THE EFFECT OF TRADJENTA ® (LINAGLIPTIN) ON INFLAMMATION, OXIDATIVE STRESS AND INSULIN RESISTANCE IN OBESE TYPE 2 DIABETIC SUBJECTS

RESEARCH PROTOCOL

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Over the past decade, following the initial suggestion that pharmacological 'harnessing' of the in vivo stability of GLP-1 may be a novel approach to the management of type 2 diabetes(1), two separate strategies to overcome the inherent drawbacks in using native GLP-1 have been investigated by academic laboratories and the pharmaceutical industry alike (2, 3). The outcome of this research has been the design of long-acting, DPP IV-resistant analogs, and/or derivatives of GLP-1, suitable for exogenous administration, and the development of inhibitors of DPP IV to enhance the endogenously released hormone.

Linaglitpin and other DPP-IV inhibitors reduce blood glucose concentrations by stimulating c-AMP dependent insulin release from the β cell, suppressing post prandial glucagon increase and by slowing gastric emptying. Linagliptin is a potent, long- acting, competitive, reversible inhibitor of DPP IV. Linagliptin once daily increases postprandial plasma intact GLP-1 levels by 1.5-to 3-fold compared with placebo. Linagliptin also decreases postprandial glucagon levels in patients with type 2 diabetes during 12 weeks of therapy, thereby lowering fasting glucose concentrations and reducing glucose excursions after a meal. Linagliptin has been studied as a monotherapy in drug- naïve patients with type 2 diabetes, and in combination with other glucose lowering medications. It is not yet known if linagliptin is anti-inflammatory.

Obesity, diabetes and inflammation

Both obesity and diabetes have been shown to be associated with oxidative stress (4-7) and inflammation (8-12). One possible reason why obesity and type 2 diabetes are associated with oxidative stress and inflammation is the state of insulin resistance. This is due to the fact that 1) resistance is associated with the presence of pro-inflammatory factors including cytokines (13); 2) insulin has been shown to exert an anti-inflammatory and anti-oxidant effects at the cellular and molecular level both, *in vitro*, and *in vivo* (14-17). A low dose infusion of insulin (2 IU/h) has been shown to reduce ROS generation by mononuclear cells (MNC), suppress NADPH oxidase expression and intranuclear NFκB binding, induce IκB expression and suppress plasma ICAM-1 and MCP-1 concentrations (17). It also suppresses intranuclear Egr-1, plasma tissue factor (TF), PAI-1 and MCP-1 concentrations (14). An interruption of insulin signal transduction would prevent the anti-inflammatory effect of insulin from being exerted. The anti-inflammatory and pro-fibrinolytic effects of insulin have also been confirmed in the setting of acute ST segment elevation myocardial infarction (18). We have previously demonstrated that macronutrient intake (glucose, protein and lipids) induce ROS generation and cause oxidative stress in humans. Glucose or a fast food mixed meal intake cause an increase in NFκB and a fall in total cellular IκBα, the two cardinal indices of inflammation at the cellular level (19-21). Mixed fast food meal also induces an increase in C-reactive protein (CRP) (19). Glucose intake has further been shown to increase the ratio of phosphorylated IκBα to native non-phosphorylated IκBα and to induce an increase in IκBα kinase (IKKα) and IKKβ. At a cellular level, the changes that occur in the MNC following a glucose challenge are very similar to those which occur after endotoxin challenge.

It is possible that obesity is a pro-oxidative and pro-inflammatory state resulting from chronically increased macronutrient intake. An increasing amount of epidemiologic data show that persistent, low-grade inflammation is an independent predictor of ischemic heart disease (22, 23), stroke (24, 25), diabetes (26, 27), and all-cause mortality (28, 29). In addition to these epidemiologic findings, experimental evidence shows that markers of chronic inflammation, such as pro-inflammatory cytokines IL-6 and TNF-α and the acute-phase reactant CRP, play a direct role in the etiology of atherosclerosis and insulin resistance (30, 31). Diet-induced weight loss decreases concentrations of CRP, IL-6, and TNF-α (8, 32-37). It is also relevant that caloric restriction in the obese and a fast in normal subjects result in a reduction

in oxidative stress and inflammatory mediators (5, 8, 38) In this regard, it is interesting to note that TNF-α and IL-1α have been shown to reduce hypothalamic GnRH and LH secretion in animals and *in vitro*.(39, 40) It is therefore possible that inflammatory cytokines may partly mediate hypogonadotrophic hypogonadism (HH) of obesity and type 2 diabetes.

Measuring Inflammation and Oxidative Stress

Inflammation at the cellular level can be described as an increase in the pro-inflammatory transcription factor, NFκB, in the nucleus and with a concomitant decrease in its inhibitors IκBα and/or IκBβ. NFκB is a heterodimer, and usually consists of two proteins, a p65 (RelA) subunit and a p50 subunit. In the basal state NFκB is bound to its inhibitor protein IκB, which restricts NFκB to the cytoplasm. Stimulation of cells by cytokines like TNFα or IL-1 or endotoxin results in phosphorylation of IκB, unbinding of NFκB from IκB and activation of NFκB with its subsequent translocation into the nucleus (41, 42). This in turn induces the transcription of pro-inflammatory cytokines (such as TNFα, IL-1 and IL-6), adhesion molecules (ICAM, VCAM), chemokines (such as MCP-1 and MIF), adhesion molecules, metalloproteinases (MMP-1, and MMP-9) and many other genes that regulate transcription, apoptosis and cell proliferation.

Oxidative stress can be defined as an increase in ROS generation. The term ROS is a collective term that includes not only oxygen-centered radicals such as superoxide ($O^{\cdot}{}_2^{-}$) and hydroxyl (OH), but also some non-radical derivative of oxygen such as hydrogen peroxide (H_2O_2), singlet oxygen and hypochlorous acid (HOCL). A critical balance exists between generation and detoxification of ROS in respiring cells of normal individuals. The various risk factors for atherosclerosis, including hyperlipidemia, hypertension, and diabetes, have in common the generation of oxidative stress. ROS are able to break off cell membrane proteins, fuse membrane lipid and proteins, hardening the cell membrane and exposing genetic material in the nucleus, leaving the DNA open for mutation or destruction. They may damage the endothelium of blood vessels, thereby leading to atherosclerosis. ROS-mediated lipid peroxidation is a cardinal step in the initiation of atherosclerosis (43, 44). NFκB is one of the transcription factors that may be controlled by the redox status of the cell (45). NADPH oxidase complex, composed of cytoplasmic ($p47^{phox}$ $p67^{phox}$, small GTP-binding p21rac) and plasma membrane components (heterodimer of gp91 & $p22^{phox}$) is a key enzyme involved in the production of ROS.(46)

Lipid Peroxidation and Protein Oxidation: Lipid peroxidation is an autocatalytic free radical-mediated chemical mechanism in which polyunsaturated fatty acids (PUFA) undergo oxidation to form lipid hydroperoxide (LHP) (47, 48). Levels of 15-isoprostane F2 in urine are useful for the non-invasive assessment of oxidant stress *in vivo* (49). There is evidence that oxidative modification of proteins may be physiologically important, serving as a "marking" step for initiating protein degradation. Oxidation of proteins by microsomal oxidases and metal-catalyzed oxidation treatment is known to produce carbonyl modifications of amino acids (50). Phe reacts with the hydroxyl radical (OH) to form the hydroxylated products ortho-, meta-, and para-tyrosine (*o*-, *m*-, and *p*-tyr). The aromatic hydroxylation of Phe can be used to detect OH formation and is considered as a marker of protein oxidation (51).

