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The novel NADPH oxidase 4 inhibitor GLX351322 counteracts glucose intolerance in high-fat diet-treated C57BL/6 mice

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EA and PW performed the experiments. PW, EW and NW designed the experiments, analyzed the results and wrote the manuscript.

ABSTRACT

In Type 2 diabetes, it has been proposed that pancreatic beta-cell dysfunction is promoted by oxidative stress caused by NADPH oxidase (NOX) over-activity. Five different NOX enzymes (NOX1-5) have been characterized, among which NOX1 and NOX2 have been proposed to negatively affect beta-cells, but the putative role of NOX4 in type 2 diabetes-associated beta-cell dysfunction and glucose intolerance is largely unknown. Therefore, we presently investigated the importance of NOX4 for high-fat diet (HFD)-induced glucose intolerance using male C57BL/6 mice using the new NOX4 inhibitor GLX351322, which has relative NOX4 selectivity over NOX2. In HFD-treated male C57BL/6 mice a two-week treatment with GLX351322 counteracted non-fasting hyperglycemia and impaired glucose tolerance. This effect occurred without any change in peripheral insulin sensitivity. To ascertain that NOX4 also plays a role for the function of human beta-cells, we observed that glucose- and sodium palmitate-induced insulin release from human islets *in vitro* was increased in response to NOX4 inhibitors. In long-term experiments (1-3 days), high glucose-induced human islet cell ROS production and death were prevented by GLX351322. We propose that whilst short-term NOX4-generated ROS production is a physiological requirement for beta-cell function, persistent NOX4-activity, e.g. during conditions of high-fat feeding, promotes ROS-mediated beta-cell dysfunction. Thus, selective NOX-inhibition may be a therapeutic strategy in Type 2 diabetes.

INTRODUCTION

Loss of pancreatic islet function is a central hallmark in the pathogenesis of Type 2 diabetes mellitus (T2DM) [1]. In addition, it may be that also beta-cell loss occurs in T2DM, and that this starts, after an initial phase of hyperinsulinemia, relatively late in the progression of the disease [2]. The mechanisms resulting in beta cell failure in T2DM are not clear, but accumulating evidence point to a central role of oxidative stress as a result of overproduction of reactive oxygen species (ROS) [3-6]. Chronic hyperglycemia/hyperlipidemia may be the driving force that leads to increased ROS production, and ROS are known to damage components of the cellular machinery, including DNA, proteins and lipids. Besides damaging islet cells, it is likely that oxidative stress is contributing to the development of peripheral insulin resistance, and to many of the vascular complications occurring in the late stages of the disease [7-11].

The excessive production and accumulation of ROS is, at least in part, due to hyperactivity of the NADPH oxidases (NOX). The NOX family consists of seven isoforms (NOX1-5 and DUOX1-2), which perform normal cellular functions at basal conditions, but when persistently activated produce harmful levels of ROS. Hyperactivity of some of the isoforms has been found to be an important driver in a number of diseases including diabetes and diabetes complications [12]. Both rat and human pancreatic beta-cells have been reported to express some of the NOX subunits [13-15], and NOX2 seems to be activated in response to glucose stimulation through a protein kinase C-dependent mechanism [13], leading to an increased intracellular calcium response and a stimulated insulin release [16-18]. However, *in vivo* studies have reported increased NOX-mediated ROS generation in diabetic rat and human islets, and that this was associated with reduced beta-cell function [19]. Thus, it may be that insulin release is stimulated in the

short term by increased ROS production, whereas a long-term NOX-activation leads to loss of beta-cell function. Not only glucose *in vitro* or hyperglycemia *in vivo* promotes beta-cell activation of NOX, but also sodium palmitate, a free fatty acid which is increased in Type 2 diabetes, and which stimulates the release of insulin in short-term experiments, but inhibits beta-cell function after a prolonged exposure period [20-22].

Activation of NOX in islet non-beta-cells may also contribute to ROS production and oxidative stress in the beta-cells. In this instance NOX activation in endothelial cells could be of importance. Patel *et al* [23] highlighted a unique framework for hyperglycemia-induced hydrogen peroxide production by NOX in endothelial cells and Hecker *et al* [24] found that an aberrant up-regulation of the isoenzyme NOX4 results in a sustained redox imbalance, which promotes persistent myofibroblast senescence and fibrosis. In this context it is of interest to note that pancreatic islets are among the most vascularized organs in the body with 1000-1500 capillaries per square millimeter, i.e. encompassing 10% of the islet tissue [25].

