhancing insulin sensitivity. Among these, metformin remains the most widely prescribed first-line treatment because of its ability to suppress hepatic glucose production and improve peripheral glucose utilization [4]. Despite its clinical value, long term metabolic control remains suboptimal in a considerable proportion of patients, and disease progression often continues even with treatment intensification [5]. These limitations underscore the need for broader therapeutic approaches that address additional dimensions of T2DM pathophysiology.

Increasing evidence now supports the concept that T2DM is not solely a disorder of glucose metabolism, but also an inflammatory and immunometabolic disease [6,7]. Elevated concentrations of pro-inflammatory mediators such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) contribute to impaired insulin signaling and worsening metabolic dysfunction [8–10]. In parallel, macrophage activation and nitric oxide production amplify inflammatory stress, thereby linking immune dysregulation to defective metabolic homeostasis [10–15]. Mitochondrial dysfunction, oxidative stress, and impaired autophagic adaptation further contribute to cellular stress and reduced metabolic resilience [13]. The high fat diet combined with low dose streptozotocin (HFD/STZ) rat model is widely employed in preclinical diabetes research because it reproduces several core features of human T2DM, including insulin resistance, partial β -cell damage, and sustained hyperglycemia [6,7]. This model also enables the evaluation of both basal glycemic status and dynamic glucose handling through oral glucose tolerance testing (OGTT), providing a relevant platform for assessing candidate antidiabetic interventions [14].

GLX10 is an investigational compound examined in the present work as a potential immunometabolic modulator. Based on preliminary observations, it may affect both metabolic regulation and inflammation-associated cellular signaling. Accordingly, the present study was designed to evaluate the antidiabetic activity of GLX10 in an HFD/STZ induced rat model of T2DM, while complementary in vitro experiments were performed to examine cytocompatibility, modulation of inflammatory mediators, and selected signaling-pathway responses in hepatic and macrophage cell systems as shown in Supplementary Figure S1.

2. Aim and Objectives

2.1. Aim: The primary aim of this study was to evaluate the antidiabetic efficacy and preclinical safety profile of GLX10 through an integrated in vivo and in vitro experimental approach relevant to type 2 diabetes mellitus.

2.2. Objectives: To determine whether therapeutic administration of GLX10 improves longitudinal random blood glucose levels and end-point OGTT outcomes in the HFD/STZ rat model of T2DM, to compare the effects of GLX10 with those observed in diabetic controls, metformin-treated animals, prophylactic GLX10-treated animals, and sequential metformin→GLX10 treatment groups, to assess terminal biochemical, hematological, organ-weight, histopathological, and pancreatic insulin immunostaining parameters as supportive safety and tissue level outcome measures, to characterize the in vitro cytocompatibility profile of GLX10 in hepatic cell lines using sulforhodamine B (SRB) assays, to evaluate the effect of GLX10 on nitric oxide production and inflammatory cytokine release, particularly IL-6 and TNF- α , in macrophage based systems, to explore selected signaling-pathway alterations relevant to inflammation, autophagy, and cellular stress responses as preliminary mechanistic indicators.

3. Materials and Methods

3.1. Study design

This study comprised two complementary components: an in vivo experimental model in rats and an in vitro mechanistic evaluation. The in vivo arm was designed to evaluate the metabolic and biochemical effects of GLX10 in a high-fat diet/streptozotocin (HFD–STZ)–induced diabetic model. To ensure an accurate assessment of the safety profile of GLX10, histopathological and hematological evaluations were conducted in a separate cohort of six normal rats that received GLX10 administration at the start of the study for a duration of 20 days. Hematological parameters were subsequently monitored at 10-day intervals throughout the 91-day study period to evaluate any time-dependent systemic effects.

At the end of the study, animals were sacrificed, and comprehensive histopathological examination was performed. This design enabled longitudinal assessment of hematological changes and end-point evaluation of tissue integrity, allowing for the characterization of potential GLX10-related toxicities independently of confounding influences associated with HFD and streptozotocin (STZ).

The in vitro arm, conducted by NAWAH Scientific (Cairo, Egypt), was used to investigate cytotoxicity, inflammatory mediators, cell death patterns, and selected signaling pathways relevant to the interpretation of the in vivo findings as shown on Supplementary Figure S2.

The original project began as a broader dose-ranging program involving 120 male Wistar rats allocated across 20 experimental groups; however, the present report focuses on the six predefined groups that provided the most

interpretable and biologically relevant findings, with 6 rats per group (total $n = 36$). This reporting strategy was adopted to ensure clarity and scientific focus while transparently acknowledging the broader exploratory phase. Animals used in this study were obtained from the VACSERA (The Holding Company for Biological Products and Vaccines) farm, Helwan, Egypt. Animals were purchased from a certified commercial supplier for research purposes; therefore, informed consent from private owners was not applicable.

3.2. Animals and housing conditions

Male Wistar rats were used in the *in vivo* study. For the current report, data from six selected groups were analyzed. Animals were housed under controlled laboratory conditions at $22 \pm 2^\circ\text{C}$, $40 \pm 5\%$ relative humidity, and a 12 h light/12 h dark cycle, with free access to food and water. Rats were group-housed in polycarbonate cages with appropriate bedding and were allowed a 7-day acclimatization period before study initiation. These conditions were maintained throughout the experiment to reduce environmental variability and improve reproducibility.

3.3. High-fat diet and diabetic induction

To promote insulin resistance, animals received a high-fat diet for 4 weeks before chemical induction and throughout the study. The dietary regimen was formulated to provide approximately 45% of calories from fat, 35% from carbohydrates, and 20% from protein. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, 25 mg/kg) freshly prepared in 0.1 M citrate buffer (pH 4.5) under chilled and light-protected conditions. Animals were fasted for 6 h before STZ administration, and 10% glucose solution was supplied for 24 h afterward to reduce the risk of acute hypoglycemia. Diabetic status was confirmed 72 h later using a fasting blood glucose threshold of >250 mg/dL.