Incretins and Inflammation

Use of exenatide in type 2 diabetes patients on insulin has shown very promising and exciting results. We studied the effect of exenatide in an observational study in our clinic patients (52). We found that in addition to improvement in glycemic control over 12 weeks, levels of CRP fell by 30% in patients. A reduction of 25-30% in fast acting insulin requirements were also observed in patients along with a significant weight loss. This study, for the first time, showed the potential anti-inflammatory action of synthetic incretins. We then did a randomized prospective placebo controlled blinded trial in patients with

type 2 diabetes(53). Type 2 diabetic patients on insulin received exenatide or placebo for 12 weeks. There was a significant reduction in reactive oxygen species generation and nuclear factor-κB binding by 22 and 26%, respectively after use of exenatide. The mRNA expression of TNFα, IL-1β, JNK-1, TLR-2, TLR-4, and SOCS-3 in mononuclear cells also decreased by 16-31%. We also showed that a single injection of exenatide has an anti-inflammatory effect.

DPP-IV and inflammation

Recent advances in the understanding of DPP IV have provided new insights into the link between inflammation and DPP IV. It has been found to have a role in activation and function of T cells. DPP IV serves as a target of efficient enzymatic inhibitors which induce autocrine production of TGF- β1 and subsequent suppression of T cell proliferation and cytokine release (54). In vivo, targeting DPP IV has provided a potent therapeutic approach for the treatment of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. It has been suggested that DPP IV inhibition of pathogenic T cells represents a novel and efficient therapy for autoimmune disease of the central nervous system by a mechanism that involves an active TGF-β1 mediated anti-inflammatory effect at the site of pathology.

We investigated the anti-inflammatory effects of sitagliptin in insulin naïve patients with type 2 diabetes. Twenty-two patients with type 2 diabetes were randomized to receive either 100 mg daily of sitagliptin or placebo for 12wk (55). Fasting glucagon-like peptide-1 concentrations increased significantly, whereas the mRNA expression in mononuclear cell of CD26, the proinflammatory cytokine, TNFα, the receptor for endotoxin, Toll-like receptor (TLR)-4, TLR-2, and proinflammatory kinases, c-Jun N-terminal kinase-1 and inhibitory-κB kinase (IKKβ), and that of the chemokine receptor CCR-2 fell significantly after 12 wk of sitagliptin. TLR-2, IKKβ, CCR-2, and CD26 expression and nuclear factor-κB binding also fell after a single dose of sitagliptin. There was a fall in protein expression of c-Jun N-terminal kinase-1, IKKβ, and TLR-4 and in plasma concentrations of C-reactive protein, IL-6, and free fatty acids after 12 wk of sitagliptin. These effects are consistent with a potent and rapid anti-inflammatory effect of sitagliptin and may potentially contribute to the inhibition of atherosclerosis. The suppression of CD26 expression suggests that sitagliptin may inhibit the synthesis of DPP-IV in addition to inhibiting its action. This contributes to lower GLP-1 concentrations in plasma after such meals. Therefore, it would be extremely interesting to determine what effect linagliptin has on GLP-1, GIP, and c-peptide concentrations.

Insulin Sensitization by Linagliptin

As mentioned above, sitagliptin exerts a potent and rapid anti-inflammatory effect as reflected in the suppression of ROS generation and intranuclear NFκB binding(55). In association with this action, we also observed the suppression of the expression of SOCS-3, a protein whose expression is induced by the pro-inflammatory cytokines, TNFα, IL-1β and IL-6 and which interferes with insulin signal transduction. This interference occurs at the IRS-1 level. The expression of SOCS-3 has been shown to be increased in the obese and is related to BMI and HOMA-IR, NFκB activity and TNFα expression(56). Furthermore, SOCS-3 is also induced by a high fat high carbohydrate meal in parallel with the increased expression of TNFα and IL-1β (57). More recently, it has been shown that weight loss following gastric bypass surgery is associated with a reduction not only in inflammatory indices but also that of SOCS-3 expression (58). Protein phosphotyrosine phosphatase-1B (PTP-1B) is another key protein which dephosphorylates proteins and interferes with insulin and leptin signaling and is also induced by the HFHC meal (59, 60). Indeed, a recent study has shown that Sitagliptin treatment for 6 weeks in patients with type 2 diabetes increases insulin sensitivity by 10% (measured as oral glucose insulin sensitivity during a meal test) (61). The same study also showed that Sitagliptin decreases endogenous glucose production in fasting as well

as post-prandial state by ~5%.While the effect of Sitagliptin on endogenous glucose output is probably caused by glucagon suppression, the mechanism underlying its insulin sensitizing effect is not known.

On the basis of the above, we suggest that sitagliptin and other DPP-IV inhibitors like linagliptin could potentially increase insulin signal transduction and increase insulin sensitivity by suppressing inflammatory mediators of insulin resistance including SOCS-3 and PTP-1B.

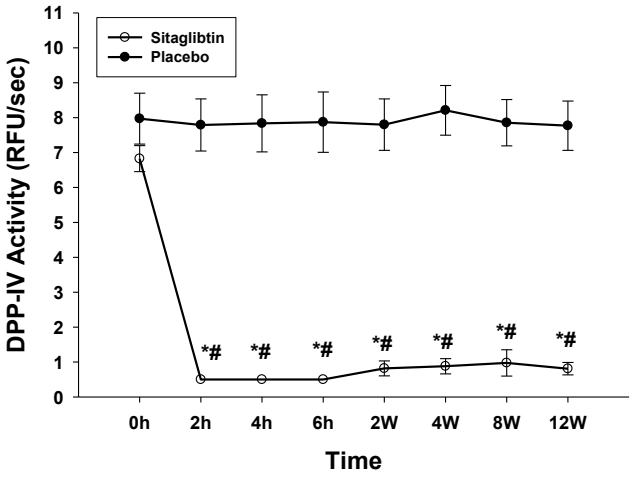
Therefore, in a study directed to investigate the potential anti-inflammatory effect of linagliptin, we would like to also test whether linagliptin exerts an insulin sensitizing effect. The methodology used will be based on the measurement of insulin sensitivity by hyperinsulinemic euglycemic clamps before and after the institution of linagliptin treatment in patients with type 2 diabetes.

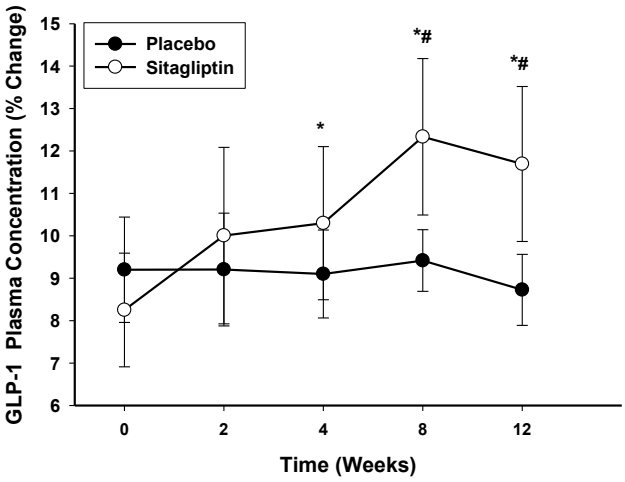
Rationale for study

Linaglitpin has been shown to result in better glycemic control in type II diabetes patients. As described above, obesity and diabetes are states of increased inflammation and are also major risk factors for atherosclerotic disease. In view of the data suggesting that DPP-IV is a pro-inflammatory mediator and that its inhibition may suppress autoimmune inflammatory mechanisms, it is likely that linagiptin may exert an anti-inflammatory effect in the human. The increase in GLP-1 concentrations may also exert an anti-inflammatory effect similar to that observed with exenatide. Indeed, our investigations on the anti-inflammatory effects of sitagliptin, the first DPP-IV inhibitor to be licensed, have demonstrated profound evidence to this effect. Our findings are summarized under preliminary data below.

Preliminary data:

In a study on obese type 2 diabetics, the administration of sitagliptin 100 mg daily for 12 weeks resulted in several novel observations. There was an inhibition of DPP-IV enzyme by 90% within 2 hours of the first dose. In addition, there was a suppression of CD26 expression. CD26 is a pro-inflammatory mediator expressed on the cell membranes and is the cellular analog of the DPP-IV enzyme. Thus, sitagliptin and possibly other DPP-IV inhibitors may also inhibit the expression and synthesis of DPP-IV. In addition, there was a reduction in plasma concentrations of FFA, CRP and IL-6; a reduction in the expression of CCR-2, IKKβ, JNK-1, TNFα (see figures below).





