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An Extract of *Artemisia dracunculus* L. stimulates insulin secretion from β cells, activates AMPK and suppresses inflammation

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Abstract

Ethnopharmacological relevance—*Artemisia dracunculus* L. (Russian tarragon) is a perennial herb belonging to the family Compositae and has a history of medicinal use in humans, particularly for treatment of diabetes.

Aim of the study—In this study a defined plant extract from *Artemisia dracunculus* L. (termed PMI-5011) is used to improve β cells function and maintain β cell number in pancreatic islets as an alternative drug approach for successful treatment of diabetes.

Materials and Methods—Mouse and human pancreatic beta cells were treated with defined plant extract of *Artemisia dracunculus* L. (PMI-5011) to understand the mechanism(s) that influence beta cell function and β cell number.

Results—We found that the PMI-5011 enhances insulin release from primary β cells, isolated mouse and human islets and it maintains β cell number. Insulin released by PMI-5011 is associated with the activation of AMP-activated protein kinase (AMPK), and protein kinase B (PKB). Furthermore, PMI-5011 suppresses LPS/INF γ -induced inflammation and inflammatory

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Conflict of interest

There is no potential conflict of interest for the authors of the work described.

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mediator(s) in macrophages. PMI-5011 inhibited Nitric oxide (NO) production and expression of inducible nitric oxide synthase (iNOS) at the protein level and also attenuated pro-inflammatory cytokine (IL-6) production in macrophages.

Conclusion—PMI-5011 has potential therapeutic value for diabetes treatment via increasing insulin release from β cells and decreases capacity of macrophages to combat inflammation.

Graphical abstract

Keywords

Botanical(s); Diabetes; Pancreatic beta (β) cells; Islets; insulin secretion; inflammation

1. Introduction

Diabetes is seventh leading cause of death in the United States. It is expected to rise up to 30.3 million people diagnosed with diabetes in 2030 (Wild et al., 2004). In adults, type 2 diabetes (non-insulin-dependent diabetes mellitus; NIDDM) usually begins as insulin resistance, a condition in which normal or elevated insulin levels fail to achieve a normal biological response. This leads to hyperplasia of β cells and hyper-insulinemia in initial stages as a compensatory mechanism, ultimately leading to exhaustion of β cells (reduced function and mass) and ultimately leading to first a relative, then absolute deficiency of insulin (Shimabukuro et al., 1998). Therefore, understanding β cell function and manipulating β cell number and function is a major challenge for diabetes therapy. β cells have been known to have a capacity for replication in rodents and in humans as well (Tyrberg et al., 2001). A variety of peptides such as INGAP, a peptide fragment of the pancreatic REG protein, GLP-1 and the GLP-1 receptor agonist exendin-4, the combination of betacellulin and activin A, and the combination of EGF and gastrin has been shown to stimulate replication/neogenesis of β cells in rodents (Egan et al., 2003; Li et al., 2004; Rosenberg et al., 2004). Also, GLP-1/exendin-4 has incretin effects, enhances insulin secretion and has anti-apoptotic effects (Bonner-Weir and Weir, 2005). Unfortunately, the success of improving β cell function and β cell number has been observed only in mice. Although, modern insulin regimens have improved the rate of micro-vascular complications, it has been clearly shown that even tight control of blood glucose does not alter the rate of macro-vascular complications, thus it is becoming increasingly apparent that alternative novel strategies are required to improve the β cell function while maintaining the β cell number in human islets for the cure of diabetes.