3.4. Experimental groups and treatment protocol

The present report includes six groups: (1) HFD + GLX10 (days 36–57), (2) post-oral metformin (days 36–63) followed by GLX10 (days 70–90), (3) HFD + metformin control (250 mg/kg), (4) normal control, (5) diabetic control (HFD + STZ + vehicle), and (6) prophylactic GLX10 (days 1–20 before STZ challenge). GLX10 was administered by subcutaneous injection at 5 units/rat twice daily. Metformin was given orally at 250 mg/kg. The study timeline included acclimatization, HFD exposure, STZ induction, diabetes confirmation, treatment phases, weekly monitoring, oral glucose tolerance testing, and terminal tissue collection.

3.5. In vivo measurements

Body weight and random blood glucose levels were recorded weekly using standardized procedures. On day 91, an oral glucose tolerance test was performed after a 6-hour fasting period using oral glucose (2 g/kg), with blood glucose levels measured at 0, 15, 30, 60, and 120 minutes. At study termination, animals were humanely euthanized under anesthesia. Blood samples were collected for biochemical and hematological analyses, and major organs were harvested for histopathological evaluation. Animals were anesthetized using intraperitoneal injection of ketamine (90 mg/kg) and

xylazine (10 mg/kg). Adequate depth of anesthesia was confirmed by the absence of the pedal withdrawal reflex prior to all invasive procedures. Serum samples were used for the assessment of alanine aminotransferase, aspartate aminotransferase, creatinine, and blood urea nitrogen levels. Ethylenediaminetetraacetic acid-anticoagulated blood samples were used for complete blood count and differential leukocyte analysis. Liver, pancreas, and kidney tissues were excised, weighed, and processed for histological examination. Specimens were fixed in 10% neutral buffered formalin, washed, dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin. Sections of 4–6 μm thickness were prepared and stained with hematoxylin and eosin according to standard protocols [16].

3.6. In vitro assays

The *in vitro* experimental package was performed by NAWAH Scientific (Cairo, Egypt) and focused on the comparison between control and GLX10-treated conditions relevant to the present study. Cell-based assays were conducted using hepatic cell lines (HepG2 and BNL), which are widely employed as *in vitro* models for metabolic and pharmacological investigations [17]. Cytocompatibility was assessed using the sulforhodamine B (SRB) assay, a well-established colorimetric method for estimating cell viability based on total cellular protein content [18–20]. Nitric oxide production was quantified indirectly by measuring nitrite accumulation using the Griess reaction, a standard colorimetric method for NO detection [21–23]. Inflammatory cytokine levels, including IL-6 and TNF- α , were measured using enzyme-linked immunosorbent assays (ELISA). Flow cytometric analyses were performed to evaluate apoptosis, autophagy, and cell-cycle distribution. Apoptosis was assessed using Annexin V-FITC/propidium iodide staining [22], while autophagy-related changes were evaluated using acridine orange staining to detect acidic vesicular organelles [21]. Exploratory pathway-oriented protein analysis was performed in RAW 264.7 macrophage-based experimental conditions to obtain preliminary insight into inflammatory and nutrient-sensing signaling responses associated with GLX10 exposure. Selected markers related to the NF- κB /mTOR axis and SAMHD1-associated signaling were assessed, including total and phosphorylation-state readouts where available. The analysis was used as a supportive exploratory component rather than as a definitive mechanistic endpoint. Accordingly, the generated signaling observations were interpreted in relation to the functional inflammatory assays, including nitric oxide inhibition and cytokine measurements, and were not used as standalone proof of pathway causality. For transparency, these data should be considered preliminary and hypothesis-generating.

3.7. Quality control and data integrity

To minimize bias and technical variability, environmental conditions, sampling time windows, and analytical procedures were standardized throughout the *in vivo* study. In the *in vitro* experiments, control conditions, standard curves, technical replicates, and fixed acquisition settings were used as part of routine quality assurance. Extracted datasets were cross-checked against the original laboratory

outputs, and derived parameters were recalculated to improve auditability and internal consistency [23].

4. Ethical considerations

All animal procedures were conducted in accordance with internationally accepted principles for the care and use of laboratory animals and were designed to minimize pain, distress, and unnecessary handling. The study adhered to the principles of the 3Rs: Replacement, Reduction, and Refinement, and was reported in line with ARRIVE 2.0 recommendations [24]. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Nawah Research Center, Cairo, Egypt. Formal approval documentation was obtained according to institutional practice; however, an approval number was not assigned. Animals were obtained from VACSERA, The Holding Company for Biological Products and Vaccines, Helwan, Egypt, and were purchased from a certified commercial biological products provider for research purposes; therefore, informed consent from private owners was not applicable. Animals were monitored under controlled housing conditions, and invasive procedures were limited to those essential for study endpoints. Appropriate anesthesia was used to minimize animal suffering. Terminal procedures were performed using a humane euthanasia approach consistent with approved institutional practice and AVMA-aligned recommendations. Specifically, euthanasia was performed using gradual-fill carbon dioxide inhalation, followed by secondary physical confirmation of death. Hazardous agents, including streptozotocin, were handled under appropriate biosafety precautions using suitable personal protective equipment and controlled preparation procedures [25].

5. Results

Overview of the analyzed study groups and sample size: The full experimental screening initially comprised 120 rats allocated across 20 groups to evaluate multiple GLX10 concentrations and treatment conditions. To the present therapeutic efficacy analysis, the Results section focuses on six key *in vivo* groups representing the most relevant arms for testing the therapeutic hypothesis of GLX10. These six groups included a total of 36 rats, with 6 animals per group. In parallel, *in vitro* reporting was restricted to the principal comparison between Control and GLX10-treated conditions. The *in vivo* groups included in the present analysis were as follows: HFD + GLX10 (D36–57), Post oral + Metformin (D36–63) + GLX10 (D70–D90), HFD + Metformin Control (250 mg/kg), Normal control (standard diet), Diabetic control (HFD + STZ + vehicle) and Prophylactic GLX10 (D1–D20 + STZ).

