AIM
Of
The Study
4. Aims

The specific aims of the study were:

- To study the effects DPP-IV inhibitors on body weight, blood glucose, plasma insulin, DPP-IV and GLP-1 levels after oral glucose loading in streptozotocin induced diabetic rats.

- To investigate the effects DPP-IV inhibitors on gastric emptying and small intestinal transit rate in streptozotocin induced diabetic rats.

- To assess the effects of chronic administration of DPP-IV inhibitors on glycaemic control, β-cell secretary functions, insulin resistance, Glycated hemoglobin (HbA1c), tumor necrosis factor-α (TNF-α), total proteins, and pancreatic islet function.

- To examine the effect DPP-IV inhibitors on liver glycogen content, GLUT-2 protein expression and hepatic SREBP-1c mRNA level; Soleus muscles glycogen content, GLUT-4 protein expressions and muscle GLUT-4 mRNA level; and pancreatic GLP-1 mRNA level.

- To determine the impact of DPP-IV inhibitors on immunohistochemistry and DNA fragmentation assay (β-cell apoptosis) in Pancreas.
Materials & Methods
5. Materials and methods

5.1. Materials
PKF-275-055 (Novartis Switzerland), vildagliptin (LAF237; 1-[(3-hydroxy-1-adamantyl) amino] acetyl]-2 cyano-(S)-pyrrolidine) & PKF-275-055, were synthesized by Novartis Switzerland. These compounds were dissolved or suspended in 0.5% carboxymethylcellulose in 0.2% Tween 80, and then orally administered. Insulin ELISA kit (SPI BIO, france, Catalog #A05105), GLP-1 ELISA kit (Linco Research, Japan, catalog #YK050, lot 091130), DPP-IV ELISA kit (R&D Systems, USA, Catalog # DC260), HbA1c assay kit (Biosystem, Spain, catalog # COD 11044), Apo-BrdU-IHC™ In Situ DNA Fragmentation Assay Kit (Biovison, USA, Catalog #K403-50), chromogen (sigma chemicals, USA), Hematoxylin and eosin (sigma chemicals, USA), streptavidin peroxidase (Genetex, USA), Tween 80 (Merck, India), phenol red (Applichem, Germany), methylcellulose (sigma chemicals, USA), trichloroacetic acid (Merck, India), tris buffer (Applichem, Germany), and 3,3′-diaminobenzidine (sigma chemicals, USA).

5.2. Animals
Healthy albino rats of Wistar strain were kept for breeding. To induce NIDDM, STZ (sigma chemicals, USA) (90 mg/kg) was administered i.p. to a group of 2 days old pups. Another group of pups received only saline. The pups were weaned for 21 days, and 6 weeks after the injection of STZ, the animals were checked for fasting glucose level (FPG) ≥ 160 mg/dl were considered as diabetic (Bonneir-Weir et al., 1981). Pups that receive saline were considered as control animals, after which they were grouped so that the blood glucose levels were uniform among the groups. All rats were housed under conventional conditions with controlled temperature, humidity and light (12 h light–dark cycle), and were provided with a standard commercial diet and water (ad libitum). All experimental procedures were conducted according to the Institutional Animal Ethical Committee (protocol no.DIPSAR/IAEC/2009/25) and CPCSEA guidelines.

5.3. Eight week chronic daily dosing study
After 6 weeks, the animals were assigned to receive vehicle or PKF-275-055 or vildagliptin at the dose level of 1, 3, and 10 mg/kg once daily for 8 weeks to evaluate dose dependant activity (Burkey et al., 2005). On the morning after final administration, blood samples were collected under fasting conditions and body weight was measured;
and the pancreas was isolated and was immersed and fixed in phosphate-buffered 10% formalin solution to prepare a paraffin section.

5.4. Blood glucose levels during the oral glucose tolerance test (OGTT) in diabetic rats

Blood samples were collected from normal and diabetic rats fasted overnight for the measurement of blood glucose levels and to which either the vehicle or the test compound had been orally administered. After 30 min, blood glucose levels were measured again, after which glucose solution (2 g/kg) was orally administered (1st OGTT). At 0.5, 1, 2 and 4 h after glucose loading, blood glucose levels were measured. At 8 h after the first glucose loading (8.5 h after drug administration), blood glucose levels were measured, and then glucose solution was orally administered (2nd OGTT). The blood glucose levels were again measured at 0.5, 1, 2 and 4 h after the second glucose loading (Akiko et al., 2008).