Epidemiologic and animal studies have shown that active compounds in plant extracts (i.e. phytochemicals) may mimic the action of insulin and suppress the activity of certain enzymes involved in glucose production (Cefalu et al., 2008). There is a historical connection between the treatments of diabetes with over 1200 traditional plants (Schmidt et

al., 2008). It was estimated that from 1981–2002, approx. 74% (48/65) of all drugs approved may have had origins in natural products, were based thereon, or mimicked them in one form or another with low toxicity (Newman et al., 2003). For example, metformin, one of the most effective and widely used drugs for the treatment of diabetes, can be linked to the traditional use of *Galega officinalis* to treat diabetes (Bailey and Turner, 1996). It is important to note that consistent documentation of a glucose or insulin lowering effect has not been shown for any specific plant extract (Ribnicky et al., 2008) because of different methods of plant extract preparations. One of the traditional plants, e.g., *Artemisia dracunculus* L. (Russian tarragon), is a wild species and a close relative of common cooking tarragon (known as French tarragon or *Artemisia dracunculus* var. *sativa*). *Artemisia* and, more specifically, *Artemisia dracunculus*, have a history of medicinal use in humans, particularly for treatment of diabetes (Swanston-Flatt et al., 1991). The *Artemisia dracunculus* extract described as “PMI-5011” is an alcoholic extract of the plant and has been shown to have significant effects to improve carbohydrate metabolism by enhancing molecular events of insulin action in skeletal muscle (Wang et al., 2008). PMI-5011 was also shown to have anti-hyperglycemic activity in animal models (Ribnicky et al., 2006). This defined plant extract may represent a novel pharmacological basis for the treatment of type 2 diabetes. The aim of the present study was to analyze the capacity of PMI-5011 to promote insulin release directly from primary β cells (NIT-1), isolated mouse pancreas islets, human pancreas islets, as well as to understand the cellular mechanism of action. This extract was studied in β cells and macrophages in relative to the activity of the widely used drug “metformin” in type 2 diabetes, the mechanism of action of which have been extensively studied (Fryer et al., 2002; Hawley et al., 2002; Zhou et al., 2001).

2. Materials and Methods

2.1. *Artemisia dracunculus* L. (PMI-5011) Extract

An alcoholic extract of *Artemisia dracunculus* L. (PMI-5011) was provided by the Botanical Core of the NIH funded Botanical Research Center at the Pennington Biomedical Research Center & the Plant Biology Department of Rutgers University (not sure we need all of this, up to you). The seed for *Artemisia dracunculus* L. was purchased from Sheffield's Seed Co., Inc. (Locke, New York) and the name of the plant was verified as correct with www.theplantlist.org. Voucher specimens are maintained at the Chrysler Herbarium of Rutgers University. The plants were cultivated at Rutgers University and the extract was produced as described previously (Ribnicky et al., 2006; Wang et al., 2008; Wang et al., 2011). Briefly, the fresh herb was extracted at 80°C with 80% ethanol for 2 hours followed by an additional extraction for 10 hours at 20°C. The extract was filtered, concentrated and freeze-dried. The dried extract was homogenized and used for experiments. The extract has been extensively characterized through the identification of the active compounds and reporting of biochemical fingerprints (Govorko et al., 2007; Logendra et al., 2006; Ribnicky et al., 2009; Ribnicky et al., 2006; Wang et al., 2008; Wang et al., 2011).

2.2. Cell Culture

NIT-1 cells were obtained from American Type Culture Collection (ATCC) VA, USA. They were maintained in Ham's F-12 medium with L-glutamine (GIBCO- Invitrogen, Grand

Island, NY), 10% fetal bovine serum (FBS), 10 mM of glucose, 1.5 g/L sodium bicarbonate, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Sigma, St. Louis, MO). Normal human islets were purchased from National Disease Research Interchange (PA, USA) and cultured in CMRL 1066 (Cellgro^R, Manassas, VA) containing 10% FBS, 5.5 mM of glucose, 2 mM glutamax (GIBCO- Invitrogen), penicillin (100 U/ml) and streptomycin (100 µg/ml). The culture of human islets was approved by Institutional review board at the Pennington Biomedical Research center (Protocol # PBRC IRB # 297 EX).

