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A Potent Immunomodulatory Compound, VGX-1027, Prevents Spontaneous and Accelerated Forms of Autoimmune Diabetes in NOD Mice and Inhibits the Immunoinflammatory Diabetes Induced by Multiple Low Doses of Streptozotocin in CBA/H Mice

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List of non-standard abbreviations:

VGX-1027: (S,R)-3-phenyl -4,5-dihydro-5-isoxasole acetic acid

CY: cyclophosphamide

NO: nitric oxide

MLD-STZ: multiple low doses of streptozotocin

PC: peritoneal cells

SMNC: spleen mononuclear cells

T1D: type 1 diabetes

Abstract

VGX-1027 ((S,R)-3-phenyl -4,5-dihydro-5-isoxasole acetic acid) is an isoxazole compound which exhibits various immunomodulatory properties. The capacity of VGX-1027 to prevent IL-1β+IFN-γ-induced pancreatic islets death *in vitro*, prompted us to evaluate its effects on the development of autoimmune diabetes in preclinical models of human type 1 diabetes mellitus (T1D). Administration of VGX-1027 to NOD mice with spontaneous or accelerated forms of diabetes induced either by injection of Cyclophosphamide or by transfer of spleen cells from acutely diabetic syngeneic donors, markedly reduced the cumulative incidence of diabetes and insulitis. In addition, VGX-1027 given either i.p. or *per os* to CBA/H mice made diabetic with multiple low doses of streptozotocin (MLD-STZ), successfully counteracted the development of destructive insulitis and hyperglycemia. The animals receiving VGX-1027 exhibited reduced production of proinflammatory mediators TNF-α, IL-1β, MIF and iNOS-mediated NO generation in both pancreatic islets and peripheral compartments. These results indicate that VGX-1027 probably exerts its antidiabetogenic effects by limiting cytokine-mediated immunoinflammatory events leading to inflammation and destruction of pancreatic islets. VGX-1027 seems worthy of being considered as a candidate drug in the development of new therapeutic strategies for the prevention and early treatment of T1D.

Introduction

Type 1 diabetes mellitus (T1D) is a T cell-mediated autoimmune disease that results from selective destruction of the insulin-producing β cells in the pancreatic islets of Langerhans. Both in human T1D and in rodent models of the disease such as the mouse made diabetic with multiple low doses of streptozotocin (MLD-STZ-induced diabetes), the NOD mouse and the diabetes-prone BB rat, the pancreatic β cells are selectively destroyed by infiltrating mononuclear cells (reviewed by Kolb, 1997).

One common mechanism by which infiltrating macrophages and autoreactive T lymphocytes promote β cell death may be related to their capacity of producing proinflammatory mediators, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-18 and nitric oxide (NO) which have all been implicated as critical players in the initiation and propagation of the disease process (reviewed by Rabinovitch and Suarez-Pinzon, 1998).

Although negating the action of proinflammatory and type 1 cytokines such as IL-1, IFN-γ, IL-12, IL-18 and TNF-α may prevent autoimmune diabetogenesis in most of these models (Rabinovitch and Suarez-Pinzon, 1998), the efficacy of similar approaches in influencing the natural course of human T1D is not known because of the lack of clinical studies with specific inhibitors of these cytokines in the human disease counterpart.

However, in spite of the promising data obtained in these rodent models, there are several drawbacks limiting the possible application of specific cytokine inhibitors in human T1D including their potential immunogenicity, the short half-life *in vivo*, the non-oral route of administration, the side effects, and the high costs of treatment. For this reason, studies aimed at discovering orally available small chemical compounds that target the synthesis and/or the action of endogenous proinflammatory cytokines are warranted. For example, a variety of small synthetic inhibitors of TNF-α have been designed to specifically inhibit the action of the cytokine at various levels, including transcriptional and post-transcriptional inhibition of TNF-α production, secretion, down-regulation of TNF-α-receptor mediated signal transduction, and inhibition of TNF-α bioactivity (Holstad and Sandler, 2001; Namazi et al., 2004; Abdul-Hai et al., 2005).

Since *in vitro* and *in vivo* data have proven a synergistic action of type 1 proinflammatory cytokines in determining β cell destruction (Rabinovitch and Suarez-Pinzon, 1998), it seems likely that compounds capable of simultaneously inhibiting most of them would prove more effective than specific neutralization of a single cytokine with the specific inhibitor. Along this line of research, *in vitro* and *in vivo* studies were carried out aimed at screening test compounds capable of inhibiting both the synthesis and the *in vivo* activity of TNF-α. In a first line of screening, a series of test compounds were analyzed for their capacity of inhibiting TNF-α synthesis from murine splenic mononuclear cells (SMNC) and peritoneal macrophages (PM) stimulated with either Concanavalin A (ConA) or lipopolysaccharide (LPS), respectively. Those test compounds achieving a significant inhibition of TNF-α in these assays were additionally

evaluated for their efficacy of counteracting TNF-α-dependent LPS-induced lethality in mice. We found that, among the compounds tested, (S,R)-3-phenyl -4,5-dihydro-5-isoxasole acetic acid- (VGX-1027) exhibited the highest potency in inhibiting the synthesis of TNF-α from macrophages and/or T cells and afforded the highest degree of protection against the lethal effects of LPS in the mice (SSG, YAL and FN, unpublished observations).

These immunopharmacological characteristics and the above mentioned pathogenic importance of TNF-α in autoimmune diabetogenesis, make VGX-1027 a suitable candidate for testing in preclinical models of human T1D and prompted us to evaluate its effects both in the NOD mouse and the mouse made diabetic with MLD-STZ. VGX-1027 exhibited powerful antidiabetogenic effects upon *in vitro*, *ex vivo* and *in vivo* conditions. In fact, it prevented IL-1β+IFN-γ-induced pancreatic islets death *in vitro* and it powerfully suppressed clinical and histological signs of the disease in both spontaneous and accelerated (Lukic et al., 1998; Like and Rossini, 1976; Kolb H, 1987; Shoda et al., 2005) forms of autoimmune diabetes in NOD mice and in the mouse made diabetic with MLD-STZ . Simultaneously, *ex vivo* studies carried out in mice with MLD-STZ-induced diabetes indicated that VGX-1027 dampened the immunoinflammatory diabetogenic processess at multiple levels, including the production of proinflammatory and cytotoxic mediators from both macrophages and pancreatic β cells.

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Materials and Methods

Reagents and drugs

Streptozotocin (STZ, S-0130), ^{3}H-thymidine, sulfanilamide, naphthylethylenediamine dihydrochloride, and cyclophosphamide (CY) were purchased from Sigma (St. Louis, MO). RPMI-1640 medium was supplemented with 1mM Hepes buffer, 5-10% fetal calf serum (FCS), as indicated, 1% sodium pyruvate, 2mM l-glutamine (all from Flow Laboratories, Irvine, GB), Penicillin/Streptomycin and $5x10^{-5}$ M 2-mercaptoethanol (Sigma). Recombinant mouse cytokines IFN-γ, TNF-α, IL-1β were from Sigma. VGX-1027 (Fig. 1) was synthesized as described previously (Eichenger et al., 1997).

The concentrations of VGX-1027 used *in vitro* were selected on the basis of preliminary experiments that evaluated the ability of the compound when added *in vitro* in the range between 1 and 100 μg/ml to inhibit TNF-α production from either Con A-stimulated murine splenic mononuclear cells or LPS-stimulated murine peritoneal macrophage. The concentration of 10 μg/ml was found to be the most effective concentration at which VGX-1027 inhibited the synthesis of TNF-α from these cells in the absence of toxicity on cell vitality that was assessed by crystal violet staining (FN, IC and SSG, unpublished observation).

The *in vivo* doses of VGX-1027 were chosen on the basis of preliminary studies carried out in the TNF-α -dependent (Mohler et al. 1993) mouse model of LPS-induced

lethality that indicated the doses of 20 mg/kg bw i.p. and 100 mg/kg bw p.o. as the most effective doses out of those tested (5, 10 and 20 mg/kg bw i.p. and 60, 100 and 120 mg/kg/bw p.o.) in significantly reducing the cumulative incidence of lethality as compared to vehicle-treated controls (FN data not shown).

Mice

Inbred CBA/H male mice that are genetically susceptible to development of immunoinflammatory diabetes following MLD-STZ were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and have then been bred at the Institute for Biological Research (Belgrade, Serbia) for more than 20 years. Adult male mice, at 6-8 weeks of age, in a body weight ranging from 25 to 30 g were used in all experiments, and each experimental group consisted of 7-8 mice.

Female NOD mice were obtained from Charles River (Calco, Italy) and were kept at the animal house of the Department of Biomedical Science of the University of Catania (Italy).

Both strains of mice were kept under standard laboratory conditions (non specific pathogen free) with free access to food and water. The handling of animals and the study protocol were in accordance with international guidelines and approved by the local Institutional Animal Care and Use Committee.

Phamacokinetic analysis of VGX-1027 in mice

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Single dose of VGX-1027 (20 mg/Kg bw) in vehicle (Na_2HPO_4 500mM) were administered via i.p. Blood samples (approximately 0.25- 0.3 ml) were collected prior to dose administration and 0.30, 1, 1.30, 2, 3, 4, 6 and 8 h after dosing via cava vein puncture and processed for plasma. Each determination consists of plasma samples pooled from 2 mice. There were 10 mice for each time point considered.

Sample preparation

Before the extraction procedure, clear and homogeneous plasma samples obtained for pharmacokinetic evaluation were thawed in a water bath at 30°C and then centrifuged at 2500×g for 10 min. Five hundred µl plasma samples pooled from different normal (VGX-1027-untreated) mice containing VGX-1027 were mixed with a 0.1 M pH 7 phosphate buffer (500 µl). Then, samples were purified by solid-phase extraction. The extraction procedure was carried out by using conditioned Oasis SPE cartridges HLB (1 ml, 30 mg) from Waters (Milford, MA), that were connected to a LiChrolut extraction unit (Merck). The stationary phase of cartridges were activated by elution under vacuum with 2 ml of a methanol/water (HPLC grade) mixture. Samples were charged on extraction cartridges, washed under vacuum with a 5 % (v/v) methanol aqueous solution (1 ml) and hence eluted twice with 1 ml of methanol acidified (pH 2.4) with trifluoroacetic acid. The elutate was collected in a glass tube and evaporated under a nitrogen stream at 40 °C by using a sample concentrator Teche DRI-BLOCK-3D. The residue was dissolved with acetonitrile (200 µl), centrifuged by a Mini Spin Eppendorf, filtered by Anotop 10 LC (0.2 µl/10mm) Whatman filters and then injected onto the chromatographic apparatus.