5.5. Plasma insulin, GLP-1 and DPP-IV levels during the OGTT in diabetic rats

Normal and diabetic rats were fasted overnight, and blood samples (basal value) were collected to measure plasma insulin, GLP-1 and DPP-IV levels. Either the vehicle or the test Compound was administered orally, and blood samples were collected 30 min later (1st OGTT-pre value). Glucose solution (2 g/kg) was then orally administered, and blood samples were collected 10 min later (1st OGTT-10 min value). Eight hours after the first glucose loading, blood samples were collected (2nd OGTT-pre value). Glucose solution was then orally administered, and blood samples were collected 10 min later (2nd OGTT-10 min value) (Akiko et al., 2008).

5.6. Blood glucose levels during the oral glucose tolerance test (OGTT) in normal rats

Blood samples were collected from normal rats fasted overnight for the measurement of blood glucose levels and to which either the vehicle or the test compound had been orally administered. After 30 min, blood glucose levels were measured again, after which glucose solution (2 g/kg) was orally administered and the blood glucose levels were measured at 0.5, 1, 2, 4, 6 and 8 h after administration (Akiko et al., 2008).

5.7. Glycated hemoglobin (HbA1c) assay
Total HbA1C content, an indicator of irreversible condensation of glucose with the N-terminal residue of the β-chain of hemoglobin A. The HbA1C concentration in blood is directly proportional to the mean concentration of glucose prevailing in the previous 6-8 weeks, equivalent to the lifetime of the erythrocytes (Bissé and Abraham., 1985) and this assay based on the procedures of a commercially available kit (Biosystem, Spain, catalog Number COD 11044).

5.8. **Homeostatic model assessment for insulin resistance**

The homeostatic model assessment (HOMA) is a method used to quantify insulin resistance and beta-cell function (Matthews et al., 1985). The approximating equation for insulin resistance, in the early model, used a fasting plasma sample, and was derived by use of the insulin-glucose product, divided by a constant.

HOMA-IR= (Glucose x Insulin)/405; HOMA-%B= (20 x Insulin)/ (Glucose-63)

Where IR is insulin resistance and %B is the β-cell function where Glucose is given in mg/dl and Insulin is given in μU/mL (both during fasting).

5.9. **Pancreatic Nitrate/ nitrite assay**

Total nitrate/nitrite content, an indicator of NO production, was estimated in serum & pancreatic tissue homogenate based on Griess reaction (Moshage et al., 1995) according to the procedures of a commercially available kit (Biovison, USA, Catalog Number K262-200).

5.10. **Estimation of Tumor Necrosis factor-α**

Quantitative measurement of Rat TNF-alpha in serum was done by Rat TNF-alpha ELISA (Ray biotech) kit as per manufacturer protocol (Ray biotech). is an in vitro enzyme-linked immunosorbent assay.

5.11. **Estimation of Total proteins**

Total Proteins in serum and pancreatic homogenate was determined by the method of Lowry et al. (Lowry et al., 1951).

5.12. **Glycogen content in liver and muscles**

Muscle or liver are ground with 5 ml. of the deproteinizing solution [Trichloroacetic acid (5 g) and Ag2SO4 (100 mg) are dissolved in water and made up to 100 ml] placed in centrifuge tube covered with a glass cap. The fluid level is marked on the centrifuge tube and is placed in a boiling-water bath for 15 min. Then the tube is cooled in running water,
filled up to the mark with deproteinizing solution to compensate for evaporation, and centrifuged at 3000 rpm. for 5 min. One ml of the clear supernatant fluid is added to 3 ml. of H₂SO₄ in a wide test tube and mixed by vigorous shaking. The mixture is heated in a boiling-water bath for exactly 6 min. and subsequently cooled in running tap water. The intensity of the pink colour produced is measured spectrophotometrically at 520 nm and the glycogen concentration read from a standard curve of glycogen (Kemp and Kits Van Heijningen., 1955).