5.1. Body weight progression throughout the study period

Descriptive analysis: Body weight was monitored longitudinally at predefined timepoints throughout the study. The Normal Control group maintained the highest body-weight trajectory across the study period, consistent with expected physiological growth under standard dietary conditions. By contrast, diabetic-model groups induced by HFD/STZ displayed altered weight maintenance patterns

after diabetes confirmation, with greater inter-animal variability than the normal group. To capture the post-confirmation pattern more comprehensively, integrated body-weight parameters were calculated over the interval from D35 to D91, including the mean body weight (Mean D35–D91) and the area under the curve (AUC D35–D91). These measures summarize both average body-weight status and cumulative body-weight exposure over time. Overall, the Normal Control group exhibited the highest and most stable growth profile, reaching the greatest terminal body weight as well as the highest post-confirmation mean and AUC values. In contrast, all diabetic-model groups showed lower overall body-weight exposure during the post-confirmation period and broader variability, particularly at later timepoints. Although treatment groups were evaluated in parallel, none of the GLX10-based or metformin-based regimens restored body weight toward the normal trajectory within the analyzed period. Accordingly, body weight in this dataset appears to function primarily as a supportive physiological endpoint rather than the main discriminator of therapeutic efficacy as shown in Supplementary Figure S3.

5.2. Inferential analysis

At each body weight timepoint, comparisons were performed using Welch's two-tailed t-test, with the Diabetic Control group used as the reference. To account for multiple comparisons, p-values were adjusted using the Holm step-down procedure across the five planned comparisons versus Diabetic Control. Statistical significance was interpreted at $\alpha = 0.05$ after adjustment. The inferential analysis showed that the Normal Control group became significantly heavier than the Diabetic Control group at later study timepoints (D77–D91), indicating sustained healthier growth toward study termination. In contrast, the treated groups did not demonstrate consistent statistically significant differences in terminal body weight relative to the diabetic control after correction for multiple testing. Although some nominal differences were observed at isolated timepoints, these did not remain significant after Holm adjustment. These findings support the view that terminal body-weight change was not the primary dimension along which treatment efficacy was differentiated in this study [26].

5.3. Random blood glucose (non-fasting) across the study period

5.3.1. Descriptive analysis

Random blood glucose (RBG) was measured repeatedly throughout the study. As the animals were maintained under *ad libitum* feeding conditions, the reported values represent random non-fasting glucose levels, except at designated fasting-related procedures such as pre-STZ and OGTT measurements. Thus, these data reflect the glycemic burden under routine housing conditions. Across the observation period, the Normal Control group remained within a low and stable glycemic range. In contrast, the Diabetic Control and HFD + Metformin Control groups exhibited persistently elevated glucose levels throughout the post-confirmation phase. Among all intervention arms, the HFD + GLX10 (D36–57) group showed the most pronounced reduction in random blood glucose after diabetes confirmation, with

late timepoint values approaching the range observed in the normal control group. Integrated post-confirmation glycemic burden, assessed by Mean D35–D91 and AUC D35–D91, was highest in the Diabetic Control and HFD + Metformin Control groups, confirming persistent hyperglycemia. By comparison, the HFD + GLX10 (D36–57) group displayed

a marked reduction in overall glycemic exposure, with a substantially lower AUC than the diabetic control and values approaching those of the normal group at later timepoints. Other intervention arms did not demonstrate a comparable reduction in integrated glycemic burden over the D35–D91 window as shown in Figure 1 [27].

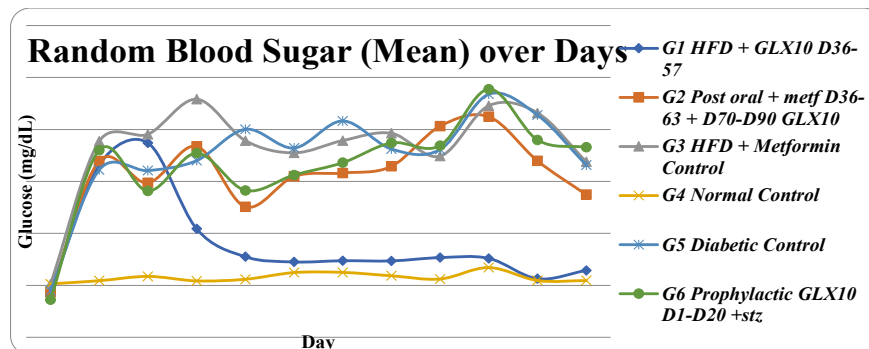


Figure 1: Random blood glucose trends across the study timeline. Longitudinal non-fasting blood glucose measurements demonstrating treatment-related changes in glycemic control

5.3.2. Inferential analysis

Welch-based inferential comparisons, with Holm-adjusted p-values, confirmed that HFD + GLX10 (D36–57) achieved a statistically significant reduction in random blood glucose relative to Diabetic Control at several late timepoints, with significance persisting through the terminal endpoint (D91). Specifically, after adjustment, this group showed significantly lower glucose values at D70, D77, D84, and D91, supporting a sustained glycemic benefit. By contrast, the HFD + Metformin, Control and Prophylactic GLX10 groups did not show consistent statistical separation from the diabetic control after correction for multiplicity. As expected, the Normal Control group remained significantly lower than the diabetic control across late timepoints. Together, these findings identify HFD + GLX10 (D36–57) as the only intervention arm demonstrating robust and sustained improvement in random glycemic control.