Control samples were prepared by adding 100 µl of an acetonitrile VGX-1027 solution at scalar concentrations. Control samples were frozen at -20 °C and then submitted to the procedure above reported to validate both the analytical method and the extraction procedure.

Extraction efficiency

The mean recovery of VGX-1027 from spiked mouse plasma samples (control samples) was evaluated to test the efficiency and reproducibility of the extraction procedure. The determination was carried out in replicate (n=3) in all samples. The extraction was conducted as described above. The responses of these standards taken by means of the extraction procedures have been compared with those of standard solution at the same concentration injected directly into the liquid chromatographic apparatus. The peak-areas were compared to that of standard aqueous samples without extraction.

The extraction efficiency was expressed as the percentage of the amount of VGX-1027 that was able to extract from control plasma samples spiked with known amount of the drug. The extraction efficiency of the method, as a function of the drug amount was reported in the Table.

HPLC experiments

The chromatographic system is a Jasco PU 1580 intelligent HPLC pump (Tokyo, Japan) equipped with a JASCO MD 1510 multiwavelenght detector (Tokyo, Japan) set at VGX-1027 $\lambda_{(max)}$ 260 nm. A 100 µl loop was used for analytical determinations. The HPLC apparatus was connected to a computer running the Jasco Borwin software program ver. 1.5 both for acquisition and data analysis. The chromatographic separation

was carried out by an Agilent Nucleosil C18 (250×4.6 mm, 5 μm) reversed-phase column with an Agilent column guard. The analytical column was thermostated to 25 °C with a block heater Gastorr GF 103 (Jones Chromatography, CO). The mobile phase was an acetonitrile/pH 2.01 water (acidified with trifluoroacetic acid) mixture (35:65 v/v). The mobile phase was delivered at a flow rate of 1.0 ml/min.

To determine the amount of VGX-1027 in mouse plasma samples a calibration curve was carried out by preparing control samples spiked with known amounts of drug added in plasma samples and plotted as a function of the chromatographic peak area (Figure 2). The calibration curve presented a r^2 value of 0.99984. The curve was constructed from five replicate measurements of five different drug concentrations over an interval of 0.1-50 μg/ml.

The specificity of the analytical method was assessed by comparison of chromatograms for the presence of interfering peaks and changes in retention times. Chromatograms of extracted plasma samples, derived from non treated animals, and spiked with low and high concentration of VGX-1027 were examined.

Clinical evaluation of diabetes

NOD mice were examined for diabetes development by twice weekly determination of glycosuria followed, when positive, by measurement of glycaemia. The NOD mice were defined as diabetics when fasting blood glucose levels exceeded 11.8 mmol/l for two consecutive days. The development of the disease in MLD-STZ-induced diabetes was evaluated by measuring blood glucose level and body weight loss on a

weekly basis. For determination of blood glucose levels in MLD-STZ diabetes, nonfasting mice were bled at the indicated time points from the retro-orbital venous plexus with heparinized capillary tubes. Plasma glucose concentration was determined by a glucose-oxidase method, using a blood glucose meter with electrode (Sensimac®, IMACO GmbH, Lüdersdorf, Germany).

Spontaneous and accelerated diabetes in NOD mice and in vivo *treatments*

To evaluate the impact of VGX-1027 on the development of spontaneous type 1 diabetes in the NOD mice, euglycaemic female 12 week old NOD mice were treated i.p. with either 20 mg/kg bw VGX-1027 or its vehicle six times a week from the 12^{th} until the 25^{th} week of age. An additional control group of mice was left untreated.

For induction of diabetes with CY, the drug was dissolved in water for injection and injected intraperitoneally (i.p.) at the dose of 200 mg/kg bw into 12 to 14 week old euglycemic NOD mice.

For adoptively transferred diabetes, $1x10^7$ spleen cells from acutely diabetic mice were injected intravenously (i.v.) to euglycemic 4-weeks old female NOD mice.

Both in CY-induced and adoptively transferred diabetes, the mice were treated i.p. with either 20 mg/kg bw VGX-1027 or its vehicle daily, six times a week starting one day after the diabetogenic challenge. Treatment was continued until the end of the studies, 2 weeks after CY-injection or 40 days after cell transfer. At the end of the experiment, these latter mice were sacrificed and pancreata specimens collected for histological analyses.

Histological evaluation of insulitis in NOD mice

Mononuclear cells infiltration of the pancreatic islets was graded in a blind fashion as described elsewhere (Nicoletti et al 1996): 0, no infiltrate; 1, peri-ductular infiltrate; 2, peri-islet infiltrate; 3, intra-islet infiltrate; 4, intraislet infiltrate associated with β cell destruction. At least 15 islets were counted for each mouse. A mean score for each pancreas was calculated by dividing the total score by the number of islets examined. Insulitis scores (IS) are expressed as mean values ± SD.

MLD-STZ-induced diabetes in CBA/H mice and in vivo *treatments*

Immunoinflammatory diabetes was induced with MLD-STZ, as described earlier (Lukic et al., 1991). Briefly, STZ was dissolved in citrate buffer pH 4.5 and injected i.p. at doses of 40 mg/kg (low dose) daily for 5 consecutive days. Day 0 was defined as the first injection of STZ.

In order to evaluate the effect of VGX-1027 on the disease development, the drug was administered as a continuous 12-day treatment, upon either an “early” or “late” prophylactic regime; while for the former, treatment of the mice started 1 day prior to MLD-STZ, for the latter it started the day after the last STZ dose. VGX-1027 was dissolved in 500 mM Na_2HPO_4 (pH 8.5), further diluted in H_2O and administered to mice either i.p. or perorally (p.o.), as indicated in the results. A daily i.p. dose of VGX-1027 was 10 mg/kg bw or 20 mg/kg bw, while 100 mg/kg bw was used for p.o. treatment. The control mice were treated under similar experimental conditions with an equivalent amount of vehicle.

Histology and immunohistochemical analysis of pancreas in MLD-STZ diabetic mice

In selected experiments of MLD-STZ-induced diabetes the pancreata from individual mice (n = 7-8 per group) were fixed in 10% formalin, embedded in paraffin, sectioned, and routinely stained with hematoxylin and eosin for histological examination by light microscopy. Multiple non-consecutive sections (4 or more) randomly selected from each pancreas (a total of 20-40 islets/animal) were analyzed.

Immunohistochemical analyses were performed on frozen 9-μm-thick pancreata sections obtained from mice with MLD-STZ-induced diabetes as described elsewhere (Lukic et al., 1991), or 5-μm-thick paraffin sections. To optimize immunohistological staining of paraffin sections, the microwave antigen retrieval technique was used (Penkowa and Hidlago, 2000). Briefly, deparafinized sections were immersed in 0.01M sodium citrate buffer (pH 6.0), boiled in a 750-W microwave oven for 10 min and cooled to room temperature. Staining was performed using primary mAbs: guinea pig anti-swine insulin Ab, strongly cross-reactive with insulin from several mammalian species, including mouse (Tian et al., 2004) (A0564, Dako, Hamburg, Germany), and rabbit anti-mouse iNOS (N 7782, Sigma). For detection, we then used the ExtrAvidin peroxidase staining kit (Sigma) with 3,3'-diaminobenzidine as substrate. Sections were counterstained with Mayer's hematoxylin and mounted in Canada balsam.

Apoptosis was assessed with a basic terminal deoxynucleotidyl transferase (TUNEL) assay using TACS™ TdT *in situ* apoptosis detection kit (R&D Systems, Oxford, United Kingdom) according to manufacturer's guidelines. The slides were then counterstained in methyl green and examined on a Zeiss Axiolab microscope. At least 4 mice per condition and 20-40 islets per mouse were examined.

Cell preparations and cultures

Pancreas, spleen and resident peritoneal cells (PC) were collected from individual mice given MLD-STZ and treated with either VGX-1027 or its vehicle, on day 15 after the first injection of STZ, as well as from normal untreated animals. The islets of pancreas, splenic mononuclear cells (SMNC), and PC, were prepared as previously described (Cvetkovic et al., 2005; Stosic-Grujicic et al. 2004). Cell culture supernatants, used for *ex vivo* detection of cytokines and nitric oxide (NO), were obtained by culturing the cells for 48 h in 24-well Limbro culture plates in 1 ml of a standard medium ($1x10^6$ PC, $5x10^6$ SMNC, or $1x10^5$ islets/well).

Culture of murine insulinoma cell lines

Mouse insulinoma MIN6 cells (with permission of Dr. J.-I. Miyazaki, Osaka University, Osaka, Japan) (Miyazaki et al. 1990), and rat insulinoma RIN-m5F cell line, were kindly donated by Dr. Karsten Buschard (Bartholin Instituttet, Copenhagen, Denmark). Cells were cultured in tissue culture flasks (Sarsted, Numbrecht, Germany) in RPMI-1640 medium containing 10% FCS, until reaching approx. 80% confluence, when they were detached by standard trypsinization procedure. Cells were washed and seeded for cytokine and NO production into 96-well flat-bottomed cell culture plates ($6x10^4$/well) in the presence of various combinations of recombinant mouse cytokines (IL-1β, IFN-γ and/or TNF-α), with or without VGX-1027. Culture supernatants were collected after 48 h. In some experiments the assessment of β cell viability after cytokine treatment was performed. The cytotoxic action of cytokines was evaluated by fluorescein

diacetate (FDA) staining, and by crystal violet colorimetric assay (Kaludjerovic et al., 2005), after 24 h and 48 h of cell cultivation, respectively, at the same conditions as described above. Nonfluorescent molecule FDA is hydrolyzed inside the viable cells to fluorescein by the intracellular esterases, the activity of which is reduced in the cells undergoing apoptosis. To discriminate live and dead cells, FDA dissolved in RPMI medium was added in cell cultures (100 ng/ml) and after 20 min on 37°C, cells were trypsinized, washed twice and analyzed on FACSCalibur flow cytometer using CellQuestPro software (Becton Dickinson, Heidelberg, Germany). FDA positive cells were considered viable. The staining with crystal violet has been used as a test for the viability of adherent cells (Kaludjerovic et al., 2005). At the end of incubation, cell cultures were washed with PBS to remove nonadherent dead cells, the remaining adherent cells were fixed with methanol and stained with 1% crystal violet. After thorough washing, the stain was extracted by the addition of 33% acetic acid, and the absorbance of the dissolved dye, corresponding to the number of live adherent cells, was measured at 570 nm in a microplate reader.