5.13. Gastrointestinal functions in diabetic rats

Either the vehicle or the test compound was administered to diabetic rat that had been fasted overnight. 30 min later (compound treatment examination), glucose solution (0.2 g/mL glucose, 0.25% methylcellulose, 1 mg/mL phenol red and 10 mg/mL charcoal) was orally administered at a volume of 15mL/kg. Under ether anesthesia, the stomach was ligated and removed, after which it was transferred to a tube and cryopreserved. The entire length of the small intestine (between the pylorus of the stomach and the end of the ileum) and the distance to the charcoal front were measured. The rats in the control group were given vehicle solution in order to measure the total amount of glucose solution injected into the stomach. At 15 minutes after administration, the pylorus of the stomach was ligated under ether anesthesia; after which the stomach was immediately removed, and small intestinal transit was checked. To measure the gastric emptying rate, 0.1 mol/L NaOH solution (5 mL) was added to the stomach sample, and they were homogenized. After centrifugation (3000 rpm, 10min), 20% TCA solution (50 mL) was added to a 500-μL aliquot of the supernatant. The mixture was then stirred and centrifuged (15,000 rpm, 10 min). A 100 μl aliquot of the supernatant was then dispensed into a 96- well assay plate, and 0.5 mol/L NaOH solution (50 μl) was added. After stirring, phenol red concentration in the sample was determined from a phenol red (0–1000 μg/mL) calibration curve at 450 nm. The gastric emptying rate (%) was then calculated using the following equation: [(mean value of the control group) - (the sample value)]/ (mean value of the control group). The small intestinal transit rate (%) was calculated using the following equation: (the distance traveled by the charcoal front)/ (the entire length of the small intestine) (Akiko et al., 2008).

5.14. RNA isolation and Real-time PCR analysis
A quantitative real-time protocol was used in which the components were combined in a 96 well plate. Reaction conditions were 10X PCR buffer [20mM Tris (pH 8.4), 50mM KCl and 2.5mM MgCl2], 20 pM oligonucleotide each primers gene-specific PCR included: GLUT – 4 (Gene ID: 25139) :- 5’-AGCGTAGGTACCAACACTTTTCT-3’ - forward primer & 5’-CCGCCCTTAGTTGGTCAGAAG-3’-Reverse primer, GLP-1 (Gene ID: 25051) :- 5’- CAACCGGACCTTTGATGACTA-3’ - forward primer & 5’- GCTGTGCAGAACC GG TGAC-3’ - Reverse primer, and SREBP1c(Gene ID: 78968) :- 5’- GGAGCCATGGATTGCACATT- 3’ - forward primer & 5’- AGGAAGGCTTCCAGAGAGGA-3’ - Reverse primer. 300μM dNTPs, 1:1000 SYBR Green I nucleic acid stain, 0.5 U Taq DNA polymerase (recombinant), and 50 ng templates cDNA in a 25 ml reaction volume. PCR products of β-actin-F 5’- TCACCCACACTGTGCCCATCTACGA-3’ and β-Actin-R 5’- CAGCGGAACGCTTGCATGCCAATGG-3’ primers gene, were used as internal standards. Thermal cycling conditions were 95°C for a 3 min denaturation step, followed by 40 PCR cycles (94°C for 30 s, 56°C for 30 s and 72°C for 1 min) and reactions were performed in a Light Cycler 480 (Roche) instrument. Fluorescence was detected at the end of the 56°C segment in the PCR step.

Melting curve analysis

After 40 amplification cycles, a melting analysis was carried out to verify the correct product by its specific melting temperature (Tm). The thermal profile for melting curve analysis consisted of a denaturation for 1 min at 95°C, lowered to 55°C for 30 s and then increased to 95°C with continuous fluorescence readings.