5.4. Oral glucose tolerance test (OGTT) at D91.

Descriptive analysis: At D91, an oral glucose tolerance test (OGTT) was performed to assess glucose handling and post-load clearance. Animals were fasted for 6 hours before glucose administration, and glucose values were recorded at 0, 15, 30, 60, and 120 min. To summarize the total glycemic

excursion, AUC_{0-120} was calculated for each animal using the trapezoidal method and subsequently summarized at the group level. The Normal Control group showed the lowest OGTT response and the most efficient return toward baseline by 120 min. In contrast, the Diabetic Control group exhibited a markedly elevated glycemic excursion, consistent with impaired glucose clearance. A measurement ceiling effect at the assay upper limit (600 mg/dL) was observed at the 60-min timepoint in the HFD + Metformin, Post-oral + Metformin + GLX10, and Prophylactic GLX10 groups, indicating severe post-load hyperglycemia in these arms. Among the intervention groups, HFD + GLX10 (D36–57) demonstrated the clearest improvement in glucose tolerance. This group showed a substantially lower OGTT AUC than the diabetic control and a markedly lower 120-min glucose level, indicating improved late-phase glucose clearance. In quantitative terms, total glycemic exposure was reduced by approximately 42% relative to diabetic control ($AUC\ 32,972.50$ vs $57,021.25$ mg·min/dL). In contrast, the metformin-based and prophylactic schedules remained associated with severe post-load hyperglycemia and high AUC values as shown in Figure 2A and 2B.

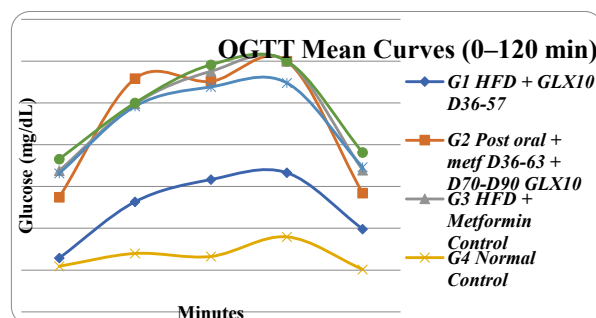
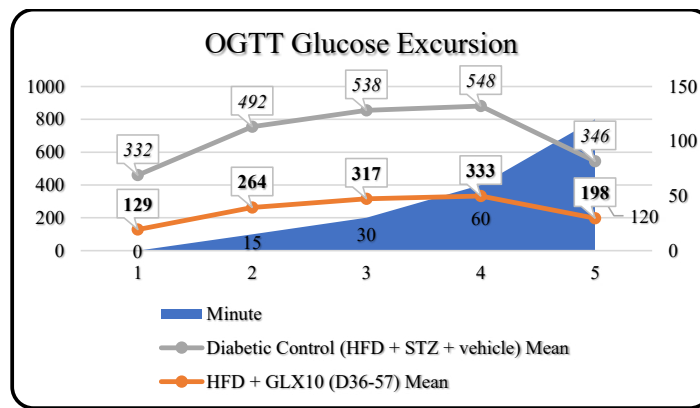


Figure 2: Oral glucose tolerance test (OGTT) analysis at Day 91

(A) Baseline and post-glucose load blood glucose levels.



(B) OGTT glucose excursion curves comparing diabetic and normal rats, including area under the curve analysis.

5.5. Inferential analysis of key in vivo endpoints

To substantiate the descriptive findings in both RBG at D91 and OGTT AUC₀₋₁₂₀, Welch's two-tailed t-tests were performed comparing each group with the Diabetic Control, followed by Holm adjustment across the five planned comparisons. Effect sizes were additionally quantified using Cohen's d. This analysis showed that HFD + GLX10 (D36-57) was the only intervention arm that reached statistical significance after adjustment for both major glycemic endpoints:

- Random blood glucose at D91
- OGTT AUC₀₋₁₂₀

The observed effects were very large in magnitude, with Cohen's d = -3.752 for RBG at D91 and d = -3.358 for OGTT AUC, supporting a robust therapeutic effect on both chronic and acute glycemic burden. In contrast, the Post oral + Metformin + GLX10, HFD + Metformin Control, and Prophylactic GLX10 groups did not achieve statistical significance versus diabetic control for either endpoint after multiple-comparison correction.

5.6. In vitro cytotoxicity and anti-inflammatory activity

Sulforhodamine B (SRB) cell viability assay: The cytotoxic potential of GLX10 was assessed in three cell lines representing distinct biological contexts: BNL (normal liver), HepG2 (hepatic carcinoma), and RAW 264.7 (murine macrophages). Cell viability was measured using the sulforhodamine B (SRB) assay, which estimates total cellular protein content as an indicator of cell density and survival following drug

exposure. Results were expressed as percentage viability relative to untreated control and presented as mean ± SEM with corresponding SD values. Across the tested concentration range, GLX10 produced greater cytotoxicity in HepG2 cells than in BNL cells, indicating increased sensitivity of the carcinoma model. At matched concentrations, HepG2 viability declined more steeply than BNL viability, consistent with the lower IC₅₀ observed in HepG2 [28]. In contrast, RAW 264.7 cells maintained relatively high viability over the tested macrophage concentration range, suggesting that the anti-inflammatory effects observed in this model were not attributable to generalized macrophage toxicity as shown in Supplementary S4.

5.7. In vitro IC₅₀ summary

Based on the NAWAH dose-response outputs, GLX10 showed a lower SRB IC₅₀ in HepG2 cells (2.68 µg/mL) than in BNL cells (4.52 µg/mL), supporting preferential cytotoxicity toward the carcinoma cell line within this dataset. For RAW 264.7 cells, a 50% loss of viability was not reached within the tested SRB range, and therefore an SRB IC₅₀ could not be calculated for macrophages under those conditions. Notably, macrophage viability remained relatively preserved across the tested range (0.19–2.5 µg/mL), with viability remaining approximately 89.8% even at the highest tested concentration. This supports the interpretation that the anti-inflammatory effects of GLX10 occurred under exposure conditions that were largely non-lethal to RAW 264.7 cells as shown in Figure 3.