Nitric oxide production

Nitrite accumulation, an indicator of NO release in the supernatant was determined using the Griess reaction as described (Cvetkovic et al., 2005). Briefly, samples of cell-free culture supernatants were mixed with an equal volume of Griess reagent (a 1:1 mixture of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). After 10 min, the OD was measured at 570 nm in a microplate reader, and compared with a standard curve of $NaNO_2$.

Measuring of cytokines in culture supernatants

Cell-culture supernatant samples were analyzed in duplicate for murine TNF-α, IL-1β, IFN-γ, and IL-10 by ELISA using anti-mouse paired antibodies (R&D System, Minneapolis, MN, USA) according to manufacturer's instructions.

Cell-based ELISA

The *ex vivo* expression of MIF in PC and islets of Langerhans was determined by cell-based ELISA according to previously described protocol (Cvetkovic et al., 2005). Rabbit anti-mouse MIF antibody (at 1:2000 dilution) was used as a primary Ab, while detecting Ab was HRP-conjugated anti-rabbit IgG (U.S.Biochemical Corporation, Cleveland, OH) at 1:2000 dilution. The data obtained by measuring the light absorbance at 492 nm were corrected for differences in cell number by staining the cells with crystal violet after the ELISA procedure.

Semiquantitative RT-PCR

Total RNA was isolated from pancreatic islets with Trizol reagent (Genosys, Woodlands, TX) according to manufacturer's instruction. RNA was reverse transcribed using Moloney leukemia virus reverse transcriptase and random primers (Pharmacia, Uppsala, Sweden). PCR amplification of cDNA with primers specific for TNF-α and β-actin as a house-keeping gene, was carried out in a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany) as follows: 30 s of denaturation at 95^{o}C, 30 s of annealing at 58^{o}C, and 30 s of extension at 72^{o}C. For each gene, preliminary experiments

were conducted to ascertain that amplification of cDNA was in the linear range under the respective cycling conditions. For TNF-α, the primers were: sense, 5'-ACGCTCTTCTGTCTACTGAAC-3'; antisense, 5'-CTTGTCCCTTGAAGAGAACC-3' and the PCR product was 304 bp. The primers for β-actin were: sense, 5'-TCCTTCTTGGGTATGG-3'; antisense, 5'-ACGCAGCTCAGTAACAG-3' and the PCR product was 358 bp. The PCR products were visualized by electrophoresis through 2.5% agarose gel containing ethidium bromide, gels were photographed and results analyzed by densitometry using KODAK 1D 3.6 software.

Statistical analysis

Results are shown as mean values ± SD. Unless otherwise specified, statistical significance of differences between groups was evaluated using ANOVA, followed by Student-Newman-Keuls test for multiple comparisons between treatment groups,, unpaired Student's *t*-test for means between two groups, or χ^2 test as indicated. In the spontaneous model of diabetes of NOD mice differences in the kinetic and cumulative of incidence of the diabetes were assessed by Logrank (Mantel-Cox). A $p<0.05$ was considered to be significant.

Results

In vitro *effects of VGX-1027 on the production of TNF-α and NO by islet β cells and on their survival*

The exposure of islet β cells to IL-1β in combination with IFN-γ and/or TNF-α is known to induce severe functional suppression and death (Rabinovitch and Suarez-Pinzon, 1998; Eizirik and Mandroup-Poulsen, 2001). Therefore, experiments were carried out to ascertain whether VGX-1027 could modulate production of proinflammatory mediators as well as survival of cytokine-induced pancreatic islet death *in vitro.* Since the sensitivity of the islets to the action of cytokines is known to vary among different species we carried out the experiments in freshly isolated pancreatic islets, rat (RIN-m5F) and mouse (MIN6) insulinoma cell lines as β cell models. Exposure of MIN6 or RIN-m5F cells to 5 ng/ml of recombinant IL-1β + IFN-γ for 48 h resulted in the production of TNF-α (Fig. 3A). Similarly, overnight cytokine exposure of freshly isolated islets, as well as RIN-m5F cells resulted in high levels of nitrite (Fig. 3B). VGX-1027 (10 μg/ml) significantly inhibited both IL-1 β /IFN-γ-induced TNF-α and nitrite accumulation (Fig. 3A and B). We also assessed the capacity of VGX-1027 to interfere with cytotoxic effects of the cytokines by examining the death and survival of β cells. Results from FDA-staining (Fig. 3C) and crystal violet assay (Fig. 3D) of MIN6 cells showed that significant increase of cell survival was observed in the presence of VGX-1027. In contrast, the protective effect of VGX-1027 was annulled by exposure to exogenous TNF-α. These *in vitro* results urged us to proceed testing of VGX-1027 in various preclinical models of T1D.

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Pharmacokinetic analysis of VGX-1027 in mice

A high sensitive and selective analytical method was developed to allow the determination of VGX-1027 in the microgram range in plasma samples. This procedure consists of the use of solid-phase extraction and reversed-phase high-performance liquid chromatography with ultraviolet detection.

Figure 4 (panel A) showed a retention time for VGX-1027 of 5.027±0.3 min. The lower limit of detection was 0.1 µg/ml, while the lower limit of quantification was 0.5 µg/ml.

The high selectivity of the chromatographic method is proven by the absence of interfering peak at the retention time of VGX-1027 (Figure 4, panel A and B).

The pharmacokinetic profile (Figure 5) of mice treated i.p. with VGX-1027 showed a peak plasma concentration of 130 µg/ml (C_{max}) that was achieved after 2 h (T_{max}). In figure 4,(panel C), it is possible to observe the presence of the VGX-1027 followed by very small chromatographic peaks which suggests that VGX-1027 is not subjected to extensive biotransformation in vivo. Of particular interest when observing the pharmacokinetic profile of VGX-1027 was the observation that concentrations of the drug well above those found to exert iummunopharmacological effects in vitro (10 micrograms/ml) were already achieved after 30 minutes from the i.p. injection of the drug and that these levels were maintained until 5 hours after its administration (Figure 3). In addition, referring to the linear part of the pharmacokinetic profile of VGX-1027 a t½ of ~90 min was calculated.

VGX-1027 prevents development of spontaneous type 1 diabetes in NOD mice and also counteracts accelerated diabetogenesis induced by CY-challenge or adoptive transfer of diabetogenic spleen cells in NOD mice

Most of the female NOD mice treated with either the vehicle of VGX-1027 (13/16, 81.3%)or that were left untreated (12/16, 75%) developed diabetes by the end of the study at the age of 25 weeks. In contrast, the prolonged treatment with VGX-1027 markedly and significantly reduced the cumulative incidence of the disease in these mice with only 5 out of 16 mice (31.3%) becoming diabetics by this period of age (Fig. 6A, $p < 0.0001$ vs both vehicle-treated controls and untreated NOD mice by Logrank [Mantel-Cox]). Importantly, the prolonged treatment with the drug appeared to be well tolerated by the mice as judged from their behavior and the general appearance. No difference in food consumption was either observed between the mice treated with VGX-1027 and the mice treated with the vehicle or left untreated (data not shown). Accordingly, there were no significant differences in body weight gain between the mice treated with VGX-1027, its vehicle or that were left untreated that remained euglycaemic throughout the study period (the diabetic mice were not considered in the analysis as they characteristically lose body weight after disease onset [data not shown]).

Concurring with the antidiabetogenic effects of VGX-1027 observed in the spontaneous model of diabetes, the drug turned out to markedly reduce the development of the disease in accelerated models of autoimmune diabetogenesis. VGX-1027 was first tested in the model of CY-induced diabetes. As expected, within 2 weeks after the challenge, most of the control NOD mice that were treated with the vehicle of VGX-1027 (15/20, 75%) developed classical signs of diabetes with two or more consecutive days of

glycosuria and fasting hyperglycaemia. In contrast, the incidence of diabetes was significantly lower in the mice that were treated with VGX-1027, with only 6/20 (30%) having developed diabetes during the same period of time (p= 0.01 *vs* vehicle-treated controls by χ^2) (not shown in the figure) .

In another model of accelerated diabetes in NOD mice, starting from 23 days after the transfer of spleen cells from acutely diabetic donors, the control mice treated with the vehicle of VGX-1027 progressively started to develop diabetes with a cumulative incidence of 81.2% (13/16) on day 40 post transfer (Fig. 6B). In agreement with the preventive effects of VGX-1027 in CY-induced diabetes, the drug was also capable of significantly reducing the development of adoptively-transferred diabetes in the NOD mice as only 25% (4/16, p=0.005 *vs* vehicle-treated controls by χ^2) of the VGX-1027-treated mice developed diabetes within the same period of time. In the VGX-1027-treated mice that developed disease there was no significant difference in the kinetics of disease development between VGX-1027 and vehicle-treated diabetic mice (30.3±3.0 *vs* 31.6± 4.1 days, respectively).

Concurring with the clinical findings, histological analyses carried out in pancreatic specimens obtained in these two groups of mice at the end of the study showed that VGX-1027-treated mice had a significantly milder form of insulitis than the vehicle-treated controls (insulitis score =1.94± 0.91 vs 2.91 ± 0.8 , respectively, p= 0.003 by one way ANOVA).