5.15. GLUT- 2 expressions in liver and GLUT- 4 expressions in soleus muscle

Western Blot Analysis: 0.3 g of each liver and muscle slices were homogenized with ice-cold 10mM Tris–HCl buffer (pH 7.4) containing 1mM EDTA 2Na, 250mM sucrose, 1mM phenylmethyl sulfonyl fluoride and 1000 U/ml aprotinin, the homogenates were centrifuged at 600 rpm for 10 min at 4 °C, and the supernatant was then centrifuged at 11000 rpm for 15 min at 4 °C. In this study, the precipitate was designated as the total membrane fraction of the liver. The protein concentration of each sample was determined using BCA Protein Assay Kit.
For determination of glucose transporter 2 (GLUT2) protein expressions in pancreas and liver; and glucose transporter 4 (GLUT4) protein expressions in skeletal muscle; each sample prepared was mixed with 1% sodium dodecyl sulfate and 50mM dithiothreitol, and the mixture was subjected to electrophoresis with 10% polyacrylamide gel and molecular standard markers according to the method of Laemmli, 1970. The separated proteins on the gel were electrotransferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk solution including 0.05% Poly (Oxyethylene) sorbitan monolaurate (Tween 20) overnight at 4 °C, the membrane was reacted with anti-GLUT-2 antibody (Abcam, UK) & anti-GLUT-4 antibody (Abcam, UK) for 2 h. Subsequently, it was incubated with horseradish peroxidase conjugated IgG (diluted 1: 2000) [Jackson immunoresearch laboratories, USA] for 2 h at room temperature. The blots were detected with chemiluminescence reagents (Western Blot). (Yoshihiko et al., 2007).

5.16. Immunocytochemistry

Whole pancreas from rats was removed under anaesthesia and fixed in 10% buffered formalin for 24 h. Tissues were dehydrated in graded series of alcohol, embedded in paraffin, sectioned at 5 micron thickness and used for immunostaining. The tissue sections were stained with haematoxylin and eosin while the remaining serial sections were used for immunostaining. Serial sections of the rat pancreas were immunostained by strepavidin-biotin peroxidase method (Hsu et al., 1981) using pre-diluted polyclonal antibodies. All sections were de-paraffinized in xylene bath to remove the excess wax. The slides were placed in two changes of absolute alcohol for 3 min each. The same procedure was repeated with 90 per cent alcohol. The slides were placed in blocking reagent in order to block the endogenous peroxidase activity for five min, which was pre-diluted with 5 volumes of 100 per cent ethanol. The slides were placed in two changes of 70 per cent alcohol for three min each. The excess alcohol around the sections was removed and the slides were quickly immersed in Tris buffer, pH 7.6 for 5 min. Two drops of tissue conditioner was added and the sections were incubated for 5 min and then rinsed in buffer solution. Pre-diluted primary polyclonal anti-guinea pig antibody to insulin (1:1,000) [Genetex, USA] raised against human insulin, anti-rabbit glucagon antibody (1:375) [Novus biologicals, USA] raised against Procine glucagon conjugated to
BSA and anti-rabbit somatostatin antibody (1:500) [Novus biologicals, USA] raised against synthetic somatostatin, were added to the sections and incubated for one hour. The secondary antibodies for insulin, glucagon and somatostatin were anti-rabbit polyclonal antibodies. After incubation for half an hour, the sections were rinsed with tris buffer, peroxidise solution was added, incubated for 30 min and later rinsed with the buffer. AEC (3-amino, 9-ethyl carbazole) chromogen substrate was added to the sections and was incubated for 15 min and rinsed with distilled water. The sections were counterstained with Harris’ haematoxylin for 45 sec to facilitate nuclear identification (Hsu et al., 1981).

5.17. DNA Fragmentation Assay
For detection and localization of apoptosis in pancreas, we used the technique of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Apo-BrdU-IHC™ In Situ DNA Fragmentation Assay Kit, Biovision, USA). Briefly, sections were deparaffinized, hydrated, and digested with proteinase K (20 μg/ml), and then added biotinylated dUTP to the 3' end of DNA fragments by incubating sections in 0.05 mol/l Tris–HCl buffer (pH 7.6) with 0.03 U/μl TdT and 0.04 nmol/μl biotin-11-dUTP at 37 °C for 1 h. The sections were rinsed in PBS. Endogenous peroxidase was blocked with 0.3% H2O2 in distilled H2O. The sections were rinsed with PBS and covered with 2% blocking solution in 0.1 mol/l sodium maleate to reduce background staining. The sections were then incubated with avidin-peroxidase complexes in PBS (1:50) for 30 min and rinsed with PBS (3×5 min). Peroxidase activity was visualized with 3,3'-diaminobenzidine until the brown product was clearly visible. The sections were then counterstained with methyl green. The positive apoptotic cells were the cells with brown nucleus (Matsuno et al., 1997).

5.18. Statistical analysis
Values are mean ± S.E.M. Significant differences between treatment groups one-way-analysis of variance (ANOVA) with post-hoc analysis using Dunnet multiple comparison test (sigma plot 11, USA). Values of P ≤ 0.05 were accepted as significant.