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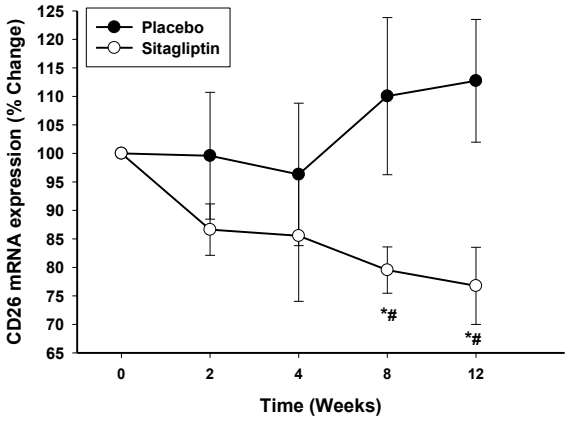
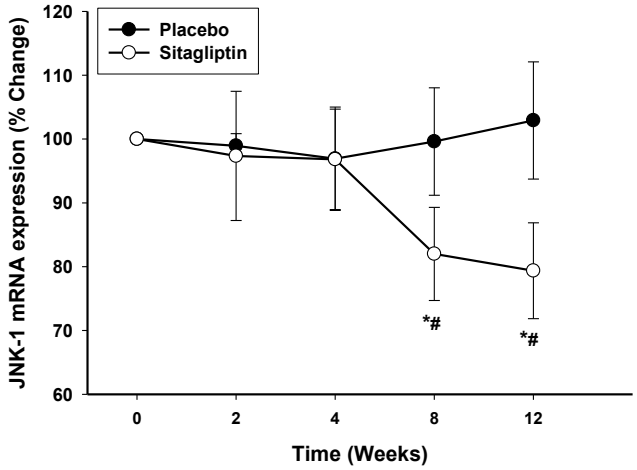
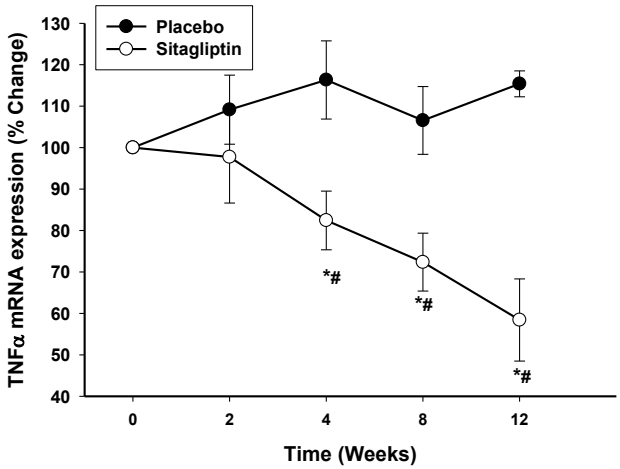
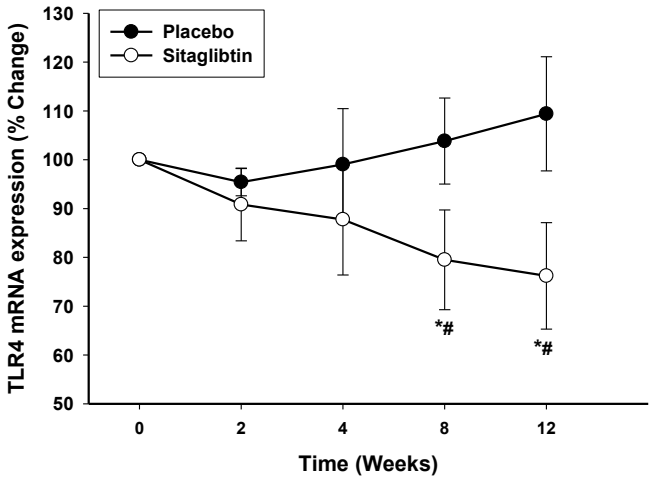
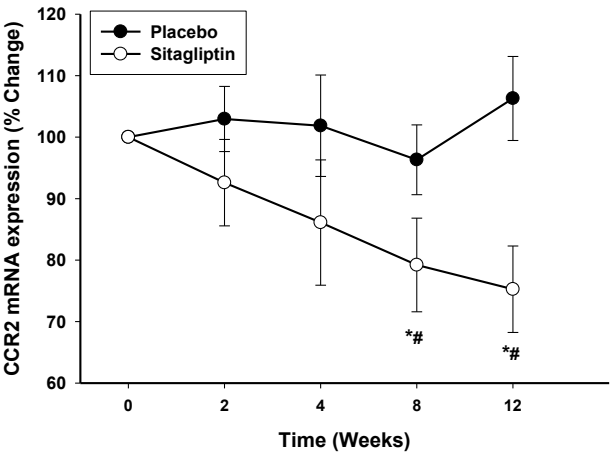


Figure 1: Change in DPP-IV activity (A), GLP-1 Concentrations (B) and CD26 mRNA in MNC (C) following placebo and Sitagliptin 100mg daily for 12 weeks in type 2 diabetic subjects. N=12 each, data are presented as mean±SE. (*, **:P<0.05 by RMANOVA (compared to baseline) in sitagliptin and placebo groups, respectively, #:P<0.05 by Two-way RMANOVA compared to control groups.









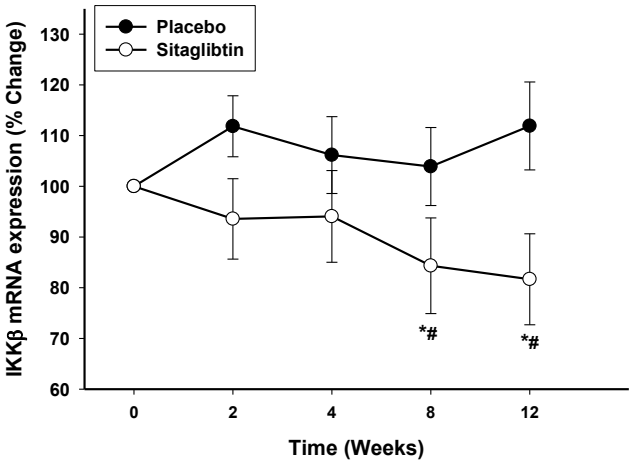
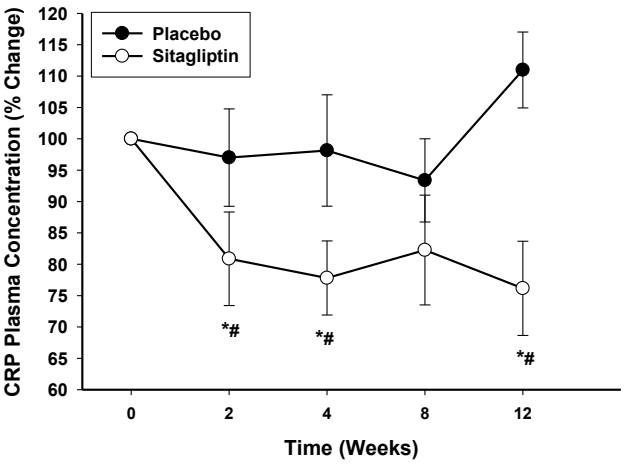
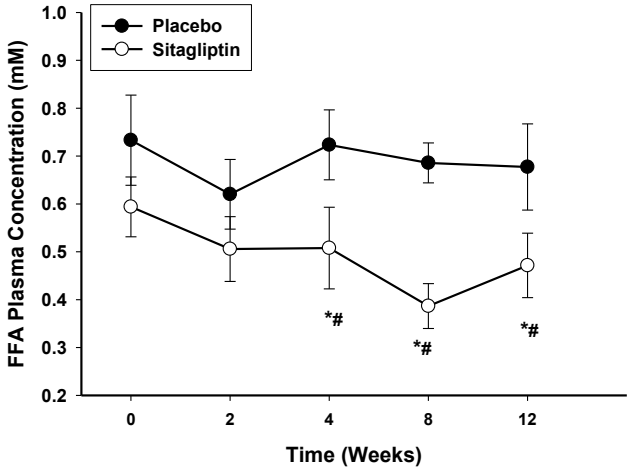


Figure 2: Percent Change in mRNA expression of JNK-1(A), TNF-α, (B) TLR-4 (C), CCR-2 (D) and IKK-β (E) in MNC following placebo and Sitagliptin 100mg daily for 12 weeks in type 2 diabetic subjects. N=12 each, data are presented as mean±SE. (*, **:*P*<0.05 by RMANOVA (compared to baseline) in sitagliptin and placebo groups, respectively, #:*P*<0.05 by Two-way RMANOVA compared to control groups.