2.3. Islet isolation

Islets of Langerhans were isolated from C57BL/6J 10-week old female mice. The protocol learned and used was from Dr. Franck Mauvais-Jarvis laboratory, Division of Endocrinology, Metabolism and Molecular Medicine, and Northwestern Comprehensive Center on Obesity (NCCO), Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA, with a slight modification (Li et al., 2009). Briefly, the method involves cannulation of common bile duct and distension of pancreas using collagenase Type IV (GIBCO- Invitrogen, Grand Island, NY) and followed by purification (Li et al., 2009). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center (Protocol # 648). Purity of islets was checked by staining islets with dithizone staining (Sigma) and cell viability by staining with DAPI and by trypan blue (Sigma). Islets were cultured in a medium same as of NIT-1 cells.

2.4. Enzyme-Linked Immunosorbent Assay for Insulin Measurement

24 h before the experiments, the culture medium was renewed. The NIT- cells, and mouse islets were washed twice with Krebs-Ringer bicarbonate (KRB) buffer, pH 7.5, containing 0.1% BSA (KRB-BSA). The cells were pre-incubated for one hour in KRB-BSA containing 1 mM glucose at 37°C, 5% CO₂ and incubated for two hours in KRB-BSA containing various concentrations of glucose and/or PMI-5011. After incubation, the medium was collected, centrifuged at 1000 rpm for 5 min and samples were stored at -20°C until analyzed. Insulin release was measured by using Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Downer Grove, IL) according to the manufacturer's instruction. The insulin content in supernatants from human islets was measured by APLCO diagnostic kit (Salem, NH). The sensitivity of the assay is 0.399 µIU/ml.

2.5. Confocal laser scanning microscopy

Confocal microscopy was performed as described previously (Aggarwal et al., 2011). NIT-1 cells and Mouse islets were plated on cover slips, fixed with 2 % formaldehyde, washed with PBS, permeabilized with 0.1% Trion-X100 and incubated with 2 % goat serum for one hour. The cells were then incubated with a primary antibody specific for Insulin (Cell Signaling Technology, Cambridge, MA) overnight at 4°C, then washed with PBS and incubated with a secondary antibody conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR) for one hour followed by three washings with PBS. Finally, cells were mounted with mounting medium from Molecular Probes (Carlsbad, CA) and images of insulin immunostained cells were acquired under an oil immersion objective (x40) with a

confocal laser microscope (Zeiss Confocal LSM510, Carl Zeiss MicroImaging Inc. Thornwood, NY, USA) equipped with an argon-krypton laser.

2.6. Cytotoxicity assay

The assay was done using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) as described previously (Aggarwal et al., 2004). Briefly, NIT-1 cells (5,000 per well), mouse islets and human islets were incubated with increasing concentrations of PMI-5011 extract in triplicate in a 96-well plate and incubated for 48 h and/or 72 h at 37°C. A MTT solution was added to each well and incubated for 2 h at 37°C. An extraction buffer (20% SDS and 50% dimethylformamide) was added, and the cells were incubated overnight at 37°C. The absorbance of the cell suspension was measured at 590 nm using a Benchmark plusTM microplate spectrophotometer (Bio-Rad, Philadelphia, PA). This experiment was repeated twice and the statistical analysis was done to obtain the final values.

2.7. Macrophage preparation and activation

Bone marrow cells from female C57/B6 mice were plated in 10-cm plates with 10 % (vol/vol) FCS in RPMI 1640 medium supplemented with recombinant mouse macrophage-colony-stimulating factor (10 ng/ml; R&D Systems). Media was changed every third day. On day 7, adherent cells were collected and used for experiments.

2.8. Nitric Oxide (NO) assay

Production of NO was determined by assaying culture supernatant for nitrate, a stable product of NO and molecular oxygen. Briefly, 100 µl of culture supernatant was allowed to react with 100 µl of Griess reagent and incubate at RT for 15 min. The optical density of the assay samples were measured at 570 nm. Fresh culture media was used as blank. Nitrate concentrations were calculated from a standard curve derived from the reaction of NaNO₂ in the assay.

