Electronic Supplementary Material (ESI) for MedChemComm.

Supplementary Data

Synthesis of Substituted 2*H*-Benzo[*e*]indazole-9-carboxylate as Potent Antihyperglycemic Agent that May Act through IRS-1, Akt and GSK-3β Pathways

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Materials and methods

1. Materials

Fetal bovine serum (FBS), trypsin, and antibiotic/antimycotic solution were procured from Gibco, USA. Dulbecco's modified Eagle's medium (DMEM), cytochalasin B, 2-deoxyglucose, protease inhibitor cocktail, RIPA buffer and all other chemicals unless otherwise noted were obtained from Sigma Chemical (St. Louis, MO). 2-Deoxy-D-[^{3}H]-glucose (2-DG) was purchased from PerkinElmer, USA. Antibody for protein kinase B (AKT) was purchased from Santa Cruz Biotechnology Inc. (USA) and antibodies to phospho-AKT (Ser-473), insulin receptor substrate-1 (IRS-1), phospho-Tyr, phospho-GSK-3β (Ser-9) and GSK were received from Cell Signaling Technology (USA).

2. Cell culture

Wild-type L6 skeletal muscle cells were maintained in DMEM supplemented with 5 mM D-glucose, 10% FBS, and 1% antibiotic/ antimycotic solution in a humidified atmosphere of air and 5% CO_2 at 37°C. Differentiation was induced by switching confluent cells to medium supplemented with 2 % FBS. Experiments were performed in differentiated myotubes 6-7 days after seeding.

3. Glucose uptake measurement

Determination of 2-deoxyglucose (2-DG) uptake was performed in differentiated L6 myotubes or HepG2 hepatoma cells. Briefly, cells were incubated in culture medium containing indicated concentrations of compound for 16h and 2-DG uptake was assessed for 5 min in

HEPES-buffer saline containing 10 mM 2-DG (0.5 mCi/ml 2-[^{3}H] DG) at room temperature. To quantify the radioactivity incorporated, cells were lysed with 0.05 N NaOH and lysates were counted with scintillation fluid in a β-counter. Nonspecific uptake was determined in the presence of cytochalasin B (50 mM) during the assay and these values were subtracted from all other values. Glucose uptake measured in triplicate and normalized to total protein was expressed as percent stimulation with respect to unstimulated cells.

4. Hepatic glucose production assay

HepG2 cells were cultured in low glucose dulbecco modified eagle medium. After 70-80% confluency, cells were subjected to 24h treatment consisting 3h serum starvation with test compound at 10 μM. Cells were washed twice with PBS and 2 ml glucose production media consisting of glucose free media was added with 25 mM sodium lactate and 2.5 mM sodium pyruvate or with 15 nM glucagon. After 4h incubation in glucose production media, media was collected, concentrated and glucose was measured using Amplex red glucose/glucose oxidase assay kit. Glucose release was normalized to total protein and activity was expressed as percent inhibition with respect to control.

5. Western blot analysis

L6 myotubes grown in 6-well plate were incubated with increasing concentrations of the compound for 16h with final 3h in serum free medium. After incubation, cells were stimulated with 100 nM insulin for 10 min and lysed in RIPA buffer. Lysates were centrifuged and protein concentration was determined using BCA assay reagent. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. After blocking, blots were incubated with indicated antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies. Immunoblots were developed using enhanced Chemiluminescence (ECL, Millipore) reagent.

Densitometric quantification of protein bands was performed using National Institute of Health (NIH) Image J software.

6. Protein-tyrosine-phosphatase-1B inhibitory assay

Protein tyrosine phosphatase-1B inhibitory activity of compounds was determined using PTP1B drug discovery kit (Enzo Life Sciences, Catalog # BML-AK822) according to the manufacturer's instruction. Briefly, the test compounds were incubated with the human, recombinant PTP1B and the enzyme assay was performed by addition of the phosphopeptide substrate containing sequence from the insulin receptor β subunit domain. The amount of free-phosphate released was measured based on the classic Malachite green assay using Biomol Red reagent and the absorbance was determined at 620 nm. Suramin was taken as standard in enzyme assay. The IC_{50} of the compound was determined by examining the effect of different concentrations of compounds.