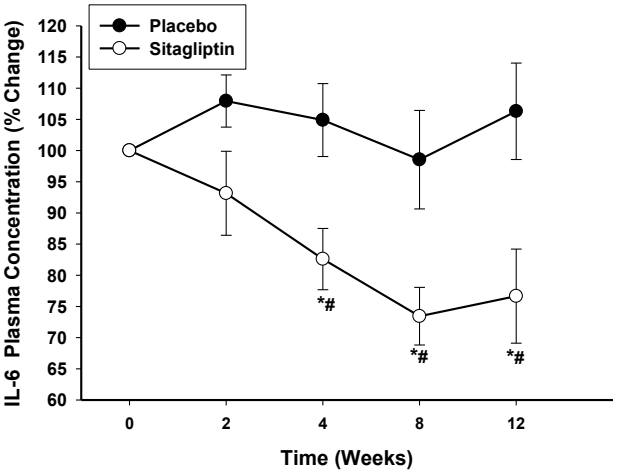


Figure 3: Change in CRP (A), FFA (B) and IL-6 (C) concentrations following placebo and Sitagliptin 100mg daily for 12 weeks in type 2 diabetic subjects. N=12 each, data are presented as mean±SE. (*, **:*P*<0.05 by RMANOVA (compared to baseline) in sitagliptin and placebo groups, respectively, #:*P*<0.05 by Two-way RMANOVA compared to control groups.

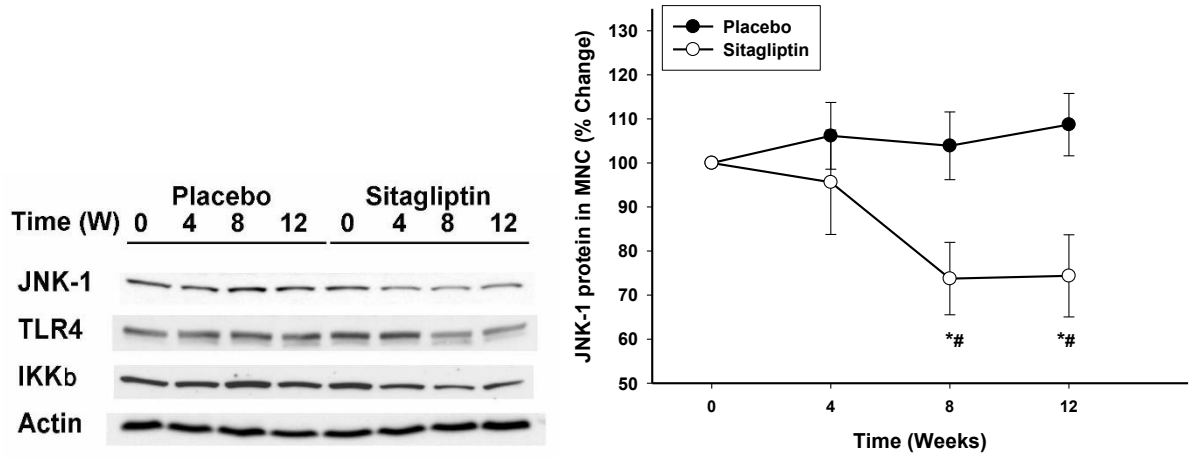


Figure 4: Representative Western blot and percent change in protein levels of JNK-1 after 12 wk of placebo and sitagliptin (100 mg/d) treatment in MNC of obese type 2 diabetic subjects (n _ 10 and 12, respectively). Data are presented as mean ±SE. *, P <0.05 by RMANOVA (compared with baseline); #, P <0.05 by two-way RMANOVA compared with placebo groups.

Study Hypothesis and Aims

Hypothesis 1: Linagliptin treatment suppresses basal and meal induced oxidative and inflammatory stress in mononuclear cells (MNC) and adipose tissue of obese patients with type 2 diabetes.
Aim 1.1: To compare reactive oxygen species (ROS) generation by MNC, protein expression of $p47^{phox}$ subunit of NADPH oxidase, in MNC's and adipose tissue prior to and following 12 weeks of linagliptin or placebo.
Aim 1.2: To compare intranuclear NFκB binding and the expression of JNK-1, TLR-4, TLR-2, CD26 and TNFα in mononuclear cells (MNC) and adipose tissue before and following 12 weeks of treatment with linagliptin or placebo.
Aim 1.3: To compare plasma hs-CRP and IL-6 concentrations and oxidized lipids in plasma (TBARS) and in urine (F_2-isoprostane) of obese type 2 diabetic patients prior to and following 12 weeks of linagliptin or placebo.
Aim 1.4: To compare post prandial (after a HFHC meal) changes in ROS generation, NFκB binding and the expression of JNK-1, TLR4, TLR-2 IL-1β and TNFα in MNC and adipose tissue prior to and following 12 weeks of linagliptin and placebo.

Hypothesis 2: Linagliptin **acutely** suppresses oxidative and inflammatory stress in mononuclear cells (MNC) of obese patients with type 2 diabetes.
Aim 2.1: to study the effect of single dose of linagliptin or placebo on oxidative stress and inflammatory mediators listed in Aim 1.1, 1.2 and 1.3 in MNC from obese type 2 diabetic patients.

Hypothesis 3: Linagliptin is an insulin sensitizer
Aim 3.1: To compare the glucose uptake induced by hyperinsulinemic-euglycemic (HE) clamp prior to and following 12 weeks of linagliptin or placebo.
Aim 3.2: To compare the endogenous glucose output and suppression of endogenous glucose production by insulin during HE clamp prior to and following 12 weeks of linagliptin or placebo.

Aim 3.3: To compare mRNA and protein expression of SOCS-3 and PTP-1B, two important insulin resistance inducers, in MNC and adipose tissue prior to and following 12 weeks of linagliptin or placebo.

Hypothesis 4: Linagliptin increases post-prandial GLP-1, GIP and c-peptide concentrations.
Aim 4.1: To measure GLP-1, GIP and c-peptide concentrations following HFHC meal prior to and following 12 weeks of linagliptin or placebo.

RESEARCH DESIGN AND METHODS

RESEARCH DESIGN:

This is a prospective, **randomized, placebo -controlled, double blind** trial. The study will be conducted at the Diabetes – Endocrinology Center of Western New York under the direction of Dr. Paresh Dandona, M.D.