VGX-1027 treatment reduces clinical signs of MLD-STZ-induced diabetes and suppresses pathohistological changes of pancreas

In order to evaluate the ability of VGX-1027 to interfere with immunoinflammatory diabetogenic pathways induced by MLD-STZ, the CBA/H mice were treated i.p. daily, for 12 consecutive days with either 10 or 20 mg/kg bw of the drug. The group of control mice that were challenged with MLD-STZ and received the vehicle from day –1 to day 10 developed persistent hyperglycemia that started from around 2 weeks after the first injection of the STZ. In contrast, mice treated under the same experimental conditions with VGX-1027 exhibited a dose-dependent reduction in blood glucose levels (Fig. 7A). This protection did not depend on continuous application of the drug, since none of the mice developed hyperglycemia throughout the entire follow-up period after treatment withdrawal. Moreover, VGX-1027 (100 mg/kg bw per day) showed similar effect when given p.o. (Fig.7B).

In order to avoid direct interference of the drug with STZ or its initial damaging effects towards β cells, we next tested the effect of treatment with VGX-1027 at a later stage of disease development upon a "late prophylactic" regime when, though the mice are still normoglycaemic, immunopathogenic processes have already been initiated by STZ (Karabatas et al., 2005). In this case, treatment with VGX-1027 was commenced immediately after the last STZ injections (from day 5 to day 16). In accordance with the results obtained when the drug was used upon "early prophylaxis", down-regulation of diabetes development was also achieved by the "late prophylactic" treatment with VGX-1027 (Fig. 7C). In addition, the slight lost in body weight observed in MLD-STZ-treated control mice during disease development (Fig. 7D) was not observed in VGX-1027-treated animals.

We next evaluated the impact of VGX-1027 on histopathological events taking place at the level of pancreatic β cells during development of diabetes in MLD-STZ-induced diabetes. Representative examples of the light microscopical evaluation of the islets are presented in Fig. 7E-J. We have previously reported the appearance of a limited but significant insulitis and subsequent depletion of β cells in mice by 10-20 days after MLD-STZ administration (Lukic et al., 1991). Consistent with our earlier studies, as early as 15 days after the injections of MLD-STZ, mild mononuclear cell infiltrates of endocrine pancreas accompanied by initial necrotic changes could be observed (not shown). From this period up to the end of the serial measurement of blood glucose levels (8 weeks), the destructive process progressed, and most of the islets lost clear margins (Fig. 7E). By contrast, the prophylactic administration of VGX-1027 abrogated the development of these changes. The pancreatic sections from VGX-1027-treated mice did not harbor a substantial infiltrate and the majority of pancreatic islets appeared normal, with no signs of inflammation (Fig. 7F). Concordantly with the clinical status of these mice, the distribution of insulin positive β cells (Fig. 7H), was normal long after treatment cessation. Moreover, histological evidence for apoptosis was consistently found in the control diabetic mice, as evidenced by TUNEL staining (Fig. 7I), whereas in the islets of VGX-1027-treated mice no such evidences for the apoptosis were found throughout the entire follow-up period (Fig 7J), indicating that the treatment with VGX-1027 preserved islets from autoimmune attack, thus enabling appropriate regulation of plasma glucose levels. These data confirmed that the antidiabetogenic effect of VGX-1027 was maintained both at the clinical and histological level.

VGX-1027 treatment down-regulates the production of proinflammatory mediators

To elucidate the mechanisms by which VGX-1027 treatment prevents disease onset, functional studies were performed on both pancreas and peripheral compartments during early progression of the disease. Hence, pancreatic islets, splenocytes and peritoneal cells were harvested on day 15 after MLD-STZ injection from mice that had been treated i.p. daily for 12 consecutive with either VGX-1027 (20 mg/Kg) or its vehicle and the levels of the diabetogenic mediators TNF-α , IL-1β, MIF and NO were examined *ex vivo*.

As shown in Fig. 8, both pancreatic and peripheral immune cells established from mice treated with 0.5 mg VGX-1027 i.p, released significantly reduced amounts of TNF-α in comparison to control diabetic animals. The inhibition by VGX-1027 treatment in PC (Fig. 8A) and SMNC (Fig. 8B) was 46.6% and 93.4%, respectively, and in the islets 91.4% (Fig. 8C). Consistent with this finding, a marked reduction in mRNA expression was detected between pancreatic islets isolated from VGX-1027-treated versus diabetic mice (inhibition of 61.3%), indicating that VGX-1027 effect occurs through down-regulation of TNF-α gene expression (Fig. 8D).

Fig. 9 shows that the animals receiving VGX-1027 produced or released less MIF, IL-1β and NO in response to MLD-STZ than vehicle treated control mice. The effect was observed both in PC (Fig. 9A,C,E) (the inhibition 29.7%, 36.4% and 60.9%) and in the islets of pancreas (Fig. 9B,D,F) (the inhibition 36.0%, 59.6% and 45.1%, respectively, in comparison to control diabetic mice). In line with the results of nitrite measurement, iNOS staining of pancreatic islets was significantly lower in mice that received VGX-1027 (Fig. 9H) in comparison to vehicle-treated diabetic control (Fig. 9G), suggesting

that the observed down-regulation of NO production was mediated through reduction of iNOS expression.

We next studied the effects of VGX-1027 on the production of two prototypical type 1 and type 2 cytokines such as IFN-γ and IL-10. As previously shown by us and others (Lgssiar et al., 2004), MLD-STZ-treated mice demonstrated significant up regulation of IFN-γ, and down regulation of IL-10 (not shown). However, neither of the cytokines was significantly affected by VGX-1027 administration. IFN-γ production by SMNC obtained from VGX-1027-treated and nontreated mice was 800±151 *vs*. 651±126 pg/ml, respectively, P=0.261, while IL-10 production by PC cultures was 351±46 *vs*. 374±24, P=0.485. Similarly, in the micromilieu of pancreatic islets there were no noticeable effects of VGX-1027 on either Th1 or Th2 cytokine production (not shown).

Discussion

VGX-1027 is an orally bioactive small isoxazole compound that is being developed for the treatment of immune-mediated pathologies. We have shown here for the first time that VGX-1027 exhibited multiple and powerful antidiabetogenic effects including prevention of β cell death and suppression of clinical and histological signs of immunoinflammatory diabetes both in spontaneous and accelerated models of the disease in the NOD mouse as well as in mice with MLD-STZ-induced diabetes.

Though pathogenic and immunotherapeutic differences occur between MLD-STZ-induced diabetes and NOD mouse diabetes, it is believed that in both models the damaged pancreatic islets ultimately become targets for cell-mediated (auto)immune reactions mounted from T lymphocytes and macrophages which migrate into the pancreatic islets. β cell destruction could result from the toxic effect of free radicals ($O_2^{\cdot-}$, H_2O_2, $NO^{\cdot}$), cytokines (IL-1, TNF-α, TNF-β, IFN-γ), and other inflammatory products released by activated macrophages and T cells, as well as β cells themselves (Like and Rossini, 1976; Kolb, 1987; Rabinovitch, 1998; Rabinovitch and Suarez-Pinzon, 1998; Lukic et al., 1998; Wachlin et al., 2003; Gurgul et al., 2004; Shoda et al., 2005). In addition, it has been shown that TNF-α and IL-1β may increase vulnerability of pancreatic β cells to autoimmune destruction by inducing the expression of Fas on the β cell surface (Wachlin et al., 2003). Therefore, the capacity of VGX-1027 to counteract the generation of TNF-α and NO by cytokine-stimulated islet β cells *in vitro* anticipates that its antidiabetogenic property relies on the downmodulation of cytotoxic mediators within the islets. Although the mechanism by which VGX-1027 regulates the level of TNF-α expression is not clear, it seems that VGX-1027-mediated rescue of islet β cells is

secondary to reduced synthesis of the cytokine rather than on antagonizing its bioactivity as the addition of exogenous TNF-α reverted VGX-1027-mediated protection.

In agreement with these *in vitro* observations, VGX-1027 showed beneficial effects in both spontaneous and accelerated forms of diabetes in NOD mice. Similarly, in MLD-STZ-induced diabetes, VGX-1027 counteracted the diabetogenic effects of the toxin even when administered upon "late prophylactic" regime after the last injection of STZ. *Ex vivo* studies carried out in this model showed that VGX-1027 acted in a bimodal manner, entailing both a direct protective effect on the β cells as well as a profound impact on key immunopathogenic events associated with disease development including diminished production of proinflammatory and cytotoxic mediators by macrophages and pancreatic cells.

An interesting immunopharmacological characteristic of VGX-1027 arising from these data is its impact on the macrophage secretory capacity of the type 1 proinflammatory cytokines TNF-α, IL-1β and MIF and as well as of the pleiotropic soluble immunomodulatory mediator NO. Type 1 cytokines of the innate immune system have been proposed as key players in initiating, coordinating and maintaining β cell destruction during autoimmune diabetogenesis (Beyan et al., 2003). NO has repeatedly been reported to synergize with cytokines in inducing apoptosis of pancreatic β cells possibly via potentiation of JNK activity and inhibition of Akt (Beyan et al., 2003). In agreement with these observations, we and others have shown that negating the action of endogenous TNF-α, IL-1β, MIF and NO either with specific inhibitors or by gene deletion prevent development of autoimmune diabetes in NOD mice and/or mice given MLD-STZ (Lukic et al., 1991; Nicoletti et al., 1994; Sandberg et al., 1994; Yang et al.,

1994; Flodstrom et al., 1999; Holstad and Sandler, 2001; Suarez-Pinzon et al., 2001; Drage et al., 2002; Wang et al., 2002; Thomas et al., 2004).

Note however that it is unlikely that prevention of diabetes by VGX-1027 is solely due to interference with priming and activation of lymphocytes, because the protective effect was also achieved by a "late prophylactic" treatment, after the initial induction of the disease.