In previous studies NOX2 and NOX1 have been suggested to negatively affect beta-cell function when persistently over-activated [19,26]. The putative role of NOX4 in islet-cells, however, has not been addressed. Previously used NOX inhibitors, such as apocynin and diphenylene iodonium, are today not considered to be selective NOX inhibitors. Instead, novel NOX inhibitors with better NOX specificity, and which are selective for specific NOX isoforms, have been developed [27]. One such NOX inhibitor is GKT-136901, which inhibits NOX1 and NOX4 more efficiently than NOX2, and which counteracts high glucose-induced oxidative stress in the kidney [28]. We presently report the generation of a new NOX inhibitor, GLX351322, which targets NOX4 preferentially over NOX2. The aim of the present investigation was to evaluate whether GLX351322 counteracts high-fat diet-induced hyperglycemia and glucose intolerance in

C57BL/6 mice. Using this NOX4 inhibitor we observed protection against beta-cell dysfunction, suggesting that a long-term NOX4-mediated stimulation of islet ROS production contributes to subsequent beta-cell failure and glucose intolerance.

METHODS

NOX inhibitors

2-(2-chlorophenyl)-4-methyl-5-(pyridin-2-ylmethyl)-1H-pyrazolo[4,3-c]pyridine-3,6(2H,5H)-dione (GKT-136901), a selective NOX4 inhibitor, was a kind gift from Dr. Harald HH Schmidt [Maastricht University, Netherlands]. GLX351322 (ethyl 2-[[2-[4-(furan-2-carbonyl)piperazin-1-yl]acetyl]amino]-5,6-dihydro-4H-cyclopenta-[b]thiophene-3-carboxylate) was a kind gift from Glucox Biotech (Stockholm, Sweden). Diphenylene iodonium (DPI) was from Sigma Aldrich.

Identification of GLX351322 as a NOX4 inhibitor

Using a high throughput screening approach 40.000 chemicals were tested for NOX4 inhibition potential in T-REx-293 cells with inducible NOX4 over-expression using an Amplex red based assay in a 384-well format. With 50 % inhibition of NOX4 as threshold, more than 700 primary hits were identified. These were re-tested using the same assay and a counter-screen applying 4 μM H_2O_2 left 90 structurally diverse compounds for dose-response investigation. Dose-response curves were obtained from 200 μM in 11 3-fold-dilution steps in duplicate and 54 compounds received an IC_{50} . The most potent compounds had an IC_{50} of approximately 1 μM . None of the compounds showed any effect on cell viability at 10 μM .

To exclude any form of general antioxidant activity of GLX351322 the DPPH assay was utilized. DPPH (2,2-diphenyl-1-picryl-hydrazyls-hydrate) is a well-known stable radical useful to determine all types of chemical reactions involving radicals [29]. A strong absorption band centered at 518 nm (violet color) decreases to pale yellow when the DPPH radical is neutralized by an antioxidant. DPPH (Sigma Aldrich) absorbance at 518 nm was determined without or in the presence of different GLX531322 concentrations. As positive control GLX481369, a substance with known redox activity, was used. The assay was performed in 96 wells plates and absorbance was determined in a plate reader.

Determination of GLX351322 IC₅₀ for inhibition of NOX2

On the basis of the high throughput screen campaign results and a first analysis of dose-response curves 12 compounds were selected and tested for selectivity against NOX2. Assay procedures are described in Wilcke *et al* [30]. Erythrocytes were isolated from whole blood by Dextran sedimentation and kryopreserved according to [31]. Before assaying, cells were thawed at 37°C and immediately pipetted into room tempered Hank's balanced salt solution (HBSS) and centrifuged (250 x g, 5 min, 20°C). Cells were washed twice in HBSS and re-suspended in HBSS at 2 x 10⁶ cells/ml. Cell count and viability was determined using Trypan Blue exclusion. One vial per assay plate was used, thawed and prepared just prior to analysis. Phorbol 12-myristate 13-acetate (PMA) was diluted in Isoluminol buffer at 4x working concentration to a final concentration of 30 ng/ml. Cells were stimulated with PMA to produce radicals from NOX2 enzyme.