STUDY POPULATION:

Forty type 2 diabetic patients in the age group of 20 – 80 years will be recruited into the study. An informed consent will be obtained. They will be randomized into 2 groups: placebo and 5 mg/day linagliptin for 12 weeks

INCLUSION CRITERIA:

- Age 20-80 years inclusive.
- Type 2 diabetes
- BMI $\geq$30 kg/m^2
- Subjects on statins, ACE inhibitors, thiazolidenediones and antioxidants will be allowed as long as they are on stable doses of these compounds and the dosage in not changed during the course of study.
- HbA1c $\leq$ 7.5%

EXCLUSION CRITERIA:

- Use of GLP-1 agonists or DPP-IV therapy in the last 3 months.
- History of Pancreatitis.
- Risk for pancreatitis, i.e., history of gallstones, alcohol abuse, and hypertriglyceridemia.
- Subjects who are taking antiplatelet or anticoagulant medications will be excluded from the fat biopsy portion of the study.
- Coronary event or procedure (myocardial infarction, unstable angina, coronary artery bypass, surgery or coronary angioplasty) in the previous four weeks
- Hepatic disease (liver function tests more than 3 times the upper limit of normal)
- Renal impairment (serum eGFR <30 ml/min)
- Any other life-threatening, non-cardiac disease
- Uncontrolled hypertension (BP > 160/100 mm of Hg)
- Congestive Heart Failure class III or IV.
- Use of an investigational agent or therapeutic regimen within 30 days of study

- Participation in any other concurrent clinical trial

STUDY DESIGN:

This study requires 8 visits to the clinic: a screening visit (week -1), a baseline visits (weeks 0, 1 and 2) and then follow up at 2, 4 and 12 weeks after starting the drug. Each visit will consist of blood draw. Subjects will be required to bring in a 24 hour urine sample on weeks 0, 4 and 12.
After the screening visit, subjects will be randomized into 2 groups. The first group will be started on 5 mg linagliptin (supplied by Boehringer Ingelheim Pharmaceuticals, Inc. (BIPI)) daily for 12 weeks. Fructosamine testing will be done at baseline, 4 and 12 weeks (and HbA1c at 0 and 12 weeks) to assess glycemic control. The second group will serve as a control group and will be placed on a placebo (mainly mannitol and corn starch, provided by BIPI in similar shape and color as the active drug) for 12 weeks. Blood samples will be obtained as described for patients on linagliptin. Each blood draw will consist of 45 ml of which 35 ml is collected in EDTA tubes and 10 ml in no-additive tubes. A single dose effect will be investigated at the first day of drug or placebo in 24 subjects (12 from each group) only and blood will be collected at 2, 4 and 6 hr following the first dose. The effect of a standard high-fat high- carbohydrate meal (HFHC) will be investigated on those 12 subjects from each group at baseline and at 12 weeks.

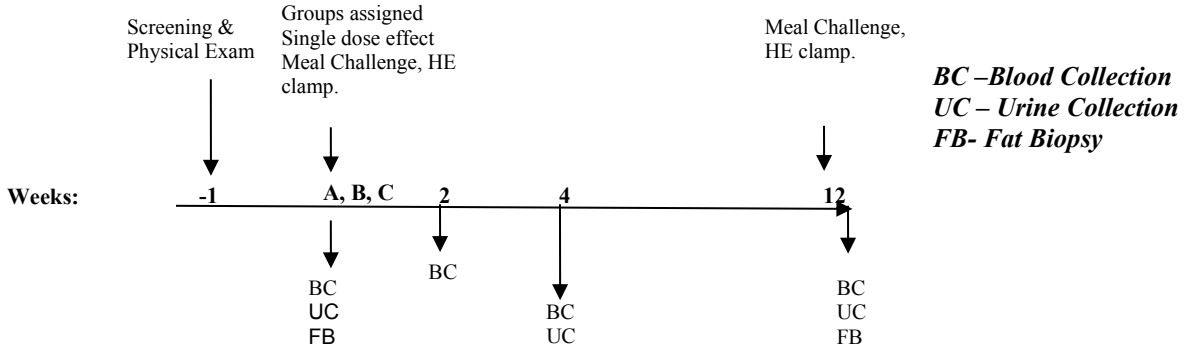


Figure 1: Study Design.

STUDY PROCEDURES BY VISIT:

Screening Day

Each subject will complete the following procedures and meet the inclusion and exclusion criteria prior to participating in the study.

1. Medical History
2. Physical Exam
3. Informed consent
4. Baseline lab draws to measure CBC, SMA, lipid profile, fructosamine and HbA1C

Visit 1: Week -2 to Week 0

Visit 1A: The participants will undergo HE clamp. They will be advised to bring 24 hour urine at this visit and research blood will be collected. All patients will have visit A. Visits B and C will be done only in 24 patients (12 in drug group and 12 in placebo group).

Visit 1B: The effect of a standard high-fat high- carbohydrate meal (HFHC) will be investigated in 24 subjects. Fat biopsy will be done before and after the meal.

Visit 1C (in 24 subjects): A single dose (5 mg or placebo) effect will be studied in 24 subjects with blood collections at 2, 4 and 6 hr following the first dose.

Visits A, B and C will be separated by one week each. All patients will be randomized to drug or placebo groups on visit 1A but only patients who are not participating in visit 1B and 1C will start the drug or placebo at the end of visit 1A (considered week 0). Patients who are participating in visits 1B and 1C will start the drug or placebo at the beginning of visit 1C (considered week 0). . Patient will return 2 weeks after starting drug or placebo. The drug will be started after these visits have been completed. Patient will return after 2 weeks.

Visit 2: Week 2
The participants will have their finger stick blood glucose records reviewed and blood will be drawn for labs. A container for 24 hour urine collection will be given. They will be advised to start collecting urine 24 hours prior to their next visit.

Visit 3: Week 4
The participants will have their finger stick blood glucose records reviewed and blood will be drawn for labs. 24 hour urine will be collected. They will be advised to start collecting urine 24 hours prior to their next visit.

Visit 4: Week 12

Visit 4A: The participants will have their finger stick blood glucose records reviewed. They will be advised to bring 24 hour urine at this visit and research blood will be collected. The participants will undergo HE clamp. All patients will have visit 4A.

Visit 4B: The effect of the HFHC meal will be investigated in 24 subjects who participated in visit 1B. Fat biopsy will be done before and after the meal.

Visits 4A and 4B will be separated by 2-5 days.

Compliance
The study coordinator will maintain a log of the entire study drug dispensed and returned.
Storage conditions: stored at room temperature in a locked cabinet.

Drug accountability: A master drug accountability log will be completed on arrival of supplies, Patient specific drug tracking logs will be completed including the following information: Date and amount of drug dispensed, initials of person dispensing study drug, date and amount of drug returned initials of

person receiving returned study drug.

Meal Challenge description

Subjects will arrive after having fasted overnight (10 hr) around 8-8:30AM. Subcutaneous fat biopsy will be done. The subjects will eat a meal that consists of 900 kcal of HFHC breakfast (egg muffin and sausage muffin sandwiches and two hash browns which contain 81g carbohydrates, 51g fat and 32 g protein). Meal must be consumed within 15 minutes. Blood samples will be obtained at baseline (before the meal) and at the following time points after the meal is consumed: 15min, 30min, 45min, 1hr, 75min, 90min, 105min, 2hr, 3hr, and 5 hr. Samples will measure fasting and post meal glucose, insulin, glucagon, GLP-1, GIP, c-peptide in plasma, and indices of inflammatory stress, specifically intra-nuclear NFκB, expression of TNFα, and JNK-1 in MNC. Blood will be collected from an intravenous cannula in a superficial forearm vein. Samples at 15, 30, 45, 75, 90, 105, and 120 min will each be 5mL. Samples at baseline (0h) and 1hr, 3hr and 5hr are 35mL each. The total amount of blood drawn during this meal challenge is 175mL. Fat biopsy will be repeated after 5 hours. Samples for GLP-1, GIP and c-peptide measurement will be collected in DPP-IV inhibitor and protease inhibitors containing tubes.