The progression of T1D in mice is marked by two general "checkpoints"; the first is associated with "benign" insulitis with limited β cell destruction, when animals remain diabetes-free, and the second corresponds to the shift to "aggressive" insulitis when β cells are destroyed to promote overt diabetes (Andre et al., 1996). The transition from benign to pernicious insulitis requires an islet cell response to TNF-α (Pakala et al., 1999) which is consistent with the recent observation that TNF-α plays a central role in the effector function of diabetogenic $CD4^+$ Th1 T cell clones (Cantor and Haskins, 2005). The marked inhibitory effects of VGX-1027 on *in vitro* and *in vivo* production of TNF-α at the pancreatic level as well as the fact that VGX-1027-induced reduction of TNF-α synthesis correlated with reduced severity of pancreatic inflammation all suggest that inhibition of TNF-α may represent an important mechanism by which VGX-1027 influences the transition to aggressive insulitis and hence to clinical appearance of diabetes. Nonetheless, since the pathogenic effect of endogenous TNF-α in NOD mice is age-dependent and primarily occurs in the early stages of the diabetogenic process (Yang et al., 1994; Pakala et al., 1999; Cantor and Haskins, 2005; Green and Flavell, 2000; Christen et al., 2001) it is possible that the antidiabetogenic action of VGX-1027 might

have depended on its presently demonstrated capacity to simultaneously inhibit the production of other diabetogenic mediators such as IL-1β, MIF and NO.

In immunoinflammatory diabetes Th1 response has been connected with a β cell destructive insulitis, whereas a Th2/3 type response was associated with the protection from the disease (Kolb, 1997; Rabinovitch and Suarez-Pinzon, 1998). Pharmacological compounds capable of deviating the predominant Th1 state to a Th2/Th3 state have been considered suitable candidates for the prevention and early treatment of the disease. However, in spite of its *in vitro* and *in vivo* antidiabetogenic efficiency as well as its capacity to prevent pancreatic and/or macrophage production of diabetogenic type 1 proinflammatory cytokines and NO, VGX-1027 failed to influence the production of the two prototypical Th1 and Th2 cytokines, IFN-γ and IL-10. Knowing the powerful stimulatory effects of IFN-γ on macrophages, these data suggest that the VGX-1027 inhibits macrophage functions in an IFN-γ-independent manner.

Two observations of particular relevance from the clinical point of view are the apparent low toxicity of the drug that was witnessed by the lack of characteristic body weight loss occurring in MLD-STZ-induced diabetes and that, unlike control mice, was not observed in VGX-1027-treated mice, and the efficacy of the drug to equally prevent MLD-STZ diabetes regardless of whether it was given i.p. or p.o. The proper evaluation of VGX-1027 in the treatment of newly diagnosed T1D patients will require preclinical studies carried out under “therapeutic” dosing to animals with established disease. It is nonetheless encouraging for the translation of these findings to the clinical setting, the

capacity of the drug to counteract the diabetogenic effects of MLD-STZ even when administered upon a "late prophylactic" regime started one day after the last of the 5 injections of the toxin had been given. As this is a period of time when early diabetogenic pathways are fully activated in this model (Karabatas et al., 2005), the above indicates that VGX-1027 is capable of delaying/reverting an already initiated process of β cell and anticipates its possible prophylactic use in individuals at risk for developing T1DM that can be selected on the basis of immunological markers associated with actively ongoing β cell destruction. In addition, if the long-term persistence of the antidiabetogenic effects of VGX-1027 observed in MLD-STZ diabetic mice after treatment interruption would also occur in humans, this would have the obvious advantage to avoid requirements of prolonged and possibly life-lasting treatment of both newly diagnosed T1D patients and individuals at risk for disease development.

Finally, the preliminary observation emerging upon *ex vivo* analyses that VGX-1027 may preferentially inhibit immunoinflammatory events leading to inflammation and destruction of pancreatic islets without influencing IFN-γ and IL-10 production may also be important for the clinical setting as it might anticipate down-regulation of the immune system that spares its physiological functioning. Preservation of the IL-12 – IFN-γ axis might presume that VGX-1027 may be less likely than other immunosuppressants to provoke compromise of innate immunity with consequential reduced immunity to opportunistic pathogens (Ware, 2005).

Taken together these findings all provide VGX-1027 with an immunopharmacological and toxicological profile worthy of being further studied for its use in the prevention and early treatment of human T1D and other autoimmune diseases.

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Footnotes

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Legends for Figures

Figure 1. **Synthesis and structure of (S,R)-3-phenyl -4,5-dihydro-5-isoxasole acetic acid (VGX-1027)**.

Figure 2**. HPLC calibration curve of mouse plasma samples spiked with known amount of VGX-1027**. The curve was constructed from five replicate measurements of five different drug concentrations over an interval of 0.1-50 μg/ml

Figure 3. ***In vitro*** **effects of VGX-1027 on the production of TNF-α, NO and survival of islet β cells.** TNF-α production was determined in MIN6 or RIN-m5F cell culture supernatants (A), and NO accumulation in fresh pancreatic islets or MIN6 cells (B). Viability of MIN6 cells was determined by colorimetric assay by FDA staining (C) or crystal violet staining (D). *$p<0.05$ refers to treatment with IFN-γ/IL-1β.

Figure 4. **Typical HPLC chromatograms of plasma samples of untreated mice** (Panel A), plasma samples of mice spiked with the drug (10 μg/ml) (panel B) and plasma samples of mice treated with VGX-1027 after 2 h from i.p. administration (panel C). The black arrow in panel B shows the time at which the peak of VGX-1027 has to appear.

Figure 5. **Plasma concentration of VGX-1027** as a function of time following IP administration in mice.

Figure 6. **Reduction of spontaneous and adoptively transferred type 1 diabetes in NOD mice by VGX-1027.** (A) Euglycaemic female 12 week old NOD mice were treated i.p. with either 20 mg/kg bw VGX-1027 or its vehicle six times a week from the 12^{th} until the 25^{th} week of age. An additional control group of mice was left untreated. Each group consisted of 16 mice. By the 25^{th} week of age the incidence of diabetes was 81.3 % (13/16) in the mice treated with the vehicle of VGX-1027, 75% (12/16) in the mice left untreated and 31.3% (5/16) in the mice treated with VGX-1027. The data are representative of two independent experiments that were merged because of interstudies variability lower than 10%. Statistical analysis was performed by Logrank (Mantel-Cox). (B): Diabetes was induced by i.v. transfer of $1x10^7$ spleen cells from acutely diabetic mice were injected i.v. to euglycemic 4-weeks old female NOD mice. The mice were treated i.p. with either 20 mg/kg bw VGX-1027 or its vehicle daily, six times a week starting one day after the diabetogenic challenge. Each group consisted of 16 mice. On day 40 post transfer, the incidence of diabetes was 81.2% (13/16) in the vehicle-treated mice and 25% (4/16) in the mice receiving VGX-1027. The data are representative of two independent experiments that were merged because of interstudies variability lower than 10%.

Figure 7. **Effect of VGX-1027 treatment on the MLD-STZ-induced hyperglycemia, body weight loss and the hystopathology of pancreatic islets.** (A, B, C): Plasma glucose levels in control CBA/H mice receiving STZ (40 mg/kg/day, for 5 consecutive days) in conjunction with 12 daily i.p. injections of vehicle (STZ), or in mice treated with STZ and VGX-1027 given as a continuous 12-day treatment, applied according to

following treatment protocols: (A): different doses of VGX-1027, 20mg/kg and 10 mg/kg bw, administered i.p. from day -1 to day +10; (B): 100 mg/kg bw of VGX-1027, administered p.o. from day -1 to day +10; (C): 20 mg/kg bw VGX-1027 administered i.p. as an "early" (STZ+ VGX-1027 early) or "late" (STZ+ VGX-1027 late) prophylactic treatment (as described in *Materials and Methods*). (D): Percent change in body weight from the start of the experiment, determined in mice treated according to protocol (C). Results from a representative experiment are presented as mean ± SD for seven to eight mice per group. *$p<0.05$ refers to treatment with MLD-STZ. (E,F): Light micrographs showing morphological profiles of pancreatic islets by day 62 after disease induction (H.E. staining); (E): Control MLD-STZ-treated animals; note atrophy and loss of islet margins; (F): MLD-STZ-treated animals after "early prophylactic" i.p. treatment with VGX-1027; note well-preserved morphology. (G, H): Immunocytochemical detection of insulin expression in pancreatic islets by day 27 after disease induction (immunoperoxidase staining); (G): Control MLD-STZ-treated animals; note paucity of insulin-containing β cells; (H): MLD-STZ-treated animals after "late prophylactic" i.p. treatment with VGX-1027; note homogenous pattern of well-preserved insulin-containing β cell mass. (I, J) : Detection of apoptotic cells in pancreatic islets by day 15 post-MLD-STZ treatment (TUNEL staining) ; note an increased number of apoptotic cells in control MLD-STZ-treated animals (I) compared to VGX-1027-treated mice (J). (E-J) Images are representative of 4 - 8 animals per group. Magnification x 400.

Figure 8. **Effect of VGX-1027 treatment on the TNF-α expression.** PC (A), SMNC (B), and pancreatic islets (C, D), were isolated from mice that were not challenged with

STZ, (STZ-untreated), or treated with STZ and vehicle (STZ+ vehicle) or STZ and VGX-1027 (STZ+VGX-1027)under "early prophylactic" i.p. regime by day 15 after disease induction. Production of TNF-α was measured in the 48h culture supernatants of cells by ELISA. Results are given as mean ± SD for five mice per group, done in duplicate. (D): The expression of TNF-α mRNA was assessed by RT-PCR. Results from the representative of three separate experiments with similar results are presented as relative expression of TNF-α mRNA in comparison to β-actin. *$p<0.05$ refers to control group treated with vehicle.