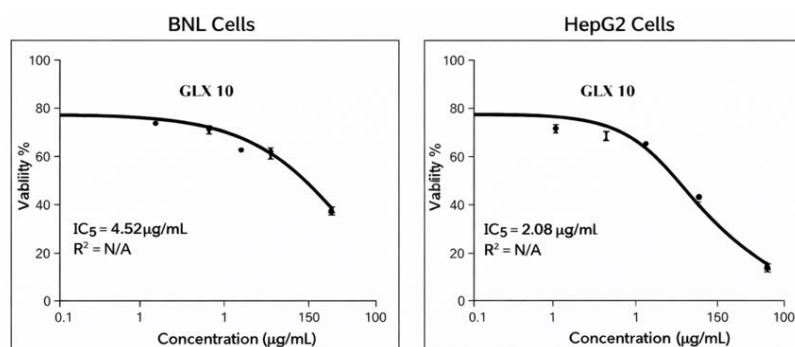


Figure 3: In vitro dose-response effects of GLX10 in hepatic cell lines. Cell viability was assessed using the sulforhodamine B assay in BNL and HepG2 cells after 72 hours of exposure. Data are presented as percentage viability relative to control. IC₅₀ values were calculated using nonlinear regression analysis.

5.8. Anti-inflammatory activity in RAW 264.7 macrophages: nitric oxide inhibition

Nitric oxide (NO) production was assessed indirectly by measuring nitrite accumulation in culture supernatants using the Griess reaction. In this assay, nitrite serves as a stable oxidative product of NO and is quantified calorimetrically. RAW 264.7 macrophages exposed to GLX10 showed a clear concentration-dependent increase in NO inhibition over 48 h. Mean inhibition increased from 18.46% at 0.19 $\mu\text{g/mL}$ to 73.17% at 2.50 $\mu\text{g/mL}$, with relatively low replicate variability, particularly at the highest concentration. The IC_{50} for NO inhibition was 0.88 $\mu\text{g/mL}$, indicating that GLX10 achieved substantial anti-inflammatory activity at concentrations

below those associated with overt macrophage cytotoxicity.

5.9. Human cytokine outputs (ELISA): IL-6 and TNF- α

Human IL-6 and TNF- α levels were quantified by ELISA under Control and GLX10 conditions. All reported values fell within the detection ranges specified in the NAWAH reports. The ELISA outputs indicated lower cytokine concentrations under GLX10 treatment than in the corresponding control condition, consistent with an anti-inflammatory effect. However, because the NAWAH outputs provided a single reported value per condition, these cytokine data should be interpreted

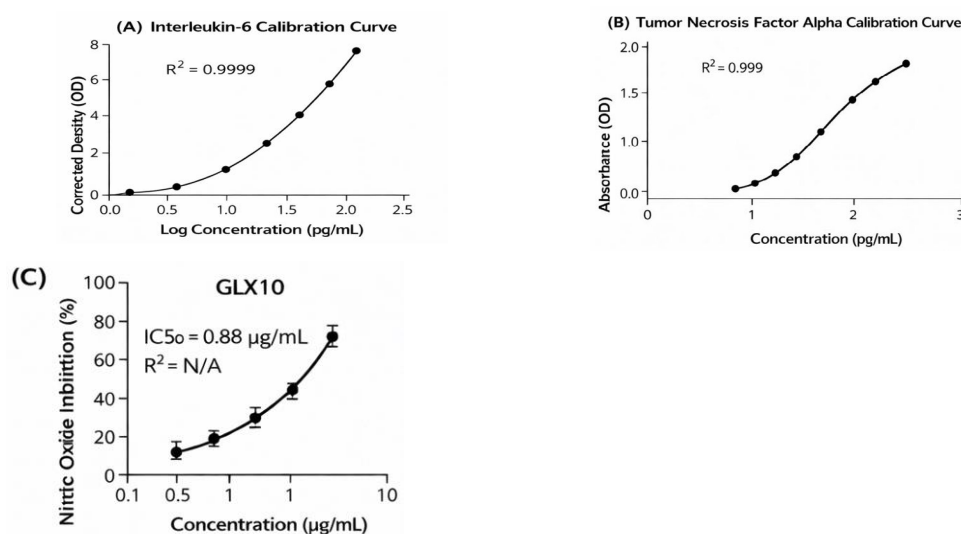


Figure 4: Cytokine quantification and nitric oxide inhibition analysis. (A) Calibration curve for human interleukin-6 enzyme-linked immunosorbent assay. (B) Calibration curve for human tumor necrosis factor alpha enzyme-linked immunosorbent assay. (C) Dose-dependent inhibition of nitric oxide production in RAW 264.7 cells after 48 hours of exposure. Data are presented as mean $\pm$ standard deviation ($n = 3$). IC_{50} value is indicated in panel (C)

5.10. Flow cytometry in BNL cells (72 h): apoptosis, autophagy, and cell cycle

Apoptosis profiling: Flow-cytometric apoptosis analysis using Annexin V/PI in BNL cells demonstrated a marked redistribution of events following GLX10 exposure. Relative to control, GLX10 shifted events away from the dominant control quadrant and increased the derived Total Cell Death from approximately 0.98% in control cells to 20.86% after 72 h of exposure. These findings indicate that GLX10 induced a substantial increase in cell death in BNL cells under the tested conditions as shown in Supplementary S5.

5.11. Autophagy-related readout

Autophagy-related acridine orange flow-cytometry outputs showed increased Mean X and Median X fluorescence metrics in GLX10-treated BNL cells compared with control, whereas the P1% gated fraction was lower in the GLX10 condition. These changes are consistent with altered lysosomal/autophagy-related fluorescence behavior following treatment as shown in Supplementary S6.