Determination of GLX351322 solubility

Solubility of GLX351322 was tested utilizing the shake-flask method in three different

media, 0.1 M phosphate buffer pH 7.4, FASSIF (synthetic fluid representing small intestinal juices when no food has been ingested) blank pH 6.5 (without lipids) and FASSIF pH 6.5. In brief, approximately 0.5 mg GLX351322 was weighed in HPLC glass vials. 0.5 ml of either media described above was added, the vials were sealed and incubated with rotation (900 rpm) at 37°C for 24 h. After the incubation, an aliquot was centrifuged at 10 000 rpm (to remove insoluble matter) and the supernatant was diluted and analyzed by LC-MS/MS.

Determination of GLX351322 chemical stability

GLX351322 (1 μ M from a 10 mM DMSO stock) was incubated in closed glass vials at 37°C in phosphate buffered saline (PBS) at pH 7 and in PBS:Propanol (1:1) at the same pH for 24 h. Aliquots of the buffer stock solutions were immediately frozen at -80°C after incubation. After thawing, the samples were analyzed by LC-MS/MS and compared to control samples.

Determination of GLX351322 metabolic stability

The microsomal metabolic stability assay utilized commercially available pooled human liver microsomes with supplemented cofactor (NADPH) to primarily facilitate cytochrome P450 reactivity against target compound. GLX351322 (1 μ M) and microsomes (0.5 mg/ml incubation concentration) are added to 150 μ l of 0.1 M phosphate buffer pH 7.4. The reaction is initiated with addition of NADPH (1 mM). The incubation times were 0, 5, 15, 40 min and the reaction was quenched, at each time point, by addition of 100 μ l acetonitrile containing Warfarin as analytical internal standard. The plate was then sealed, centrifuged and frozen at -20°C until LC-MS/MS analysis.

Determination of GLX351322 plasma protein binding

In brief, 0.2 ml of the plasma test solution (typically 10 μ M final compound concentration) was transferred to the membrane tube in the RED (Rapid Equilibrium Dialysis) insert. 0.35 ml isotonic phosphate buffer pH 7.4 was added to the other side of the membrane. The sample was incubated with rapid rotation (900 rpm) at 37°C for 4 h to achieve equilibrium. The plasma test solution was incubated at 37°C for 4 h and then frozen to prevent any degradation. Prior to LC-MS/MS analysis the samples were mixed with equal volumes of control buffer or plasma as appropriate to maintain matrix similarity for analysis. Plasma proteins were then precipitated by the addition of methanol (1:4) containing Warfarin as analytical internal standard. The plate is then sealed, centrifuged and the supernatant is analyzed by mass spectrometry (LC-MS/MS).

Determination of GLX351322 transport

Caco-2 cell permeability study was performed in accordance with published protocols [32]. The experiment was started by applying a prewarmed (37°C) GLX351322 solution of 1 μ M on the apical side of the filter insert chamber. Directly after the termination of the experiment the membrane integrity was checked by transepithelial electrical resistance (TEER) measurement and by measurement of mannitol permeability.

Determination of oral pharmacokinetics in rat

Sprague Dawley rats (males, 8-12 weeks) were acquired from Harlan Europe. The animals were weighed day 1 and mean weight was calculated for determination of dose for the experiment. 3x3 rats per group were used and animals were dosed p.o. with 10 mg of GLX351322 per kg. Plasma samples were taken at 0 time and after 10 min, 30 min,

1 h, 1,5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 18 h, 24 h, 48 h, and 72 h. The samples were analyzed by UPLC-MS/MS. Data was processed and analyzed using MassLynx (Walters Corp.), Graphpad Prism 4 (Graphpad Inc.) and WinNonlin (Pharsight Corp.).

The animal experiment was approved by the local ethical committee Malmö/Lund; license M152-09.