Figure 9. **Effect of VGX-1027 treatment on the expression of MIF, secretion of IL-1β, NO production and iNOS expression.** PC (A, C, E), and pancreatic islets (B, D, F), were isolated from the same group of mice as described in Fig. 5. (A, B): MIF expression was determined by cell-based ELISA as described in *Materials and Methods.* (C, D): Production of IL-1β was measured in the 48-h culture supernatants of cells by ELISA. (E,F): Nitrite accumulation was measured in the 48-h culture supernatants of cells by Griess reaction. Results are given as mean ± SD for five mice per group, done in triplicate. *$p<0.05$ refers to treatment with MLD-STZ. (G, H): Immunocytochemical detection of iNOS expression in pancreatic islets by day 15 after disease induction (immunoperoxidase staining); (G): Control MLD-STZ-treated animals; note strong staining of iNOS-containing cells; (H): MLD-STZ-treated animals after early prophylactic i.p. treatment with VGX-1027; note the absence of iNOS-containing cells. Magnification x 400.

Table 1

Extraction efficiency of VGX-1027 in various control plasma samples spiked with known amount of the drug[a].

Drug Concentration (µg/ml)	Extraction Efficiency (%)
0.1	70.8±2.5
1	76.9±1.5
5	85.0±0.9
10	90.4±0.3

[a] Each value is the average of three different experiments ± standard deviation.

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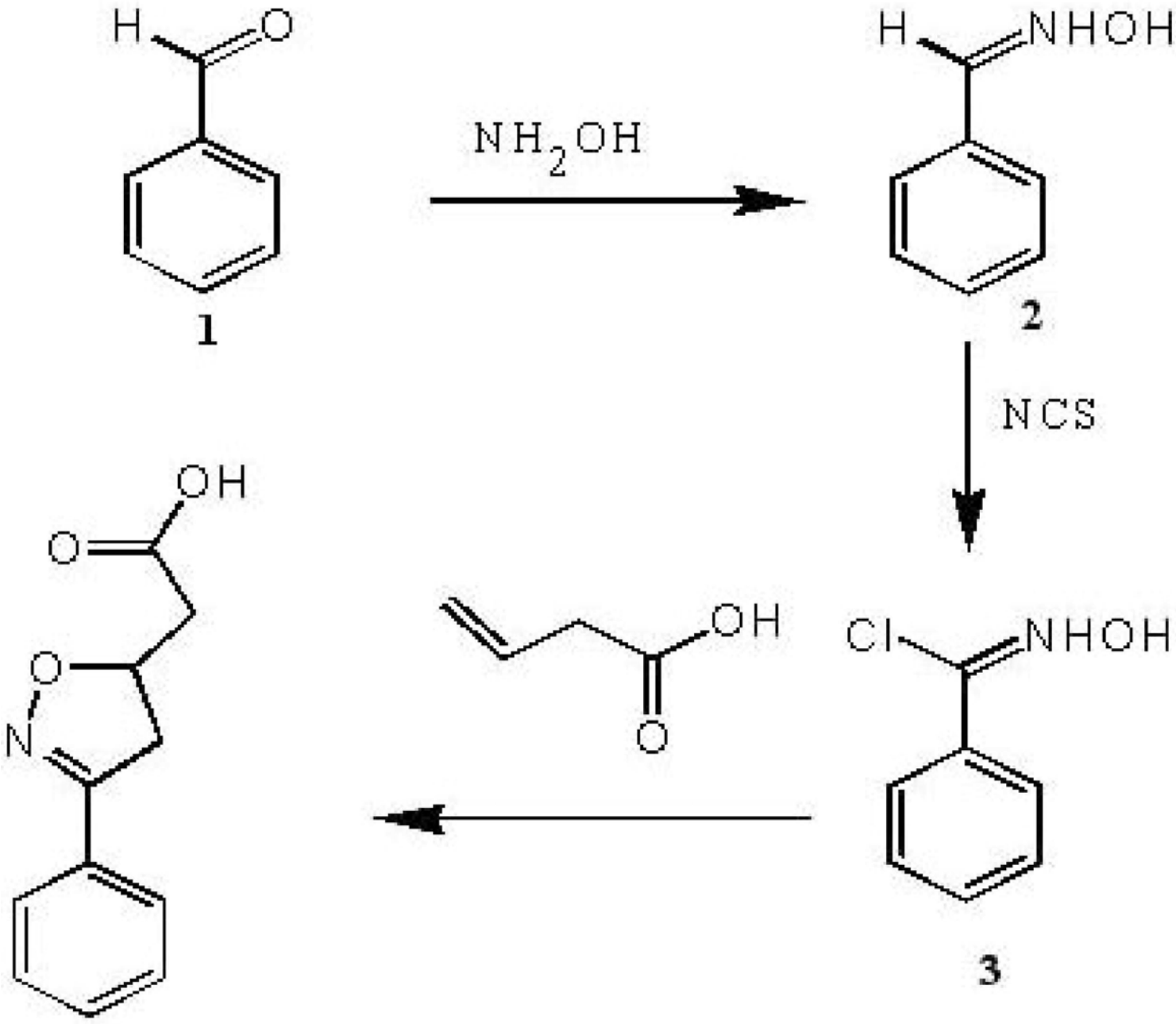

Fig 1

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Fig 2

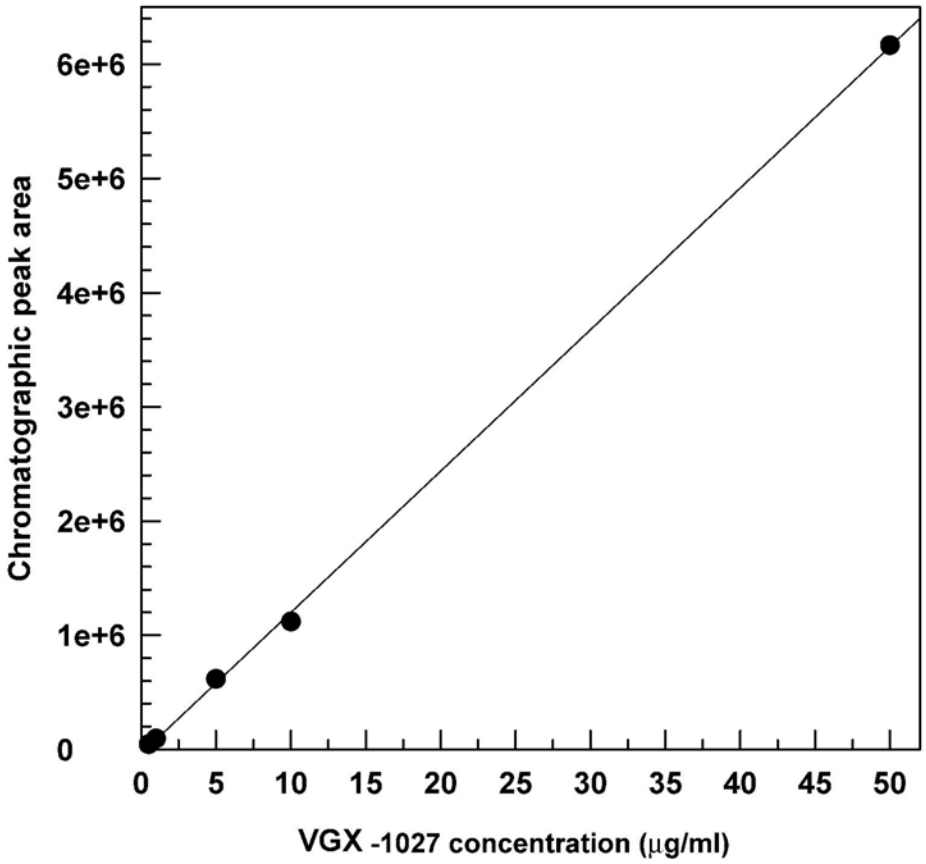

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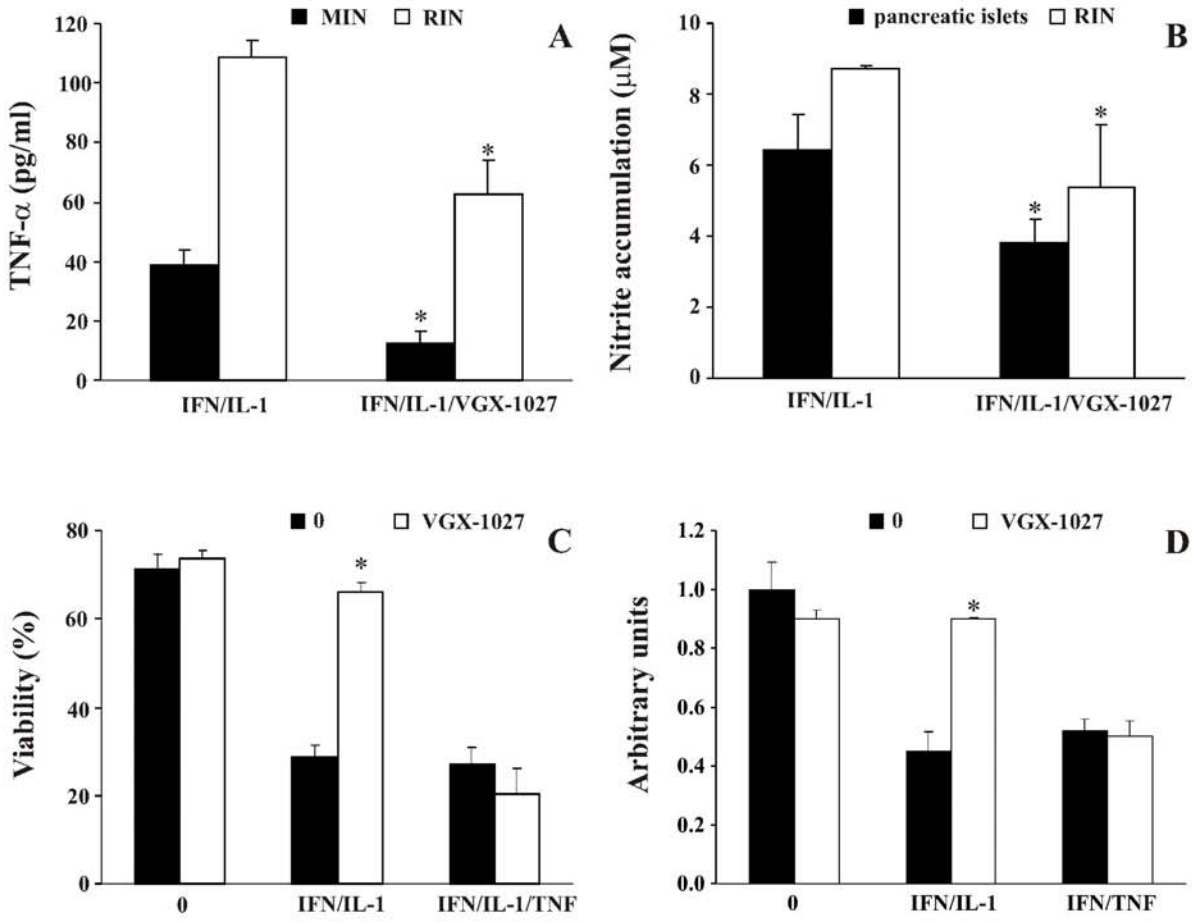
Fig 3.