7. Animals

Male albino Wistar rats and male Sprague Dawley rats of 8 to 10 weeks of age and 160 ± 20 g of body weight), and C57BL/KsJ-*db/db* mice of body weight 40±10 g available at the National Laboratory Animal center of the institute were used for the present study. The work with all these animals was cleared by Institutional Animal Ethics Committee (IAEC) and was conducted in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) formed by the Government of India in 1964. The animal were housed in polypropylene cages in the animal house, with temperature 23±3 ^{o}C, relative humidity 60-70%, light 300 lux at floor level with regular 12h light cycle; noise level 50 decibel; ventilation 10-15 air changes per hour. The animals were provided standard pellet diet and drinking water during the experimental period.

8. Glucose tolerance in normal rats

Male albino Wistar rats were used for the study. The blood glucose level of each animal was checked by glucometer using glucostrips (Boehringer Mannheim) after 16h starvation. Animals showing blood glucose levels between 60–80 mg/dl were divided into groups of six animals in each. Animals of experimental group were administered on suspension of the compound orally (made in 1.0 % gum acacia) at a dose of 100 mg/kg body weight. Animals of control group were given an equal amount of 1.0 % gum acacia. A sucrose load (10.0 g/kg) was given to each animal orally exactly after 30 min post administration of the test sample/vehicle. Blood glucose profile of each rat was again determined at 30, 60, 90 and 120 min post administration of sucrose by glucometer. Food but not water was withheld from the cages during the course of experimentation.

9. Glucose tolerance in STZ-induced diabetic rats

Sprague Dawley rats were made diabetic by injecting STZ (60 mg/kg in 0.1M citrate buffer pH 4.5) to overnight-fasted rats, intraperitoneally. Blood glucose level was checked 48h later by glucostrips and animals showing blood glucose values between 144 and 270 mg/dl were included in the experiment and considered as diabetic. The diabetic animals were divided into groups consisting of five animals in each group. Animals of experimental groups were administered on compound suspension orally (made in 1.0% gum acacia) at a dose of 100 mg/ kg body weight. Animals of control group were given an equal amount of 1.0% gum acacia. A glucose load of 2.5 g/kg body weight was given after 30 min of compound administration. After 30 min of post glucose load blood glucose level was checked at 30, 60, 90, 120, 180, 300 and 1440 min, respectively. Food but not water was withheld from the cages during 0 to 300 min (5hr) of experimentation and after this period the animals were allowed to feed. The

blood glucose level of each animal was again determined after 24 hr to ensure hyperglycemic status of the animals. Blood was sampled from the tail vein of rats and blood glucose level of each animal was checked by glucometer using glucostrips.

10. Antihyperglycemic activity evaluation in db/db mice

The animals were divided into different groups of six animals each. Group I was regarded as the control group and treated with vehicle, whereas the remainder was termed as compound-treated groups and dosed daily with the compound at 100 mg/kg dose level for 15 days. Blood glucose level of each animal was measured daily using a glucometer. On day 10 and day 15, an oral glucose tolerance test (OGTT) of each animal was performed after an overnight fast. The baseline blood glucose level was monitored at 0 min, followed by an oral glucose load of 3 g/kg body weight. The blood glucose levels were again checked at 30 min, 60 min, 90 min, and 120 min post-glucose administration. Blood was withdrawn from the retro-orbital plexus of the eye for the estimation of lipid profile using respective assay kits. Food but not water was withheld from the cages during 0 to 300 min (5hr) of experimentation and after this period the animals were allowed to feed. The blood glucose level of each animal was again determined after 24 hour to ensure hyperglycemic status of the animals.

11. Statistical analysis

Values are given as mean ± SE. Analysis of statistical significance of differences in measurements between samples was done by one-way ANOVA with Dunnets post hoc test (GraphPad Prism version 3). *P <0.05, **P <0.01, ***P <0.001 was considered statistically significant.