Human Islets

Human pancreatic islets were kindly provided by Prof. Olle Korsgren (Dept. of Radiology, Oncology and Clinical Immunology, Uppsala University Hospital, Uppsala, Sweden), through the Uppsala facility for the isolation of human islets from Scandinavian brain-dead individuals. After isolation, the islets were cultured free-floating in Sterilin dishes in CMRL 1066 medium (ICN Biomedicals, Costa Mesa, CA, USA) containing 5.6 mM glucose, 10% fetal calf serum and 2 mM L-glutamine for 1-5 days. All cells were kept at 37°C in a humidified atmosphere with 5% CO₂.

Insulin release

Islets were incubated for 1 h in either 1.7 mM glucose, 17 mM glucose or 17 mM glucose + 1 mM sodium palmitate solubilized in 2% bovine serum albumin at 37°C and in HEPES-balanced Krebs-Ringer bicarbonate buffer (KRBH) buffer. Insulin concentrations were measured using an Insulin ELISA Kit (Mercodia).

ROS production in human islets

After a 24 h culture period in 5.6 or 28 mM glucose, human islets were loaded for 60 min at room temperature with the free radical indicator carboxy-H₂DCFDA (5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, 10 μM, Life Technologies,

Stockholm, Sweden). Thereafter the cells were transferred to culture dishes containing 5.6 or 28 mM glucose with or without 10 μ M GLX531322 and 10 μ M DPI, and incubated for another 60 min at 37 °C. Hoechst stain, which labels cell nuclei, was added during the last 20 of this incubation. The islets were then washed and placed on the stage of an inverted confocal microscope (Nikon C1) and analyzed for green (DCF) and blue fluorescence (Hoechst). Intensities were determined using Adobe Photoshop and ratios between green and blue signals were calculated as a relative measure of oxidative stress.

Evaluation of cell viability

The cell viability of human islet cells was assessed after culture with IL-1 β (20 ng/ml) + IFN- γ (20 ng/ml) or with 20 mM glucose for three days. Cell viability was measured by staining cells with propidium iodide (20 μ g/ml) and bisbenzimidazole (5 μ g/ml) for 20 min at 37°C. The medium was replaced with PBS and the red and blue fluorescence was detected using the Kodak 4000 MM image station. The ratio of red to blue was taken as a relative measure of cell death (necrosis and late apoptosis) and was quantified using Carestream MI Digital Science ID software, version 5.0.6.20.

Animals and high-fat diet treatment

Four weeks old male C57Bl/6J mice were purchased from Scanbur AB (Sollentuna, Sweden). When five weeks of age mice were divided into two groups with 20 mice in each: one given a control diet (CD) and one given a high-fat diet (HFD). The high-fat diet (D12492, Research Diets) contained 60 kcal% fat, whereas the normal diet (D12450B, Research Diets) contained only 10% kcal% fat. After 7 weeks of diet, both groups were randomly divided into two subgroups, one receiving GLX351322 (3.8 mg/day/kg body

weight) in the drinking water and one group receiving no supplementation. The amount of GLX351322 in the drinking water was continuously adjusted according to water intake and body weight of the four groups. To determine the blood glucose concentrations, blood was withdrawn from the tip of the tail and analyzed using the Freestyle Mini System. The same person collected all blood samples for blood glucose determinations. The animal experiments were approved by the local animal ethics committee.

Blood glucose tolerance test

The mice, after having fasted for approximately 8 hours, were given a single dose of 2.5 g/kg body weight of 30% w/v D-glucose intravenously. Blood was withdrawn from the tail, ~1 µl, and measured with Freestyle Mini System (Abbot, TheraSense Inc). Blood glucose was determined prior to injection and then at 10, 30, 60, and 120 min after injection.

Insulin sensitivity test

The mice were given an i.p. injection (1.6U/kg body weight) of the insulin analog Actrapid (Novo Nordisk, Bagsværd, Denmark). Blood glucose was determined on blood samples from the tail, before injection and 15, 60, 120 and 180 min later using the Freestyle Mini System. The animals had free access to food before the insulin injection and were transferred to new cages without food during the measurements.