2.9. Western Blotting

Protein extracts from NIT-1 cells were prepared by lysing the cells in SDS lysis buffer (250 mM Tris-Cl, pH 6.5, 2% SDS, 4% β-mercaptoethanol, 0.02% bromophenol blue, 10% glycerol) containing protease and phosphatase inhibitors. Standard SDS-PAGE and Western blotting procedures were used to analyze the cell extracts (Aggarwal et al., 2011). Nitrocellulose blots were probed with anti- phospho -AMPK, anti-AMPK, anti- phospho -ACC, anti-phospho-AKT, (Cell Signaling Technology, Cambridge, MA) antibodies. Anti-beta actin antibody (Millipore, Billerica, MA) was used as a loading control.

2.10. Statistical analysis

Data are expressed as means ± standard deviation/or standard error. Statistical analyses were done by using GraphPad Prism (software version 5.0. VA) using simple linear regression analysis and unpaired Student's *t* test. A *p* value of < 0.05 was considered significant.

3. Results

3.1. PMI-5011 stimulates insulin release from NIT-cells and mouse islets

We first analyzed the dose response of PMI-5011 to promote insulin release from the NIT-1 cells in culture. Experiments were carried out in a glucose concentration (1 mM); PMI-5011 induced a pronounced insulin release in dose-dependent manner, with a 1.30 ± 0.15 fold increase at 5 $\mu\text{g/mL}$ ($p = 0.035$) and 2.78 ± 0.51 fold increase at 10 $\mu\text{g/mL}$ ($p = 0.003$) concentration when compared to insulin release in the 1 mM glucose concentration (Figure 1A) ($p < 0.05$). NIT-1 insulin secretor cells are well characterized β cells (Hamaguchi et al., 1991) and responded to glucose (Figure 1A, inset). Isolated islets from mice were also functional and responded to glucose-stimulated insulin release (Figure. 1B, inset). PMI-5011 induced insulin release with a 5.31 ± 0.7 fold increase at 10 $\mu\text{g/mL}$ ($p = 0.0116$) concentration when compared to the insulin release in 1mM glucose concentration, ($p < 0.05$) (Figure 1B). Confocal microscopic analysis (Figure 2) demonstrated that NIT-1 cells as well as isolated mouse islets express insulin when treated with PMI-5011 (10 $\mu\text{g/mL}$) and higher concentrations of glucose (15 mM).

3.2. PMI-5011 is not toxic to the NIT-1 cells and mouse islets

It is important to demonstrate that β cells have conserved mechanisms for regulating insulin function and mass, as it may be detrimental for a compound to result in uncontrolled insulin secretion (Bouwens and Rooman, 2005; Eberhard et al., 2010). Therefore, we determined the effect of PMI-5011 on NIT-1 cells and isolated islets containing β cells on cell proliferation. Our results showed that PMI-5011 does induce neither cell proliferation nor cell toxicity up to 72 h either in NIT-1 cells or in isolated islets in culture (Figure 3 A–B).

3.3. PMI-5011 activates cellular target in energy balance in β cells and suppresses inflammation in macrophages

The AMP-activated protein kinase (AMPK) is associated with multiple cellular energy mechanisms (Hardie, 2004; Kemp et al., 2003; Long and Zierath, 2006). AMPK α activation turns-on catabolic pathways to generate ATP and turns-off anabolic pathways that requires ATP to function (Long and Zierath, 2006). The mechanism of action of PMI-5011 involves the activation of AMPK α as evidenced from time-dependent increase in phosphorylation of AMPK and its downstream substrate, acetyl-CoA carboxylate (ACC) and PKB, also known as AKT protein (Figure 4A). Figure 4B demonstrates that PMI-5011 leads to the phosphorylation of the AMPK (Thr172) protein, the ACC (Ser79) protein and the AKT (Ser473) protein as potential targets in the PMI-5011 signaling pathway, whereas the known anti-diabetic drug, metformin also promotes the phosphorylation of the AMPK (Thr172) protein, the ACC (Ser79) protein but not the AKT (Ser473) protein. Also, chronic inflammation has become a well-accepted risk factor for diabetes associated with energy balance. Stimulated resident macrophages and passenger leukocytes secrete pro-inflammatory cytokines such as IL-1 β , TNF α and IFN γ , which lead to islet dysfunction and apoptotic death by the iNOS/NO pathway (Narang and Mahato, 2006; Ris et al., 2002). Therefore, we examined if PMI-5011 has any effect on inflammation in macrophages. For this, bone marrow derived –macrophage cells were treated with various concentrations of PMI-5011 (10–30 $\mu\text{g/mL}$) and stimulated with LPS/IFN γ to create an inflammatory