LABORATORY PROCEDURES

1. **MNC isolation:** Blood samples (30-40ml) is collected in Na-EDTA and carefully layered on Lympholyte medium (Cedarlane Laboratories, Hornby, ON) according to manufacturer's instructions. Samples are centrifuged and two bands separate out at the top of the RBC pellet. The MNC band was harvested and washed twice with Hank's balanced salt solution (HBSS). This method provides yields greater than 95% MNC preparation.
2. **ROS generation measurement by chemiluminescence:** Five hundred μL of MNC (2×10^5 cells) are delivered into a Chronolog LumiAggregometer cuvette. Luminol is then added, followed by 1.0 μL of 10 mM formylmethionyl leucinyl phenylalanine (fMLP). Chemiluminescence is recorded for 15 minutes. Our method, developed independently(62, 63), is similar to that published by Tosi and Hamedani (64). In this assay system, the release of superoxide radical as measured by chemiluminescence, has been shown to be linearly correlated with that measured by the ferricytochrome C method. The interassay coefficient of variation of this assay is 8 %. We have further established that the biological variation in reactive oxygen species generation in normal subjects is approximately 6 % for readings obtained 1 to 2 weeks apart. Similarly, the variation in reactive oxygen species generation in the obese over a period of 1 to 2 weeks is less than 8 %.
3. **Quantification of IKKβ, JNK-1, TNFα, IL-6, IL-1β, TLR4, TLR2, SOCS-3, PTP-1B, CCR-2 and CD-26 Expression:** The mRNA expression is measured in MNC and adipose tissue by RT-PCR: Total RNA was isolated using commercially available RNAqueous®-4PCR Kit (Ambion, Austin, TX). One μg of total RNA is reverse transcribed using Advantage RT-for-PCR Kit (Clontech, CA). Real Time RT-PCR was performed using Stratagene Mx3000P QPCR System (La Jolla, CA), Sybergreen master mix (Qiagen, CA) and gene specific primers (Life Technologies, MD).The specificity and the size of the PCR products are tested by adding a melt curve at the end of the amplifications and by running it on a 2% agarose gel. All values were normalized to the expression of a group of housekeeping genes including actin, ubiquitin C and cyclophilin A.
4. **Western blotting:** MNC and adipose tissue total cell lysates are prepared by suspending the cells in 150 μL of lysis buffer (10 mM Tris-HCl, 150mM NaCl, 0.2 % Triton X and proteases and phospatases

inhibitors). After 30 min incubation on ice, samples are centrifuged at 12000 Xg for 10 min, supernatants are collected and total protein concentrations are determined. Sixty μg of total cell lysate are boiled in 2X SDS buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.06 % bromophenol blue and 200 mM DTT), proteins separated by SDS-PAGE and then transferred to PVDF membrane. Polyclonal or monoclonal antibodies against $p47^{phox}$, JNK-1, IKK-β, SOCS-3, PTP-1B and TLR-4, (Abcam, Cambridge, MA) and actin (Santa Cruz Biotechnology, CA) will be used and the membranes are developed using super signal, chemiluminescence reagent (Pierce Chemical, IL). Densitometry is performed using molecular analyst software (Biorad, CA) and all values are corrected for loading with actin.

5. **NFκB DNA binding:** Nuclear NFkB DNA binding activity is measured by electrophoretic mobility shift assay (EMSA). Nuclear extract is prepared from MNC and fat tissue by high salt extraction as described by Andrews et al. Protein concentrations are determined using BCA protein assay (Pierce, IL). NFkB gel retardation assay is performed using double-stranded oligonucleotide consensus sequence for the NFkB-binding site (Santa Cruz Biotechnology, CA) radiolabeled with γ-P^{32} by T4 kinase. Seven and a half μg of the nuclear extract are mixed with the incubation buffer (10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 1mM EDTA, 5% glycerol) containing 0.5μg poly dI-dC and 0.2 mg/mL salmon sperm DNA to a final volume of 20 mL and the mixture is preincubated on ice for 15 min. Labeled oligonucleotides (60,000 cpm) are added, and the mixture is incubated at room temperature for 20 min. Samples are then applied to wells of 6% nondenaturing polyacrylamide gel. The gel is dried under vacuum and exposed to x-ray film. Densitometry is performed using Bio-Rad molecular analyst software (Hercules, CA). The specificity of the bands is confirmed by supershifting these bands with specific antibodies against Rel-A (p65) and p50 (Santa Cruz Biotechnology, CA) and by competition with cold oligonucleotides. Alternativley, binding to DNA can be performed using TransAM NFκB (p65) kit (Active motive, Carlsbad, CA) on NE.
6. **Plasma glucose, insulin and FFAs measurements:** Insulin levels are determined using an ELISA kit from Diagnostic Systems Laboratories Inc. (Webster, TX). Glucose levels are measured in plasma by YSI 2300 STAT Plus glucose analyzer (Yellow Springs, Ohio). FFAs levels are measured by a colorimetric assay (Roche, Richmond, VA) Active GLP-1 will be measured using ELISA kit (Millipore, MA) from samples collected in DPP-IV inhibitor (Millipore, MA) to prevent GLP-1 degradation. GLP-1 assay sensitivity is 1.5pM.
7. **15-isoprostane F_{2t}** (also known as 8-epi-PGF2α or 8-iso-PGF2α) in urine samples and TBARS will be measured by an ELISA kit from Oxford Biomedical Research (Oxford, MI).
8. **Plasma concentrations of inflammatory cytokines:** Concentrations of IL-6 and TNFα are measured in plasma using sensitive and high sensitive ELISA kits (R&D Systems, MN). ELISA will be used to measure C-reactive protein (American Diagnostica Inc., CT) and DPPIV activity (reported as relative fluorescence units/sec (RFU/sec).
9. **Measurement of whole body glucose uptake and endogenous glucose production by HE clamp:** Subjects will come to the clinical research center after an overnight 10 hour fast. The patient's blood sugar before the clamp is started should be ≤10mmol/L. Patients with blood sugar >10mmol/l will be re-scheduled. Oral hypoglycemic medications will be held on the morning of HE clamp. Long acting insulin will be held the night before the clamp. HE clamp will be done using radioactive glucose (3-^{3}H-glucose) as previously described (65, 66). Subjects will arrive at 8 AM for HE clamp with a prime-continuous infusion of 3-^{3}H-glucose. The 3-^{3}H glucose prime will be 20 μCi x (fasting plasma glucose/100); the continuous infusion will be given at the rate of 0.20 μCi/min for 420 minutes (180 minutes for isotopic equilibration and 240 minutes during the insulin clamp). Insulin clamp will be started at 180 min with a priming dose of short acting human insulin given over 10 minutes and then

an infusion at the rate of 80mU/m^2. 20% glucose infused through the cephalic vein will be started at 5 minutes and glucose infusion rate will be titrated to maintain blood glucose concentration at the fasting glucose level in the obese and at 5.5mmol/l in T2D. Plasma samples for titrated glucose will be obtained at 10 minute intervals starting 30 minutes before the clamp, to quantitate rates of whole body glucose disposal and suppression of endogenous (primarily hepatic) glucose production. Blood sample will be collected for insulin and free fatty acids (FFA) concentrations every 30 minutes during the clamp. Calculations will be done using Steele's equation(67).

Calculations: Under steady-state postabsorptive conditions, the rate of endogenous glucose appearance (Ra) will be calculated as the 3[^{3}H] glucose infusion rate (disintegrations per minute per minute) divided by the steady-state plasma 3[^{3}H] glucose-specific activity (disintegrations per minute per milligram). During the euglycemic insulin clamp, the rate of Ra will be calculated using Steele's equation, using a distribution volume of 250 ml/kg. Endogenous (primarily hepatic) glucose production (EGP) will be calculated by subtracting the mean exogenous glucose infusion rate during the last 60 minutes (after steady state has been achieved) from Ra. The rate of insulin-mediated total body glucose disposal (Rd) will be determined by adding the rate of residual EGP to the exogenous glucose infusion rate. Insulin sensitivity will be expressed as Rd/lean body mass.

10. **Fat aspiration procedure:** Subjects who are taking antiplatelet or anticoagulant medications will be excluded from the fat biopsy portion of the study. Subcutaneous fat tissue aspiration will be performed on abdomen at a 10 cm distance from umbilicus. The skin will first be prepared and a sterile drape will be placed around the appropriate area. Under local anesthesia, 500mg-3g of fat tissue will be obtained. The adipose tissue will be centrifuged to remove blood and fluid contaminants. The upper adipose tissue will be collected into a separate sterile tube, washed twice with cold sterile Phosphate Buffered Saline (PBS) and centrifuged to remove the PBS. Total RNA, nuclear extracts and total cell lysates will be prepared from the adipose tissue.