RESULTS

Generation and characterization of the novel NOX4 inhibitor GLX351322

GLX351322 (ethyl 2-[[2-[4-(furan-2-carbonyl)piperazin-1-yl]acetyl]amino]-5,6-dihydro-4H-cyclopenta [b]thiophene-3-carboxylate) (Fig. 1) was identified as a NOX inhibitor on the basis of a high throughput screening campaign involving a library of 40,000 compounds [30]. GLX351322 has previously been found to inhibit hydrogen peroxide production from tetracycline inducible NOX4 over-expressing cells with an IC₅₀ of 5 μ M, an IC_{max} of 85 % and a Hill coefficient of 0.83 [30] (Table 1). After investigating GLX351322 for solubility, chemical stability, metabolic stability, protein binding and membrane passage (Table 1), the compound was tested for selectivity against NOX2 and found to be an order of magnitude less active against NOX2 (Table 1). In a 24 h health assessment in mice GLX351322 showed no signs of adverse effects after i.p. or p.o. administration (results not shown). When i.p. administration of the compound was extended to 11 days there were still no signs of adverse reactions (results not shown). According to data presented in Table 1, GLX351322 shows moderate chemical stability (>50%) at physiological pH over a 24 h incubation at 37°C. Plasma protein binding studies indicate very high binding ($f_u \approx 0.03$) and good plasma stability. The results from metabolic stability studies suggest a moderate risk for high first pass metabolism. However, due to the high protein binding, which may limit the degree of tissue exposure, the risk is considered lowered. Caco-2 permeability studies indicate high permeability over biological membranes and a rapid uptake from the intestine. This was confirmed in the rat pharmacokinetic study. Determination of the pharmacokinetics

of GLX351322 in rats after oral administration showed a rapid uptake into the blood stream and a $t_{1/2}$ of 3 h.

Antioxidant activity of GLX351322, as assessed by DPPH absorbance, was not observed (Fig. 2). As a positive control the redox active substance GLX481369 was used to demonstrate the titration curve of a redox active substance. This excludes the possibility that GLX351322 acts as a general antioxidant.

Effects of GLX351322 on high-fat diet-induced glucose intolerance in mice

The NOX-inhibitor GLX351322 was given to male C57BL/6 mice in the drinking water at 3.8 mg/kg/day, to determine whether high-fat diet-induced glucose intolerance is affected. We found that GLX351322 supplemented via the drinking water during the last two weeks of a nine-week long high-fat diet period did not affect the weight increase of the CD and HFD mice (Fig. 3A). The non-fasting blood glucose levels of high-fat diet mice were higher than control mice (Fig. 3B). The two-week GLX351322 treatment decreased the blood glucose of HFD mice, but not of CD mice (Fig. 3B).

The glucose tolerance of the different study groups was assessed by an i.v. glucose injection followed by determinations of blood glucose levels at 0, 10, 30, 60 and 120 min. We observed that GLX351322 did not affect glucose tolerance of mice fed a control diet (Fig. 4A). The glucose tolerance test of HFD mice was, however, markedly improved by GLX351322 at all time points (Fig. 4B). Furthermore, calculations of the area under the curve (AUC) showed that GLX351322 significantly improved glucose tolerance in high-fat diet mice (Fig. 4C). GLX351322 did not exert any effect on the AUC in CD mice.

An insulin sensitivity test was performed and we observed that GLX351322 did not affect the insulin sensitivity of CD mice (Fig. 5A). HFD mice displayed a marked

reduction in insulin sensitivity (Fig. 5B). The insulin sensitivity was not affected by the GLX351322 treatment (Fig. 5B).

Acute effects of the NOX inhibitors GKT-136901 and GLX351322 on high glucose- and palmitate-stimulated human islet insulin release *in vitro*

We next studied whether acute NOX4 inhibition affects islet insulin release *in vitro*. GKT-136901 has previously been characterized as a NOX1/4 selective NOX inhibitor [28], and since NOX1 is not expressed in human islets (Fig. 6), it is likely that this inhibitor targets only NOX4. We observed that 10 μ M of GKT-136901 inhibited glucose-stimulated insulin release from human islets during short-term (1-h) incubations (Fig. 7A). GLX351322 at a concentration of 2 μ M tended to decrease human islet insulin release at a high glucose concentration, but this did not reach statistical significance (Fig. 7B). Sodium palmitate is thought to stimulate insulin release by interaction with the FFA receptor FFAR1/GPR40 and via increased long-chain Acyl-CoA esters, and long-term palmitate effects are known to be deleterious for beta-cells [22,32,33]. We observed that palmitate-stimulated insulin release was decreased by GLX351322 at a concentration of 2 μ M (Fig. 7B), a concentration at which NOX4, but not NOX2, is partially inhibited. These findings add further support to a role for the NOX4 enzyme in both glucose- and palmitate-induced insulin release, and that islet NOX-generated ROS are involved in signaling events leading to calcium mobilization and insulin release, as previously suggested [17].