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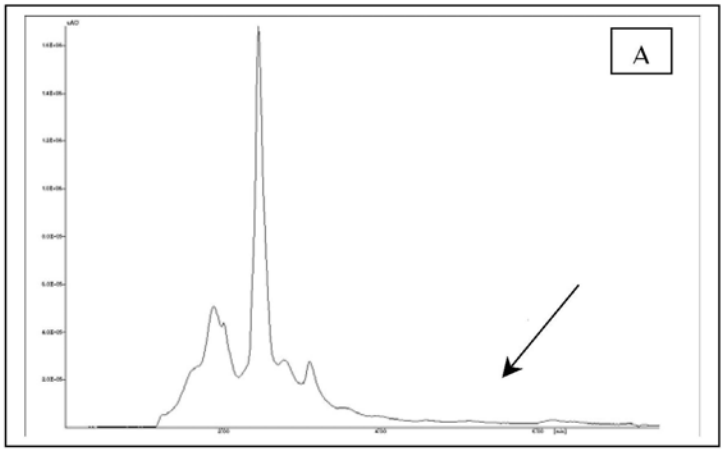

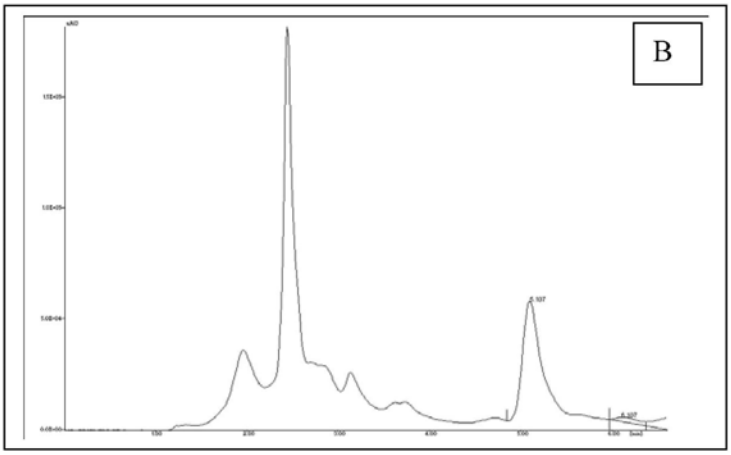

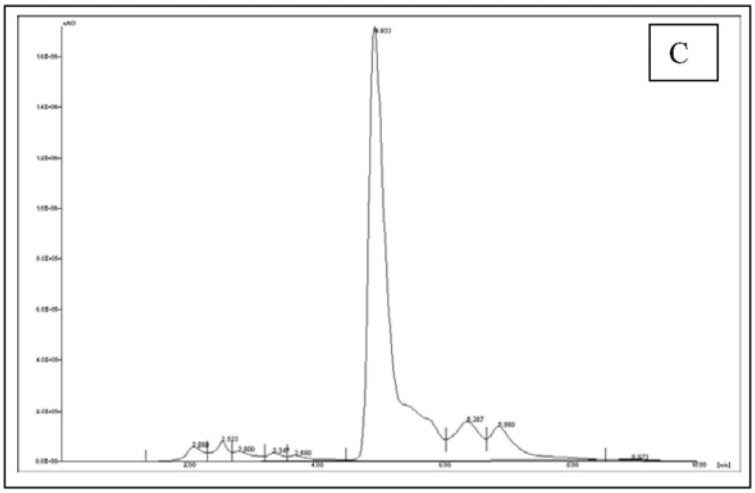

Fig. 4

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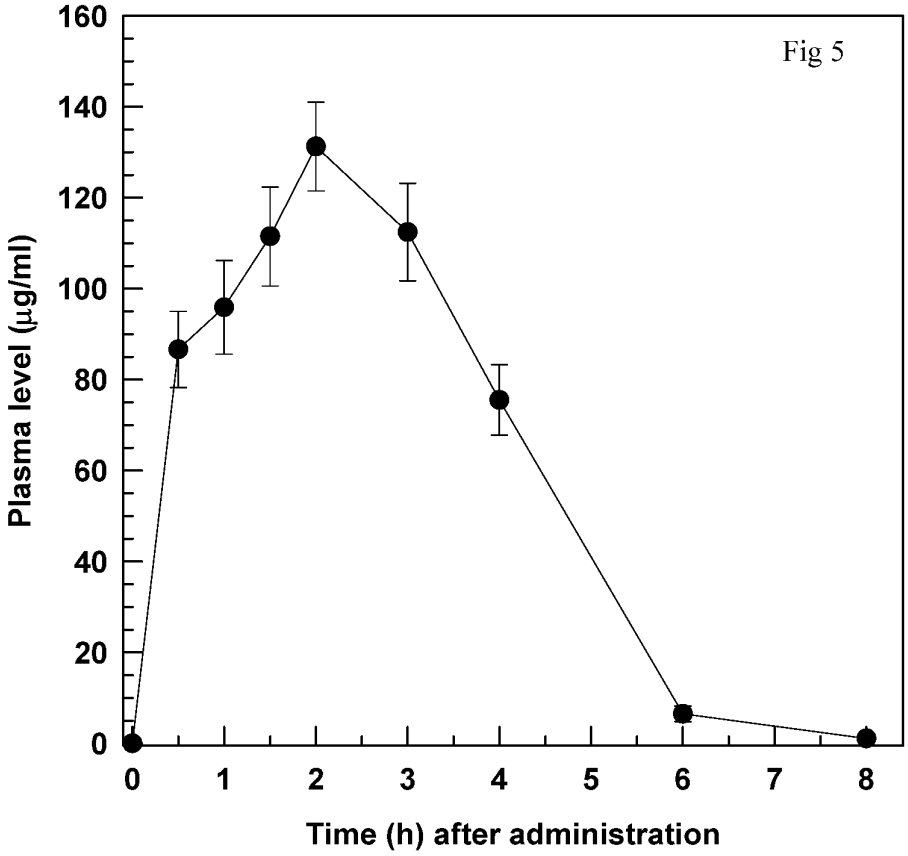

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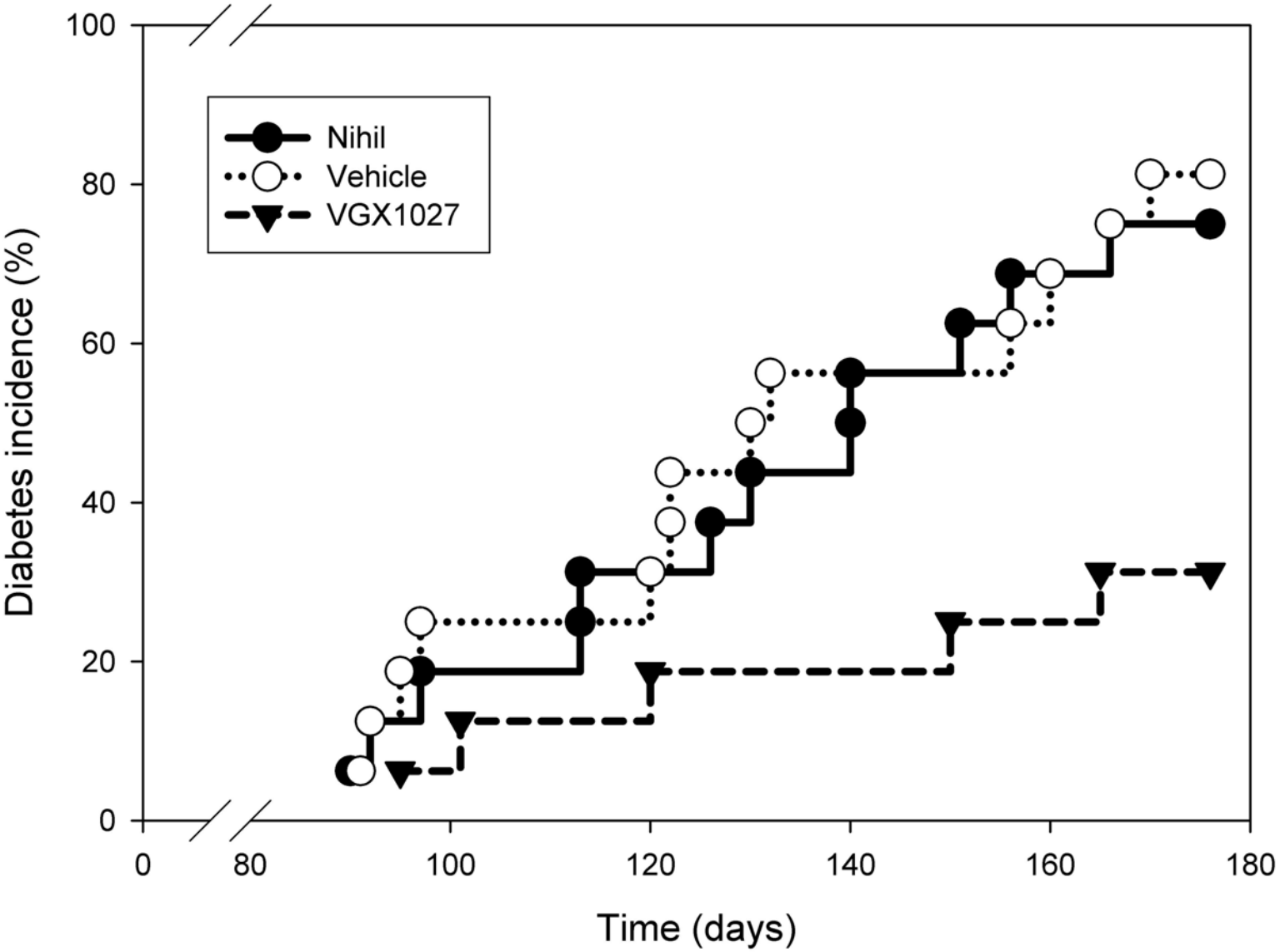
Fig 6A