environment in cell culture. We observed that treatment with PMI-5011 inhibited NO production in macrophages (Figure 4C) and also inhibited expression of the iNOS protein levels (Figure 4D). Metformin was used as positive control and also down regulated NO production and iNOS expression in bone marrow derived macrophage cells. PMI-5011 treatment also inhibited pro-inflammatory cytokine IL-6 production under similar experimental conditions as examined by ELISA (Figure 4E), further supporting the anti-inflammatory properties of PMI-5011 in macrophage.

3.4. PMI-5011 stimulates insulin release from human islets and maintains human islet cell mass

We determined the effect of PMI-5011 on the insulin release in human β cells and β cell proliferation. Groups of islets were placed into static incubation assays (Figure 5A). They were exposed to 2.8 mM or 16.8 mM glucose or increasing concentrations of PMI-5011 ($\mu\text{g/mL}$, along with 2.8 mM glucose) and insulin release in supernatants was quantified. Insulin release in response to glucose challenge under static incubation was expressed as insulin secretion index (ISI). The ISI is the ratio of the corrected average insulin release at 16.8 mM glucose or at increasing concentrations of PMI-5011 ($\mu\text{g/mL}$) to the corrected average insulin release at 2.8 mM glucose. ISI (%) was calculated using following equation: $\text{ISI} \times 100$. Figure 5A shows mean values of insulin secretion of glucose (2.8 mM and 16.8mM) and of increasing concentration of PMI-5011 (10 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$) for all analyzed samples. ISI was calculated for each culture and mean values are illustrated on Figure 5B. We observed that in both groups islets showed increased glucose-induced as well as PMI-5011-induced insulin secretion. We then determined the effect of PMI-5011 on the human islet cell mass using the MTT assay which clearly demonstrated that there was no significant increase and toxicity in the islet cell mass with the treatment of PMI-5011 for 72 hours Figure 5C. Purity of islets (Figure 5D) was assessed by dithizone staining of the Islets as described previously (Ricordi, 1991).

4. Discussion

Type 1 and Type 2 diabetes result from absolute or relative deficiencies in insulin secretory function resulting from alterations in β cell function and β cell number. Epidemiologic and animal studies have shown that compounds from natural products present in certain foods can modulate β cell apoptosis and perhaps enhance the insulin function (Modak et al., 2007). Because of the lack of scientific/clinical efficacy, precise mechanisms of action and safety data with natural botanical sources, these natural products have not been recommended for routine use in modern medical practices (Cefalu and Brantley, 2008). However, our data are the first to demonstrate that a well characterized extract of *Artemisia dracunculus* L. (PMI-5011), as observed in *in vitro* experiments, can trigger insulin release from primary β cells (NIT-1), isolated islets from mouse pancreases, and isolated human pancreatic islets without any toxicity/or change in β cell number. It has previously been demonstrated in *in vitro* and *in vivo* pre-clinical models that PMI-5011 enhances insulin sensitivity and insulin receptor signaling, and improves insulin levels (Wang et al., 2011; Zuberi, 2008). However, our studies showed the novel finding that PMI-5011 enhances the insulin release from the primary source, i.e. β cells. Therefore, these findings along with the previous reports further

strengthen our understanding of the mechanisms by which PMI-5011 exerts anti-diabetes properties.