Long-term effects of GLX351322 on human islet ROS production and cell death in response to high glucose

Human islets were cultured for 24 h in 5.6 or 28 mM glucose to promote increased oxidative stress. The islets were then loaded with H₂DCFDA for 60 min at room temperature, followed by incubation for 60 min with or without 10 μ M GLX351322 or 10 μ M DPI at 37°C. Islet cell DCF-fluorescence at basal and high glucose conditions was reduced in response to the non-specific NOX inhibitor DPI (Fig. 8). GLX351322 inhibited basal ROS production and partially also high glucose-induced DCF-fluorescence (Fig. 8), suggesting that other NOX enzymes besides NOX4 promote ROS production.

We next studied whether prolonged NOX4-generated ROS production *in vitro* participates in high glucose-induced beta-cell death. For this purpose, we cultured human islets for 3 days at different concentrations of the NOX inhibitor GLX351322. We observed that high glucose-induced islet cell death was efficiently counteracted by GLX351322 (Fig. 9).

DISCUSSION

We presently report that the novel NOX4 inhibitor GLX351322 counteracts high-fat diet-induced glucose intolerance in C57BL/6 mice, and that this occurs without any obvious improvement in peripheral insulin sensitivity. This finding may be explained by protection against ROS-induced beta-cell dysfunction by a fashion that differs from that in insulin sensitive peripheral tissues. In our study GLX351322 counteracted partially ROS production, high-glucose-induced islet cell death and the release of insulin in response to a high concentration of glucose *in vitro*, which fits well with the notion that oxidative stress is deleterious, and that beta-cell rest leads to long-term preservation of beta-cell function *in vivo* [35].

In our study NOX4 inhibition *in vivo* appears to target pancreatic islets and not peripheral insulin target tissues. Previous studies have observed that increased oxidative stress promotes peripheral insulin resistance [11], and that systemic reduction of ROS/advanced glycation end products/NOX activity reduces insulin resistance [36-42]. The reason for the presently observed lack of effect of GLX351322 on peripheral insulin sensitivity is not known, but might relate to the isoform selectivity of GLX351322 as a NOX inhibitor or the level of ROS inhibition and/or expression of NOX enzymes in peripheral target tissues as compared to pancreatic islets. For example, islets may be more sensitive to increased glucose levels and ROS than peripheral target tissues due to differences in glucose transporter expression and antioxidative defense systems. According to our *in vitro* data, we observe that GLX351322 inhibits NOX4 8-fold more effectively than NOX2. Thus, the lack of effect of GLX351322 on peripheral insulin sensitivity might also be explained by a low degree of NOX2 inhibition.

Recent RNA-seq results show that NOX4 mRNA contents are low in human islets, and even more so in EndoC-betaH1 beta-cells. Nevertheless, the NOX4 inhibitors GKT-136901 and GLX351322 inhibited acute human islet insulin release in response to glucose and palmitate, and GLX351322 counteracted high-glucose induced islet cell death, which suggests that NOX4, either expressed in the beta-cells or in other islet cells located close to the beta-cells, promotes significant effects on islet function *in vitro*. Furthermore, it is possible that NOX4 levels could be considerably higher *in vivo* than *in vitro*, especially at diabetic conditions [43]. Indeed, it is known that NOX4 is highly expressed in endothelial cells [44], and that islets are highly vascularized *in vivo* [25], but that islet endothelial cells are lost during *in vitro* culture [45]. In line with this, it can be envisaged that a high production of ROS in the microenvironment surrounding the islet blood vessels, which is further enhanced by diabetic conditions, could promote

negative effects on the beta-cells [46]. Thus, selective GLX351322-induced inhibition of NOX4 *in vivo* might mediate restoration of beta-cell function even though the beta-cell *in vitro* expression of NOX4 is low.