5.12. Cell-cycle profiling

Cell-cycle analysis further showed that GLX10 exposure was associated with a reduction in the G1 fraction from 75.27% to 47.09%, together with a marked increase in the Sub-G1 fraction from 3.38% to 26.76%. The S-phase fraction was lower and only modestly represented. Collectively, these findings are compatible with treatment-associated disruption of cell-cycle distribution and an increase in the sub-G1 population, consistent with enhanced DNA fragmentation/cell death.

5.13. Exploratory signaling readouts related to SAMHD1, NF- κB , and mTOR pathways

Exploratory signaling analysis in RAW 264.7 macrophage-based conditions suggested that GLX10 exposure was associated with directional changes in selected inflammatory and nutrient-sensing pathway markers. Compared with the control condition, GLX10-treated cells showed a pattern consistent with reduced activation of phosphorylation-state readouts linked to the NF- κB /mTOR axis, including phospho-NF- κB p65 and selected mTOR downstream markers. In parallel, changes in SAMHD1-related readouts

were observed, suggesting a possible relationship between GLX10 exposure and immunoregulatory signaling status as shown in Figure 5.

Importantly, these signaling findings should be interpreted cautiously. They are presented as preliminary supportive observations that align with the functional anti-inflammatory outputs observed in macrophage assays, particularly

nitric oxide inhibition and reduced cytokine release. They should not be considered definitive evidence of direct pathway causality. Further confirmatory experiments using independent biological replicates, uncropped immunoblot documentation, validated loading controls, and orthogonal pathway-validation approaches are required. Full-length, uncropped Western blot images are provided in the supplementary materials.

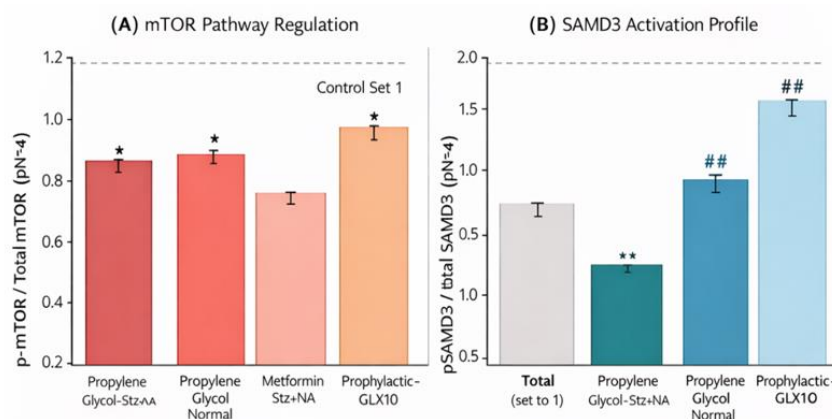


Figure 5: Exploratory Western blot-based signaling observations in RAW 264.7 macrophage-related conditions. Representative pathway-oriented protein readouts were used to explore selected inflammatory and nutrient-sensing signaling changes associated with GLX10 exposure

5.14. Integrated in vivo / in vitro results-level synthesis

Taken together, the dataset demonstrates a directional convergence between the in vivo metabolic findings and the in vitro anti-inflammatory/signaling readouts. Among the six in vivo groups analyzed, HFD + GLX10 (D36–57) produced the clearest therapeutic separation from the diabetic control, particularly in the two principal glycemic endpoints: reduced random blood glucose at D91 and reduced OGTT AUC_{0–120}. This metabolic improvement was paralleled in vitro by multiple anti-inflammatory-aligned findings under GLX10 treatment, including concentration-dependent inhibition of nitric oxide production in RAW 264.7 macrophages, lower IL-6 and TNF- α outputs in ELISA measurements, and reduced phosphorylation-state signaling across the mTOR/NF- κ B axis, together with altered SAMHD1 status. From a results-level perspective, these observations indicate a multi-level pattern of alignment between systemic glycemic improvement in the animal model and suppression of inflammatory/metabolic signaling outputs in cell-based assays. The in vitro findings therefore provide supportive experimental context for the in vivo efficacy signal, without extending beyond the directly measured outcomes of the present study.

5.15. Histopathological Findings

Histopathological examination of tissue specimens from GLX10-treated animals demonstrated largely preserved normal architecture across all evaluated organs. Tissue processing and staining were performed using standard Hematoxylin and Eosin (H&E) techniques. Liver: Microscopic evaluation revealed intact hepatic architecture, with well-preserved hepatocytes and normal portal triads. There was no evidence of hepatocellular degeneration, necrosis,

inflammatory infiltration, or vascular abnormalities, indicating the absence of treatment-related hepatotoxicity. Skeletal muscle fibers: exhibited normal morphology and organization, with no signs of myofiber degeneration, necrosis, inflammatory cell infiltration, or fibrosis. These findings suggest that GLX10, including its intramuscular component, did not induce local tissue injury or myotoxic effects. Pancreatic sections: showed predominantly preserved acinar architecture. Mild peri ductal fibrosis and occasional vascular congestion were observed in limited areas; however, these changes were minimal and not associated with inflammatory infiltration, acinar necrosis, or islet cell damage. Overall, the pancreatic tissue remained structurally intact, suggesting the absence of significant pathological alterations. Lymph node sections: demonstrated preserved histological organization, with a well-defined cortical structure and normal lymphoid follicle distribution. No evidence of lymphoid depletion, necrosis, or abnormal hyperplasia was observed, indicating a balanced immunological response without pathological overstimulation as shown in Supplementary S8.