DATA ANALYSIS

This is a prospective study to evaluate oxidative stress before and after treatment with linagliptin. The similarities between the study groups, baseline values for subject's demographics will be compared using appropriate parametric tests. Transformations of the data on order to meet statistical assumptions may be considered. All statistical analysis will be carried out using SPSS software (SPSS Inc, Chicago, Illinois). Comparison of each endpoint will be made using an analysis of covariance model, with baseline medications, change in BMI and change in HbA1c as covariates. The primary endpoint of the study is to detect a change in JNK-1 protein in MNC before and after linagliptin use as compared to placebo. We have chosen this as the primary endpoint because JNK-1 is an inflammatory mediator that also interferes with insulin signaling. Our prior data with sitagliptin show a 24% fall (with SD of 24%) in JNK-1 following 12 weeks of treatment in type 2 diabetics(55). There was a 6% non-significant increase in JNK-1 protein in the placebo group in that study. Conservatively estimating a difference in change in JNK-1 cellular concentrations of 24% among the two groups, a sample size of 20 patients per group (assuming a drop-out rate of 15%) should provide adequate power ($\beta = 0.2$) to detect a significant difference ($\alpha = 0.05$), provided the standard deviation of the residuals is not equal to or greater than the mean difference (see table below).

Sample Size Estimate

Difference in the mean % change from baseline	5%	10%	15%	20%
10%	5	17	36	62
15%	3	8	17	28
20%	3	5	10	**17**

Justification of drop-out rate: The analysis will adjust for only one baseline covariate: baseline medications. Two other covariates are change in BMI (which is not expected with linagliptin or placebo) and change in HbA1c. Sulfonylureas, metformin and subcutaneous insulin are not likely to induce insulin sensitization or decrease inflammation. Subjects currently on GLP-1 agonists or DPP-IV inhibitors will be excluded from the study. Thiazolidinediones can induce insulin sensitization as well as decrease inflammation. The use of thiazolidinediones should be equally distributed among the two groups. Less than 20% of our research subjects ore on thiazolidinediones. Therefore it is likely that adjustment for thiazolidinediones should decrease sample size by <5%. Based on this justification and on our previous experience in diabetes trials, we have estimated that our drop-out rate will be 15% of 20 patients (3 patients per group).

The secondary endpoints for the study will be comparison of the relative change from baseline in a) inflammatory mediators in serum and adipose tissue, b) single dose effect of linagliptin on inflammation and oxidative stress, c) HFHC induced inflammation in serum and adipose tissue and d) insulin sensitivity and hepatic glucose output; before and after using Linagliptin for 12 weeks.

FACILITIES

LABORATORY: The Diabetes-Endocrinology Center of Western New York Research Laboratories at SUNY at Buffalo, contain 6 laboratories with space totaling approximately 1600 Qs ft designated for research purposes. These labs are dedicated for the P.I. research. There is a cold room, dark room and four labs have fume hoods. In addition, six offices (600 Qs ft) are attached to the labs for lab staff.

CLINICAL: The clinical activities of Diabetes/Endocrinology center of Western New York, founded by the P.I. 16 years ago, have grown to dominate the diabetes-metabolism scene in upstate New York. Approximately 2,000 new patients referral are seen every year while approximately 3,000 patients with diabetes are under our long term care. Over 500 patients have been started on insulin pumps and currently 4 patients are started on insulin pumps every month. Four satellite centers have been started covering various areas in our region where our team provides care fulfilling an important need. The unit employs 35 people including 6 attending physicians, RNs, CDEs, RDs and research staff. The center is the hub of the endocrinology fellowship program at the university (3 fellows/year). The P.I. is the director of this program. There are nine examination rooms in the center and the clinical unit is provided with 3 CRC beds which are utilized for prolonged intensive investigative protocols. The center is the main base for the training of the university fellowship program in endocrinology; P.I. is the head of the division of endocrinology at SUNY Buffalo.

MAJOR EQUIPMENT: Real time PCR Cepheid Smart Cycler, A Shimadzu RF-1501 spectroflurometer; a Shimadzu BioSpec-1601UV spectrophotometer; a Shimadzu LC-10 model HPLC with diode array, autosampler, UV-Vis/fluorescence and electron capture detectors and data processing

module; Savant Speed vacuum; a Sorvall Super T21 high speed refrigerated centrifuge and a CR312 centrifuge, each with a variety of fixed angle and swinging bucket rotors; two Fisher Model Automatic water jacketed CO_2 incubator for cell cultures; Elzon 180 cell counter; a Vibracell VC40 ultrasonic tissue disrupter with microtip probe; three Olympus inverted microscopes, each with a camera attached to it; several sizes and types of gel apparatuses and power supplies for performing 1D and 2D PAGE and separation of RNA/DNA; UVP (Epi-Chem darkroom) gel documentation system that detects chemiluminescenc/fluorescence/UV; Bio-Rad gel dryer apparatus; hybridization oven; Ericomp thermal cycler; UV crosslinker; Tecan Fluostar microplate reader; Packard cyclone phosphoimager; two Chrono-log Lumi-aggregometers; Nichols institute system luminometer; Millipore Ultrapure water purification system; Wallac 1409 beta counter; a variety of water baths, vortex mixers, stirrors, hotplates and sonicators.

Assessment of adverse events

Definitions of adverse events

Adverse event

An adverse event (AE) is defined as any untoward medical occurrence, including an exacerbation of a pre-existing condition, in a patient in a clinical investigation who received a pharmaceutical product. The event does not necessarily have to have a causal relationship with this treatment.

Serious adverse event

A serious adverse event (SAE) is defined as any AE which results in death, is immediately life-threatening, results in persistent or significant disability / incapacity, requires or prolongs patient hospitalisation, is a congenital anomaly / birth defect, or is to be deemed serious for any other reason if it is an important medical event when based upon appropriate medical judgement which may jeopardise the patient and may require medical or surgical intervention to prevent one of the other outcomes listed in the above definitions.

Patients may be hospitalised for administrative or social reasons during the study (e.g. days on which infusion takes place, long distance from home to site,). These and other hospitalisations planned at the beginning of the study do not need to be reported as a SAE in case they have been reported at screening visit in the source data and have been performed as planned.

Adverse Events of Special Interest

Based on regulatory recommendation that is based on the experience with other compounds in the DPP-4 inhibitor class, the following events should be defined as adverse events of special interest.

- Hypersensitivity reactions such as angioedema, angioedema-like events, and anaphylaxis
- Skin lesions such as exfoliative dermatitis, and exfoliative rash
- Renal adverse events (e.g. renal failure, >=2x increase of creatinine from baseline)
- Pancreatitis

Hepatic adverse events such as hepatitis, liver injury, and increased liver enzymes. Hepatic injury is defined by the following alterations of liver parameters: For patients with normal liver function (ALT, AST and bilirubin within normal limits) at baseline an elevation of AST and/or ALT >3 fold ULN combined with an elevation of bilirubin >2 fold ULN measured in the same blood draw sample.

Refer to the appendix 1 for a list of prefered terms (PT's) that are considered as AEs of Special Interest.

Protocol-specified significant events are to be reported in an expedited manner similar to Serious Adverse Events, even if they do not meet any of the seriousness criteria.

Intensity of adverse event

The intensity of the AE should be judged based on the following:

• Mild: Awareness of sign(s) or symptom(s) which is/are easily tolerated

• Moderate: Enough discomfort to cause interference with usual activity

• Severe: Incapacitating or causing inability to work or to perform usual activities

Causal relationship of adverse event

Medical judgment should be used to determine the relationship, considering all relevant factors, including pattern of reaction, temporal relationship, de-challenge or re-challenge, confounding factors such as concomitant medication, concomitant diseases and relevant history. Assessment of causal relationship should be recorded in the case report forms.

Yes: There is a reasonable causal relationship between the investigational product administered and the AE.
No: There is no reasonable causal relationship between the investigational product administered and the AE.

Worsening of the underlying disease or other pre-existing conditions

Worsening of the underlying disease or of other pre-existing conditions will be recorded as an (S)AE in the (e)CRF.

Changes in vital signs, ECG, physical examination, and laboratory test results

Changes in vital signs, ECG, physical examination and laboratory test results will be recorded as an (S)AE in the (e)CRF , if they are judged clinically relevant by the investigator.