Investigation of cellular targets involved in the energy balance mechanism could enhance our understanding of diabetes and lead to the development of novel strategies for the prevention of diabetes and its complications (Moller, 2001). Our results clearly demonstrate that PMI-5011 leads to the activation of AMPK α , the phosphorylation of ACC, and the phosphorylation of AKT in insulin sensitive β cells. It might be possible that PMI-5011 works in insulin like manner because insulin stimulates glucose uptake via the PI3K/AKT pathway and AMPK activation stimulates glucose utilization (Bertrand et al., 2006). Several pharmacological approaches that improve insulin sensitivity and lower blood glucose have been developed in past decade. The most widely used drug to treat diabetes is metformin which improves glucose homeostasis in Type 2 diabetes patients. The mechanistic action of metformin involves the activation of AMPK α , leading to increased phosphorylation of ACC, not AKT and is associated with multiple cellular energy mechanisms (Bertrand et al., 2006; Fryer et al., 2002; Hawley et al., 2002; Long and Zierath, 2006) which was confirmed with our results. Further, since β cells have glucose-sensing property, the glucose-induced changes in energy state of β cells and are associated with insulin release (Detimary et al., 1998; Detimary et al., 1995). Therefore, activation of AMPK α in β cells with PMI-5011 and with metformin may act as a fuel sensor for insulin release from β cells (Long and Zierath, 2006). However, prolonged treatment of β cells with metformin (> 24h) results in β cell apoptosis (Kefas et al., 2004), while PMI-5011 doesn't induce any β cell death up to 72 hours in primary β cells, mouse islets and in human islets. Thus, it is intriguing to postulate that activation of AKT in β cells with PMI-5011 may promote long term survival and prevent apoptosis in β cells (Edinger and Thompson, 2002).

Prevention of dysregulation of islet function and islet number requires abrogation of cytokine-mediated islet dysfunction and islet cell death triggered by inflammatory and immune responses. Activation of resident macrophages and invading leukocytes to secrete pro-inflammatory cytokines, promotes activation of iNOS and production of nitric oxide (NO) leading to islet dysfunction and islet cell death (Koeck et al., 2009; Welsh et al., 1995). Our data clearly demonstrate that PMI-5011 has anti-inflammatory properties in macrophages stimulated with cytokines (LPS/IFN γ). These effects agree with other studies showing inhibition of cytokine induced iNOS/NO production leads to protection of islet function and islet cell death (Bertera et al., 2004; Koeck et al., 2009). AMPK activation also, plays a role in preventing islet apoptosis during the recovery phase from NO damage, restores cellular energy homeostasis and anti-apoptotic in physiological state (Meares et al., 2010).

In conclusion, our studies provide novel data on the role of *Artemisia dracuncululus* L. (PMI-5011) in mitigating the carbohydrate metabolism dysfunction associated with diabetes. Figure 6 illustrates the proposed mechanistic action of PMI-5011 in β cells. Specifically, this data is the first to demonstrate that the extract of *Artemisia dracuncululus* L. (PMI-5011) can enhance β cell function and restore β cell mass in pancreatic cells.

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Figure 1. Effect of PMI-5011 on insulin release from NIT-1 cells and isolated mouse islets in vitro A–B; Experiments were conducted in KRB containing (1mM) glucose concentration with 2.5 μ g/ml, 5 μ g/ml and 10 μ g/ml of PMI-5011. Inset: dose-dependent effect of glucose on insulin release from A; NIT-1 cells B; isolated islets (n = 5). Data are presented as mean $\pm$ SE values of three independent experiments.