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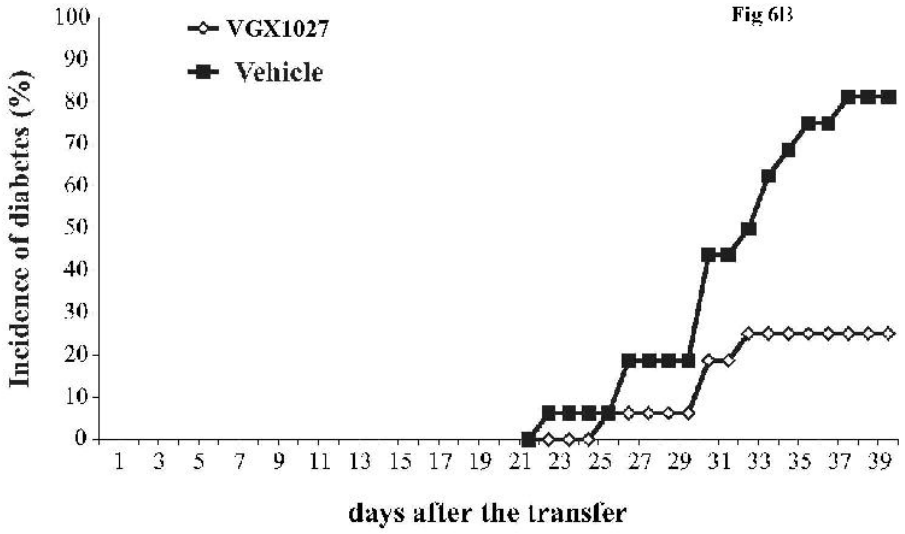

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Fig 7.

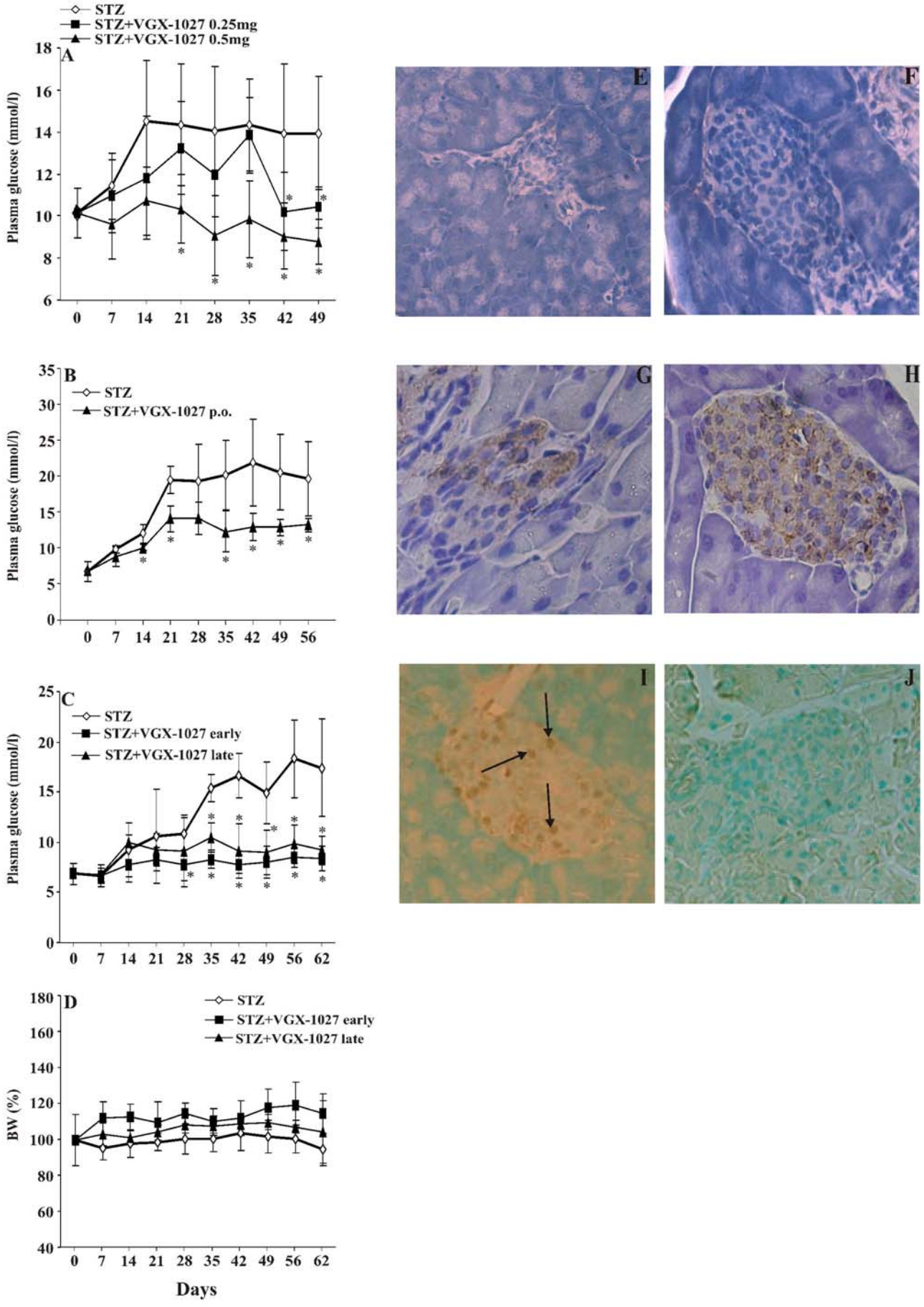

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Fig 8.

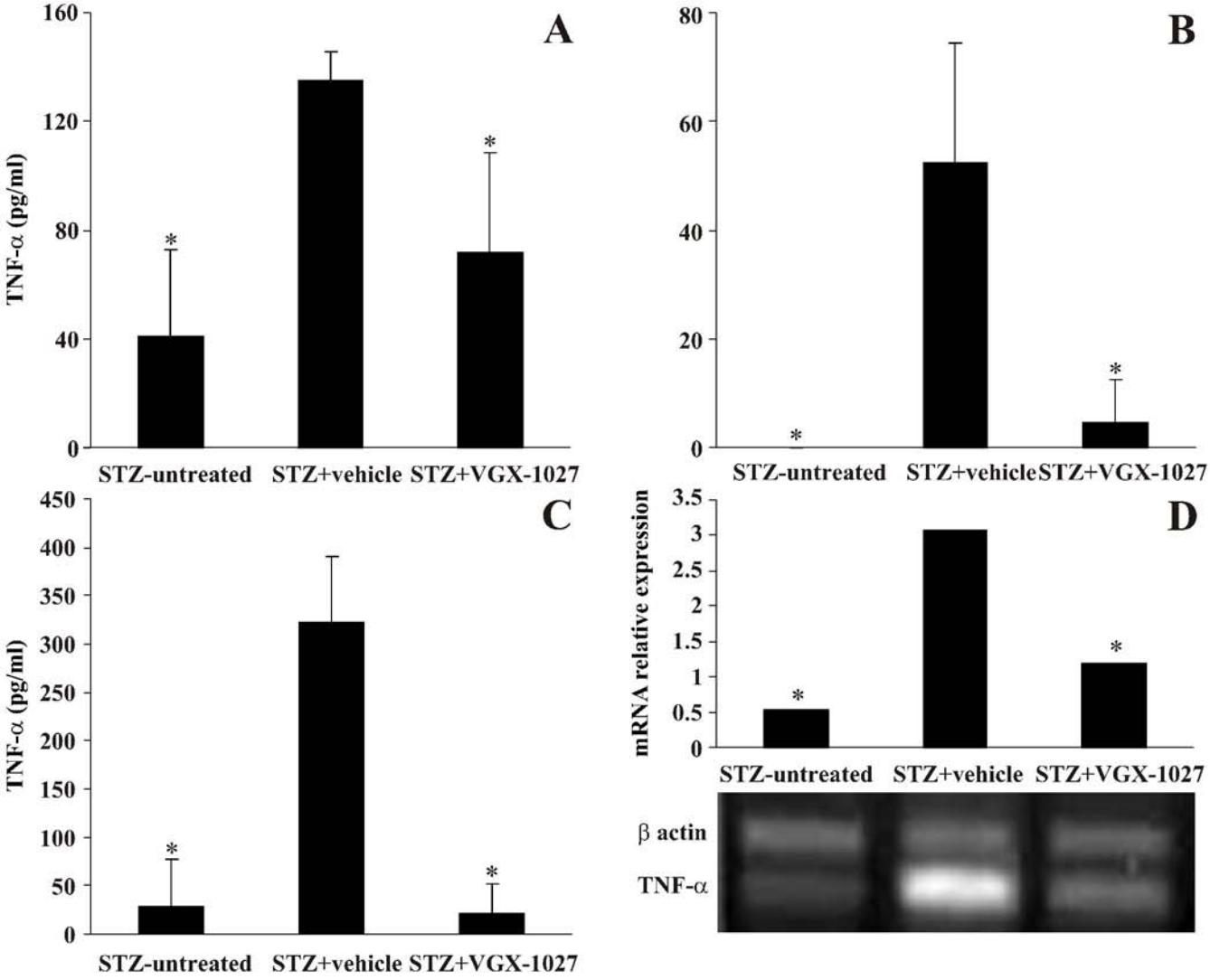

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Fig 9.