NOX4 differs from the other NOX enzymes in that it can generate both hydrogen peroxide and superoxide, and not only superoxide as most other NOX enzymes [47]. Moreover, NOX4 activity is mainly regulated at the mRNA expression level, and the protein localizes to other subcellular sites than NOX1/2/3/5. Interestingly, NOX4 gene expression appears to be controlled by miR-25 [43] and miR-146a [48], two microRNAs that are affected by aging and diabetes, respectively. The above-mentioned differences between NOX4 and NOX2 are compatible with the notion that NOX4 could play a specific role in islet dysfunction in diabetes.

In summary, our *in vitro* data suggest that NOX4 activity stimulates the release of insulin in response to a high glucose concentration. This is in accordance with previous studies supporting a stimulatory role of ROS in insulin secretion [16-19]. However, a recent study reported instead that NOX2-derived ROS antagonize glucose-induced insulin release [49]. Thus, results are conflicting as to whether NOX-derived ROS stimulate or inhibit insulin release, and it has been proposed that it is the intracellular source/location of the ROS produced that dictates the outcome [50]. As GLX351322 presently inhibited insulin release, it may be that NOX4 belongs to the ROS-generators that potentiate, rather than inhibit, the release of insulin. GLX351322 protected also against high glucose-induced islet cell death, aligning to the notion that human beta-cells, at least in part, die from hyperglycemia-induced oxidative stress [51]. Finally, GLX351322 counteracted beta-cell dysfunction *in vivo*, resulting in an improved glucose tolerance. Thus, prolonged NOX4-fueled ROS production and hypersecretion of insulin may be deleterious for the beta-cell, and it is possible that the use of NOX4 isoform-

selective inhibitors might achieve targeted effects on the beta-cell. According to a recently proposed model insulin resistance and obesity in T2DM are not primary events, but instead secondary to beta-cell hypersecretion of insulin [52]. Factors that promote hypersecretion of insulin via increased ROS production were suggested to be not only free-fatty acids, but also environmental compounds such as artificial sweeteners, pollutants and hormones [52]. This model predicts that inhibition of ROS production/hypersecretion of insulin ameliorates not only beta-cell dysfunction and death, but also peripheral insulin resistance. Such a chain of events does not fully concur to the finding of this study, but it may be speculated that a longer time period than 2 weeks is necessary for a normalization of the insulin release to restore peripheral insulin sensitivity.

In conclusion, GLX351322 is a novel NOX4 selective inhibitor that ameliorates glucose intolerance in high-fat diet-treated mice. GLX351322 might achieve a better glucose homeostasis by not only reducing oxidative stress, but also by promoting beta-cell rest.

DECLARATION OF INTEREST

Patent: Wilcke M, Walum E, Wikström P. Thiophene-based compounds exhibiting nox4 inhibitory activity and use thereof in therapy. 2013 Patent application number PCT/EP2013/055218

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Table 1**Characteristics of GLX351322**

IC ₅₀ , NOX4 inhibition in TRex 293 cells	5 µM
IC _{max} , NOX4 inhibition	85 %
Hill coefficient, NOX4 inhibition	0.83
IC ₅₀ , NOX2 inhibition in hPBMC cells	40 µM
Solubility in KP, FASSIF blank and FASSIF	2.7 µM, 1.4 and 18,9
Chemical stability, parent compound remaining after 2 and 24 h	64 % and 55 %
Metabolic stability, t _{1/2} , human liver microsomes	25 min
Plasma protein binding, % free fraction	0.03
Permeability, Caco-2 cells, Papp a-b	31 x10 ⁻⁶ cm/s
Oral pharmacokinetics, rat, t _{1/2} and AUC	2.9 h and 1620 nMxh

LEGENDS TO THE FIGURES

Figure 1. Structure of GLX351322.

Figure 2. GLX351322 does not affect DPPH absorbance. DPPH was incubated with decreasing concentrations (200-0.003 μ M) of GLX351322 or GLX481369 (positive control) and absorbance at 518 nm was measured after 60 min.