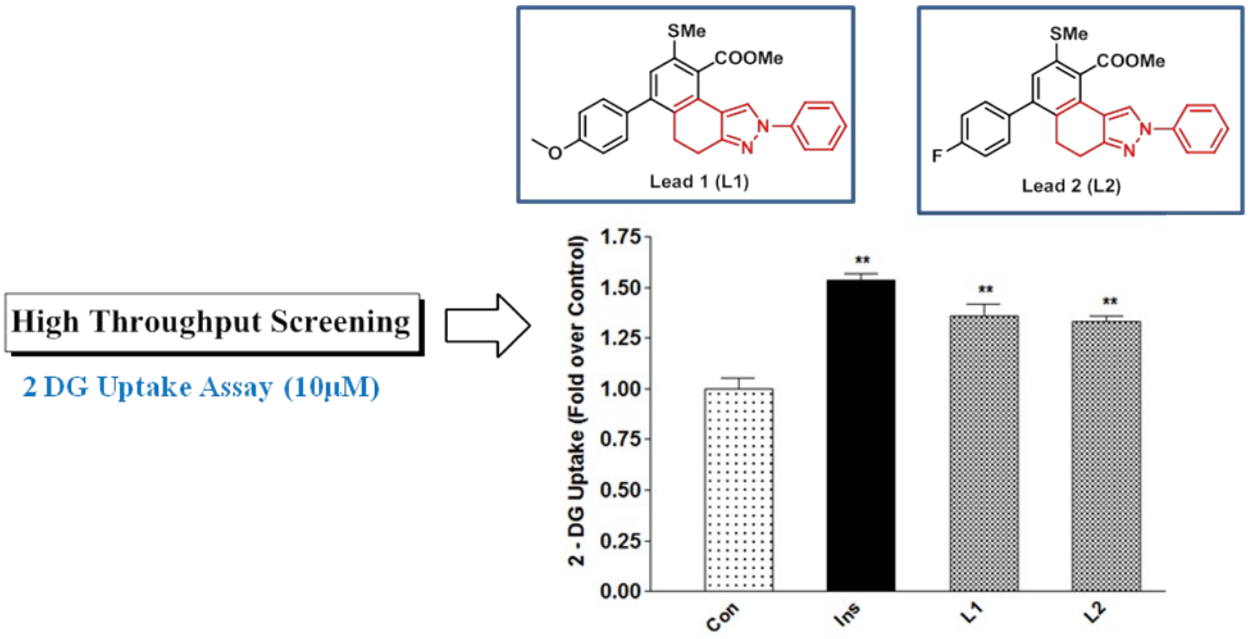


Figure S1: Lead molecules identified through 2-deoxyglucose uptake assay. Significance: **p < 0.01 relative to control condition.

2-deoxyglucose uptake by L-6 muscle cell lines :

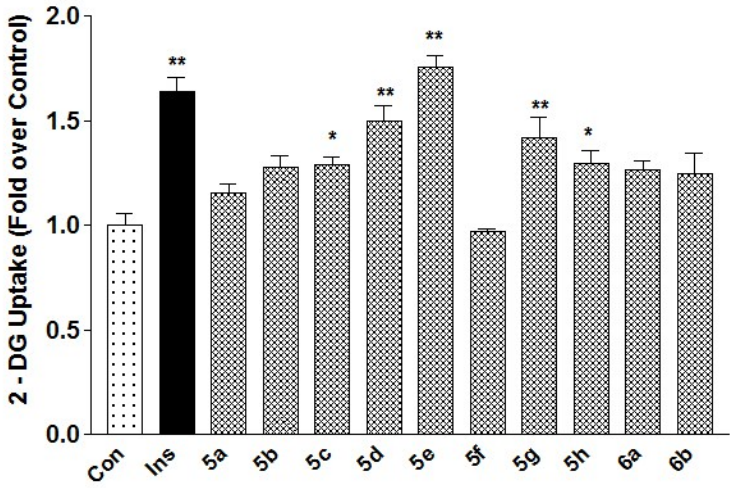


Figure S2: Effect of **5a-h** and **6a-b** on ^{3}H-2-Deoxyglucose uptake by L6 myotubes.

Significance: *p < 0.05, **p < 0.01 relative to control condition.