Responsibilities for SAE reporting

The Sponsor shall report (i.e., from signing the informed consent onwards through the trialdefined follow-up period) all SAEs and non-serious AEs occurring at the same time and/or which are medically related to the SAE and Adverse Events of Special Interest by fax or other secure method to the BI Unique Entry Point in accordance with timeline specified in the pharmcovigilance agreement

For each adverse event, the investigator will provide the onset date, end date, intensity, treatment required, outcome, seriousness, and action taken with the investigational drug. The investigator will determine the expectedness of the investigational drug to the AEs as defined in the Listed Adverse Events section of the Boehringer Ingelheim's (BI's) Investigator Brochure for the Product or BI Drug Information e.g. Summary of Product Characteristics (SmPC) or Product Information (PI) for the authorised Study Drug provided by BI.

Appendix 1

Following preferred terms are considered as Adverse Event of Special Interest

Hepatic events
Acute graft versus host disease in liver
Acute hepatic failure
Alanine aminotransferase abnormal
Alanine aminotransferase increased
Ammonia abnormal
Ammonia increased
Ascites
Ascites
Aspartate aminotransferase abnormal
Aspartate aminotransferase increased
Asterixis
Autoimmune hepatitis
Bacterascites
Bacterascites
Bile output abnormal
Bile output decreased
Biliary cirrhosis
Biliary cirrhosis primary
Biliary fibrosis
Bilirubin conjugated abnormal
Bilirubin conjugated increased
Bilirubin excretion disorder
Biopsy liver abnormal
Blood bilirubin abnormal
Blood bilirubin increased
Blood bilirubin unconjugated increased
Caput medusae
Child-Pugh-Turcotte score increased
Cholaemia
Cholestasis
Cholestatic liver injury
Cholestatic liver injury
Cholestatic pruritus
Chronic hepatic failure
Chronic hepatitis
Coma hepatic
Cryptogenic cirrhosis
Cytolytic hepatitis
Duodenal varices
Foetor hepaticus
Galactose elimination capacity test abnormal

Galactose elimination capacity test decreased
Gamma-glutamyltransferase abnormal
Gamma-glutamyltransferase increased
Gastric varices
Gastric varices haemorrhage
Guanase increased
Hepaplastin abnormal
Hepaplastin decreased
Hepatectomy
Hepatic artery flow decreased
Hepatic atrophy
Hepatic calcification
Hepatic cirrhosis
Hepatic congestion
Hepatic encephalopathy
Hepatic encephalopathy prophylaxis
Hepatic enzyme abnormal
Hepatic enzyme decreased
Hepatic enzyme increased
Hepatic failure
Hepatic fibrosis
Hepatic function abnormal
Hepatic hydrothorax
Hepatic hydrothorax
Hepatic infiltration eosinophilic
Hepatic lesion
Hepatic mass
Hepatic necrosis
Hepatic pain
Hepatic sequestration
Hepatic steatosis
Hepatic vascular resistance increased
Hepatitis
Hepatitis acute
Hepatitis cholestatic
Hepatitis cholestatic
Hepatitis chronic active
Hepatitis chronic persistent
Hepatitis fulminant
Hepatitis fulminant
Hepatitis toxic
Hepatobiliary disease
Hepatobiliary scan abnormal
Hepatocellular foamy cell syndrome
Hepatocellular injury
Hepatomegaly

Hepatopulmonary syndrome
Hepatorenal failure
Hepatorenal syndrome
Hepatosplenomegaly
Hepatotoxicity
Hyperammonaemia
Hyperbilirubinaemia
Hyperbilirubinaemia
Hypercholia
Hypertransaminasaemia
Icterus index increased
Ischaemic hepatitis
Jaundice
Jaundice cholestatic
Jaundice hepatocellular
Kayser-Fleischer ring
Liver and small intestine transplant
Liver disorder
Liver function test abnormal
Liver induration
Liver injury
Liver operation
Liver palpable subcostal
Liver scan abnormal
Liver tenderness
Liver transplant
Lupoid hepatic cirrhosis
Lupus hepatitis
Mitochondrial aspartate aminotransferase increased
Mixed liver injury
Mixed liver injury
Molar ratio of total branched-chain amino acid to tyrosine
Nodular regenerative hyperplasia
Ocular icterus
Oedema due to hepatic disease
Oedema due to hepatic disease
Oesophageal varices haemorrhage
Perihepatic discomfort
Portal hypertension
Portal hypertensive enteropathy
Portal hypertensive gastropathy
Portal triaditis
Portopulmonary hypertension
Radiation hepatitis
Renal and liver transplant
Retrograde portal vein flow

Retrograde portal vein flow
Reye's syndrome
Subacute hepatic failure
Total bile acids increased
Transaminases abnormal
Transaminases increased
Ultrasound liver abnormal
Urine bilirubin increased
Urobilin urine present
Varices oesophageal
X-ray hepatobiliary abnormal

Following preferred terms are considered as Adverse Event of Special Interest

Hypersensitivity reactions

Allergic bronchitis
Allergic cough
Allergic oedema
Allergic respiratory disease
Allergic respiratory symptom
Alveolitis allergic
Anaphylactic reaction
Anaphylactic shock
Anaphylactic transfusion reaction
Anaphylactoid reaction
Anaphylactoid shock
Angioedema
Bronchial hyperreactivity
Bronchospasm
Circulatory collapse
Circumoral oedema
Conjunctival oedema
Corneal oedema
Cough
Drug eruption
Drug hypersensitivity
Epiglottic oedema
Erythema
Eye oedema
Eye swelling
Eyelid oedema
Face oedema
First use syndrome
Gingival oedema

Gingival swelling
Gleich's syndrome
Hereditary angioedema
Hypersensitivity
Idiopathic urticaria
Kounis syndrome
Laryngeal oedema
Laryngotracheal oedema
Lip oedema
Lip swelling
Oculorespiratory syndrome
Oedema mouth
Oropharyngeal swelling
Palatal oedema
Periorbital oedema
Pharyngeal oedema
Pruritus
Pruritus allergic
Pruritus generalised
Rash
Rash erythematous
Rash generalised
Rash pruritic
Scleral oedema
Shock
Small bowel angioedema
Swelling face
Swollen tongue
Tongue oedema
Toxic skin eruption
Tracheal oedema
Type I hypersensitivity
Type I hypersensitivity
Urticaria
Urticaria
Urticaria cholinergic
Urticaria chronic
Urticaria papular
Urticaria papular
Wheezing

Following preferred terms are considered as Adverse Event of Special Interest

Pancreatitis
Cullen's sign

Hereditary pancreatitis
Ischaemic pancreatitis
Oedematous pancreatitis
Pancreatic abscess
Pancreatic necrosis
Pancreatic phlegmon
Pancreatic pseudocyst
Pancreatic pseudocyst drainage
Pancreatitis
Pancreatitis acute
Pancreatitis chronic
Pancreatic haemorrhage
Pancreatitis haemorrhagic
Pancreatitis necrotising
Pancreatitis relapsing
Pancreatorenal syndrome

Following preferred terms are considered as Adverse Event of Special Interest

Renal events

Acute phosphate nephropathy
Acute prerenal failure
Anuria
Azotaemia
Continuous haemodiafiltration
Dialysis
Haemodialysis
Neonatal anuria
Nephropathy toxic
Oliguria
Peritoneal dialysis
Renal failure
Renal failure acute
Renal failure neonatal
Renal impairment
Renal impairment neonatal

Following preferred terms are considered as Adverse Event of Special Interest

Skin lesions

Acute generalised exanthematous pustulosis
Cutaneous vasculitis
Dermatitis bullous
Dermatitis exfoliative
Dermatitis exfoliative generalised
Drug eruption
Epidermal necrosis
Erythema multiforme
Exfoliative rash
Lip exfoliation
Mucocutaneous ulceration
Mucosal exfoliation
Oral mucosal exfoliation
Penile exfoliation
Skin erosion
Skin exfoliation
Skin necrosis
Stevens-Johnson syndrome
Toxic epidermal necrolysis
Toxic skin eruption
Vaginal exfoliation
Vaginal ulceration
Vulval ulceration
Vulvovaginal ulceration

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