Figure 3. GLX351322 ameliorates HFD-induced hyperglycemia. (A) Weight curve of C57BL/6 mice given a normal diet or a high-fat diet from 5 to 14 weeks of age. After 7 weeks of control (CD, 20 mice) and high-fat diet (HFD, 20 mice) (age 12 weeks) mice were divided into four groups; CD or HFD with or without GLX351322 (3.8 mg/kg/day) and followed for another 2 weeks. GLX351322 supplementation in the drinking water did not affect the weight increase during the 2-week treatment period. (B) Non-fasting blood glucose of CD or HFD mice treated for 2 weeks with GLX351322. Blood glucose was analyzed after 5 weeks (10 weeks of age) and after 7 weeks (12 weeks of age) after start of HFD. After the 7 weeks of HFD mice were randomly divided into four groups; CD or HFD with or without GLX351322 (3.8 mg/kg/day) and followed for another 2 weeks. * denotes $p < 0.05$ vs. HFD mice without GLX351322 treatment using Student paired t-test.

Figure 4. Glucose tolerance test of CD and HFD mice treated with GLX351322. After two weeks of GLX351322 treatment CD (A) and HFD (B) mice were fasted for 8 hours and injected intraperitoneally with glucose (25 g/kg). Blood glucose levels were analyzed at the time points given in the Figure. Results are means $\pm$ SEM for 10 mice in each group. *

denotes $p < 0.05$ vs. HFD mice without GLX351322 treatment using Student independent t-test. (C) Data from Figures 3A and 3B were recalculated to Area Under Coordinates (AUC, C(I)). * denotes $p < 0.05$ vs. HFD mice without GLX351322 treatment using Student t-test.

Figure 5. Insulin sensitivity test of CD (A) and HFD (B) mice treated for 2 weeks with GLX351322. Insulin (1.6 U/kg Actrapid) was injected intraperitoneally and blood glucose was analyzed at the time points given in the Figure. Results are means $\pm$ SEM for 10 mice in each group.

Figure 6. Levels of mRNA coding for different NOX enzymes in human islets and EndoC-betaH1 cells. Expression levels at basal conditions of different NOXes as assessed by RNA-seq. Results are expressed as reads per kilobase per million (RPKM) and are normalized for exon length Results are means $\pm$ SEM for 3 independent observations and are modified from [53]. * denotes $p < 0.05$ using Student's t-test.

Figure 7. Effects of GKT-136901 (A) and GLX351322 (B) on human islet glucose- and palmitate-stimulated insulin release. (A) Human islets were incubated 60 min at 1.7 mM glucose (Control LG), 1.7 mM glucose plus 10 μ M GKT-136901 (LG GKT), 17 mM glucose (Control HG) and 17 mM glucose plus GKT-136901 (HG GKT). (B) Human islets were incubated for 60 min with (Palmitate) or without (Control) 0.5 mM sodium palmitate. All groups were incubated at 17 mM glucose. GLX351322 was added at a concentration of 2 μ M. Results are means $\pm$ SEM for 7-8 observations. * denotes $p < 0.05$ vs. corresponding control using Student paired t-test.

Figure 8. GLX351322 and DPI reduce human islet ROS production. Islets were pre-cultured for 24 h in 5.6 or 28 mM glucose. After loading of the fluorescent dye H₂DCFDA (10 μ M) at room temperature, the islets were returned to culture conditions for a final 60 min incubation with or without GLX351322 (10 μ M) and DPI (10 μ M). Hoechst stain (10 μ g/ml) was added during the final 20 min of the incubation. Islets were then analyzed by confocal microscopy. Results are means $\pm$ SEM for three independent experiments in which 2-4 islets per group were analyzed. * denotes $p < 0.05$ using Student's unpaired t-test.

Figure 9. Effects of GLX351322 (2 and 10 μ M) on human islet cell death in response to high glucose. After a 3-day culture period in the presence of 20 mM glucose (high glucose) islets were labeled with propidium iodide and Hoechst and then analyzed in a Kodak 4000MM Image station for blue and red fluorescence. The ratio red/blue fluorescence was calculated and normalized to the control. Results are means $\pm$ SEM for 5 independent observations * denotes $p < 0.05$ vs. corresponding control using Student paired t-test.

A **B** **C**

A **B**