Figure 2. Visualization of insulin in NIT-1 cells and isolated mouse islets by confocal microscopy NIT-1 cells (A–C) and islets (D–E) were analyzed for the insulin by immunocytochemistry. A) Secondary antibody (Texas red) only, B) Glucose (15 mM); positive control C) PMI-5011 (10 µg/mL), D) Glucose (15 mM); positive control, E) PMI-5011 (10 µg/mL). Red stain: Localization of insulin; Blue Stain: Localization of nucleus

Figure 3. Effect of PMI-5011 on cell toxicity on NIT-1 cells and isolated mouse islets in vitro
 A) NIT-1 cells (10,000/well), and/or B) isolated mice islets, dissociated with Accumax and equal number were incubated in the absence or presence of increasing concentrations of PMI-5011 ($\mu\text{g/mL}$) and equivalent amount of DMSO at the highest concentration of PMI-5011 for 72 h and number of viable cells examined by MTT. All points are mean $\pm$ SE of triplicate wells of three independent experiments.

Figure 4. Effect of PMI-5011 on AMP-activated protein Kinase and protein kinase B (PKB) phosphorylation in NIT-1 cells

A) NIT-1 cells were incubated in the absence or presence of 10 μ g/mL PMI-5011 for the indicated times. B) NIT-1 cells were incubated in the absence or presence of 10 μ g/mL PMI-5011, or metformin. Then, Total cell lysates were subjected to Western blot analysis by using anti-pThr172 AMPK α , anti-pSer79 ACC, anti-pSer473 AKT, anti-AMPK α , anti-AKT and anti-GAPDH antibodies. The images are representative image from three independent experiments. C) Anti-inflammatory effect of PMI-5011 on macrophage. Bone marrow derived macrophages were treated with different concentration of PMI-5011 (10–30 μ g/ml) followed by stimulation with LPS/IFN γ (0.1 μ g/50 U/ml). Post 20 h of treatment, cell supernatant was used for NO estimation using Griess reagent and D) cell lysate was processed for immunoblot analysis for iNOS (BD Bioscience). Beta-actin was used for equal protein loading control. E. Levels of IL6 were determined by ELISA (BD Bioscience) in cell supernatant. Metformin (10 mM) was used as control in this experiment. Values are means + SD of three experiments.

Figure 5. A) Effect of PMI-5011 on insulin release from isolated human islets

Islets were pre-incubated for one hour in Krebs-Ringer bicarbonate (KRB) buffer containing 0.1% BSA, 2.8 mM glucose at 37°C, in a 5% CO₂ and then incubated for one hour in high concentration of glucose (16.7 mM) and different concentration of PMI-5011. The concentration of PMI-5011 (10 μg/mL, 30 μg/mL and 50 μg/mL). The insulin content was measured by APLCO diagnostic kit (Salem, NH). The sensitivity of the assay was 0.399 μIU/ml. Results are reported as mean ± SE, as μIU/ml of stimulation. Data is considered significant when $p < 0.05$. **B) Insulin secretion indexes for glucose and PMI-5011**

treatment. Comparative review of insulin secretion indexes for isolated human islets as response to low and high glucose and increasing concentration of PMI-5011 stimulation. **C.** Effect PMI-5011 on human islets toxicity. Isolated human islets, dissociated with Accumax and equal number were incubated in the absence or presence of increasing concentrations of PMI-5011 (μg/mL) and equivalent amount of DMSO at the highest concentration of PMI-5011 for 72 h and number of viable cells examined by MTT. All points are mean ± SE of triplicate wells of three independent experiments. **C.** Staining of human islets with diphenyl-thiocarbazon dye. Isolates islets were stained with diphenyl-thiocarbazon dye and photographs were taken with a microscope NIKON, Diaphot 300.

Figure 6. Proposed mechanistic action of PMI-5011 in beta cells

On one side PMI-5011 activates cellular target of energy balance (AMPK), its substrate, ACC and cell survival target AKT in β cells. On another side PMI-5011 decreases the secretion of pro-inflammatory cytokines, NO/iNOS in macrophages. Collectively, PMI-5011 leading to insulin secretion from β cells and protection of β cells and contributes to preserve metabolic homeostasis of insulin and β cells.