5.16. Hematological parameters

Longitudinal hematological measurements remained largely within the normal physiological range throughout the study period. However, a gradual increase in neutrophil percentage and platelet count became evident after Day 20, reaching maximal values at Day 60 before partially declining during later follow-up. The summarized hematological findings are presented in Figure 7. **Biochemical parameters:** Biochemical and organ-weight measurements showed only modest changes over time. Serum creatinine, AST, and liver weight increased gradually through Day 60 but remained

within the expected range for rats, followed by partial normalization by the end of follow-up. These findings are summarized in Figure 6.

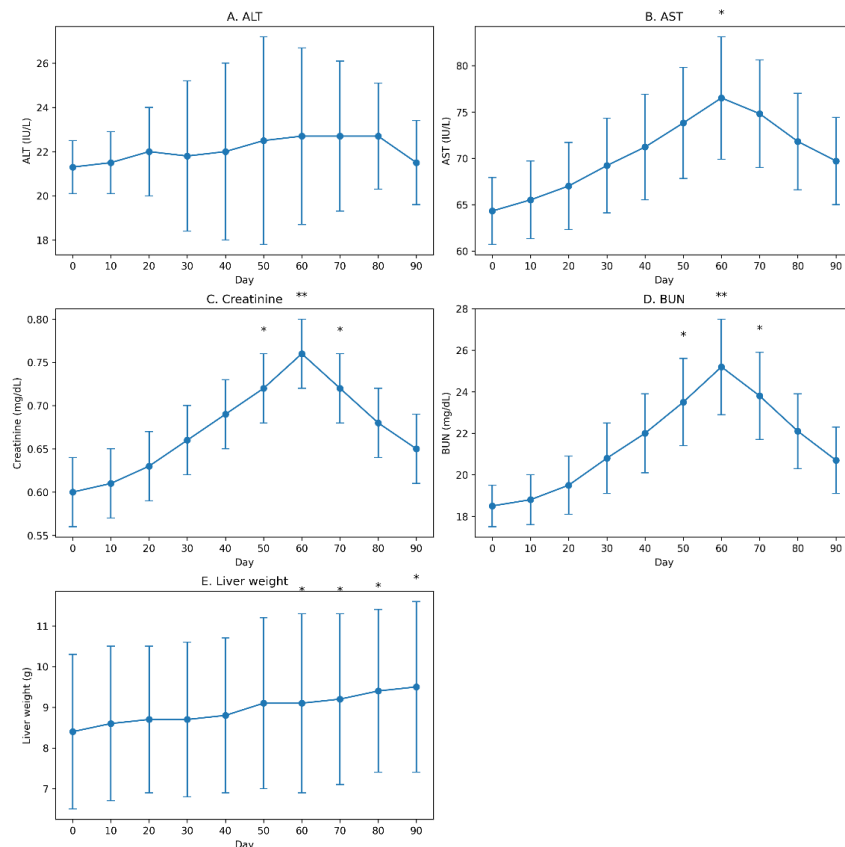


Figure 6: Longitudinal biochemical and organ-weight profile following GLX10 administration in normal rats. (A) Alanine aminotransferase, (B) aspartate aminotransferase, (C) creatinine, (D) blood urea nitrogen, and (E) liver weight were monitored from Day 0 to Day 90. Data are presented as mean $\pm$ standard deviation ($n = 6$).

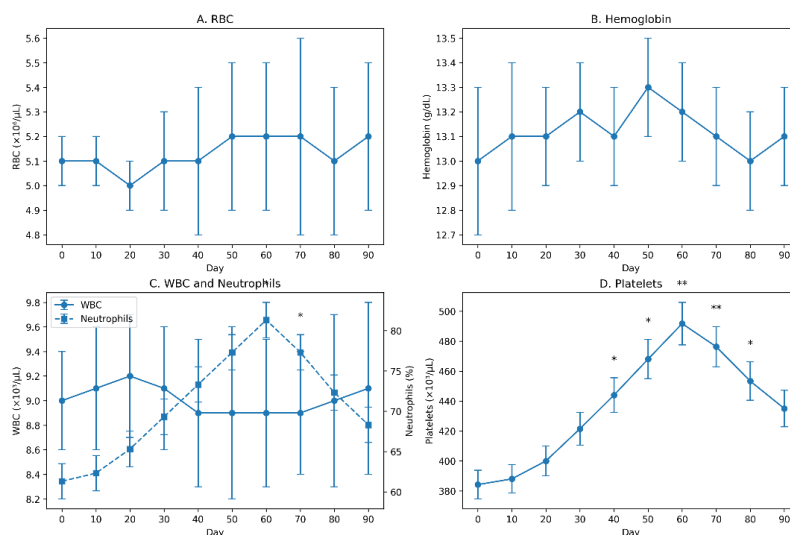


Figure 7: Longitudinal hematological profile following GLX10 administration in normal rats. (A) Red blood cell count, (B) hemoglobin, (C) white blood cell count and neutrophil percentage, and (D) platelet count were monitored from Day 0 to Day 90. Data are presented as mean $\pm$ standard deviation ($n = 6$). Statistical comparisons were performed versus baseline values. * $p < 0.05$, ** $p < 0.01$

In summary, GLX10 demonstrated differential in vitro activity across the tested cell systems, with greater sensitivity observed in HepG2 cells than in BNL cells, while RAW 264.7 macrophage viability was largely preserved across the anti-inflammatory concentration range. Under these conditions, GLX10 produced concentration-dependent inhibition of nitric oxide production and was associated with lower IL-6 and TNF- α outputs. Exploratory pathway-oriented observations further suggested directional changes in signaling markers related to inflammatory and nutrient-sensing pathways, including the NF- κ B/mTOR axis; however, these signaling findings should be interpreted as preliminary and hypothesis-generating rather than definitive mechanistic proof. Overall, these in vitro findings provide supportive biological context for the in vivo reductions in random blood glucose and OGTT AUC observed in the HFD + GLX10 (D36–57) group.

6. Discussion

The present findings indicate that GLX10 is associated with meaningful improvement in glycemic control in a widely used HFD/STZ rat model of type 2 diabetes mellitus. The most pronounced efficacy signal was observed in the therapeutic GLX10 group, which showed reductions in both end-study random blood glucose and integrated OGTT glucose exposure when compared with diabetic controls. This is particularly important because OGTT reflects dynamic glucose handling rather than a single static measurement [14].

The in vitro findings provide biologically relevant support for these in vivo observations. The reduction in nitric oxide production, together with decreased IL-6 and TNF- α release in macrophage systems, is consistent with attenuation of inflammatory activation. This is particularly relevant because chronic low-grade inflammation is increasingly recognized as a central contributor to insulin resistance and progressive metabolic dysfunction in T2DM [8–10,15,28–30]. In this context, GLX10 appears to extend beyond a purely glucose-lowering profile and may instead act through a broader immunometabolic mechanism.

The exploratory Western blot-based observations provide preliminary pathway-level support for the anti-inflammatory phenotype observed in macrophage-based assays. The directional reduction in phosphorylation-associated readouts linked to NF- κ B and mTOR signaling is biologically consistent with the observed reduction in nitric oxide production and lower IL-6 and TNF- α outputs. However, these findings should not be interpreted as definitive mechanistic proof. Rather, they suggest a plausible signaling framework that requires formal validation. Future studies should confirm these observations using replicated immunoblot experiments, uncropped blot documentation, validated loading-control normalization, densitometric quantification, and complementary assays such as qPCR, immunofluorescence, reporter-based NF- κ B activity assays, pharmacological pathway inhibition, and tissue-level validation in metabolically relevant organs [18]. Nevertheless, mechanistic interpretation should remain cautious. The current data support an association

between GLX10 treatment, improved glycemic control, and reduced inflammatory readouts, but they do not establish direct causality. Likewise, although the broader discussion of mitochondrial regulation, autophagic adaptation, and immunometabolic reprogramming offers a biologically plausible framework, these concepts should presently be regarded as hypothesis-supporting interpretations rather than definitive mechanistic conclusions.

Several limitations should be acknowledged. The sample size was modest ($n = 6$ per group), and the present article emphasizes the principal comparison groups within a broader exploratory research program. In some cases, OGTT values approached assay ceiling limits, which may have reduced discrimination between more severe phenotypes. Moreover, direct assessments of insulin sensitivity, β -cell functional reserve, and pharmacokinetic/pharmacodynamic behavior were not included. The apparent stress-related effects observed in BNL cells also warrant additional concentration-dependent investigation to define the therapeutic window and safety profile more clearly [31].

Despite these limitations, the overall pattern of results supports continued investigation of GLX10. The therapeutic GLX10 regimen produced the clearest in vivo antidiabetic signal, while the accompanying cellular findings support an immunometabolic mode of action that is both biologically plausible and experimentally testable. On this basis, GLX10 merits further preclinical development with expanded mechanistic validation and translational characterization.

7. Conclusion

GLX10 was associated with improved glycemic control in an HFD/STZ-induced rat model of type 2 diabetes mellitus, as demonstrated by lower end-study random blood glucose levels and reduced OGTT AUC_{0–120} compared with diabetic controls. Complementary cell-based findings showed reduced nitric oxide production and lower IL-6 and TNF- α output, together with preliminary pathway-oriented observations suggesting possible involvement of inflammatory signaling networks. These findings provide supportive but not definitive mechanistic context for the observed in vivo glycemic improvement.

Collectively, these results provide a preclinical rationale for the continued investigation of GLX10 as a potential immunometabolic therapeutic candidate, while also emphasizing the need for further mechanistic, safety, and translational studies.

Future Research Directions

Future studies should include larger cohorts and more refined dose-response analyses to strengthen statistical robustness and improve confirmatory interpretation. Direct measurement of insulin sensitivity and insulin secretion, including insulin tolerance testing or clamp-based methodologies, will be important to clarify the mechanisms underlying glycemic improvement. Priority should be given to confirmatory pathway-validation studies, including fully replicated Western blotting with uncropped blot availability,

validated loading controls, densitometric normalization, and orthogonal assays to verify NF- κ B/mTOR involvement in both macrophage systems and metabolically relevant tissues.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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Credit Author Statement

Sherif Salah and Mohamed Sherif Salah contributed to study conceptualization, development of the scientific framework, experimental design, data acquisition, and drafting of the original manuscript. Khalid F. Kassem contributed to critical revision of the manuscript, as well as medical and scientific guidance and interpretation of the findings. Ahmed A. Naser, Hossam M. Afifi, Omar M. Hafez, and the team at Nawah Scientific Center for Research contributed to data acquisition, experimental execution, and technical support. All authors contributed to data interpretation, reviewed the manuscript critically for important intellectual content, and approved the final version for publication.

Ethical Considerations

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Nawah Scientific Center for Research, Cairo, Egypt. Animals were maintained under controlled environmental conditions and monitored regularly throughout the study period. Procedures related to diabetes induction, blood collection, and treatment administration were performed using standardized protocols designed to minimize stress, discomfort, and experimental variability. At study termination, euthanasia was carried out by gradual-fill carbon dioxide inhalation followed by secondary physical confirmation, in accordance with accepted humane euthanasia guidelines. Clinical trial number: not applicable, as this study does not involve human participants and is based on in vivo animal models and in vitro experimental systems.

Confidentiality Statement

This manuscript includes data related to the investigational candidate GLX10. Certain methodological details, including specific formulation and dosing parameters, are presented at a generalized level because of their proprietary nature. Additional technical details may be made available upon reasonable request and subject to appropriate confidentiality arrangements.

Supplementary Materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. Detailed statistical methods are provided in Supplementary Methods S1. Additional datasets are

available at: <https://doi.org/10.5281/zenodo.19775183>

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