MTT Assay :

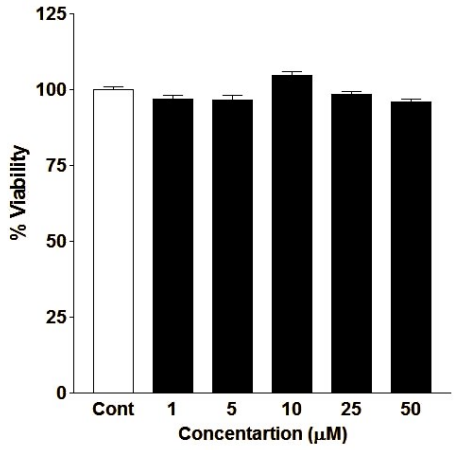
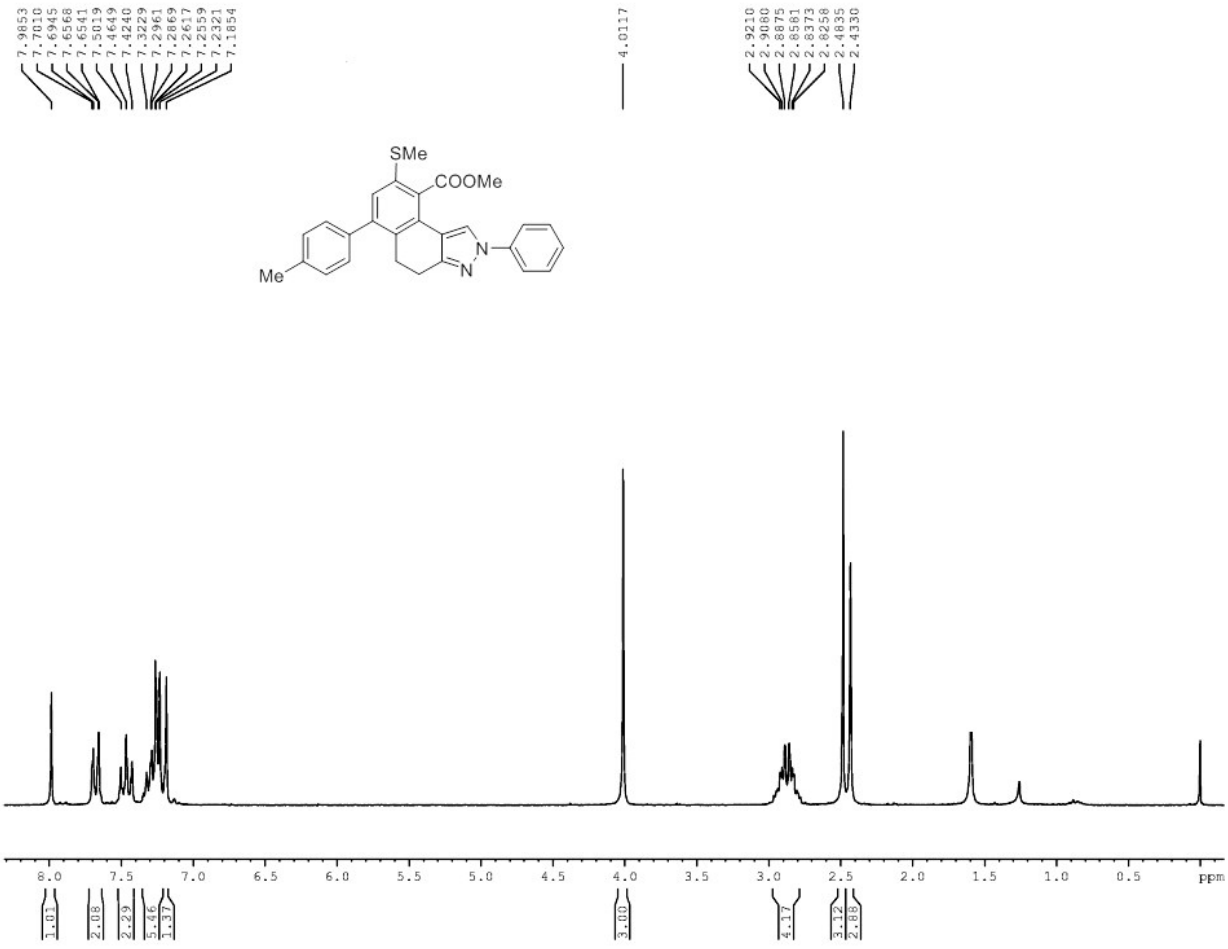


Figure S3: Effect of **5e** on cell viability in L6 myotubes.

^{1}H NMR and ^{13}C NMR Spectra



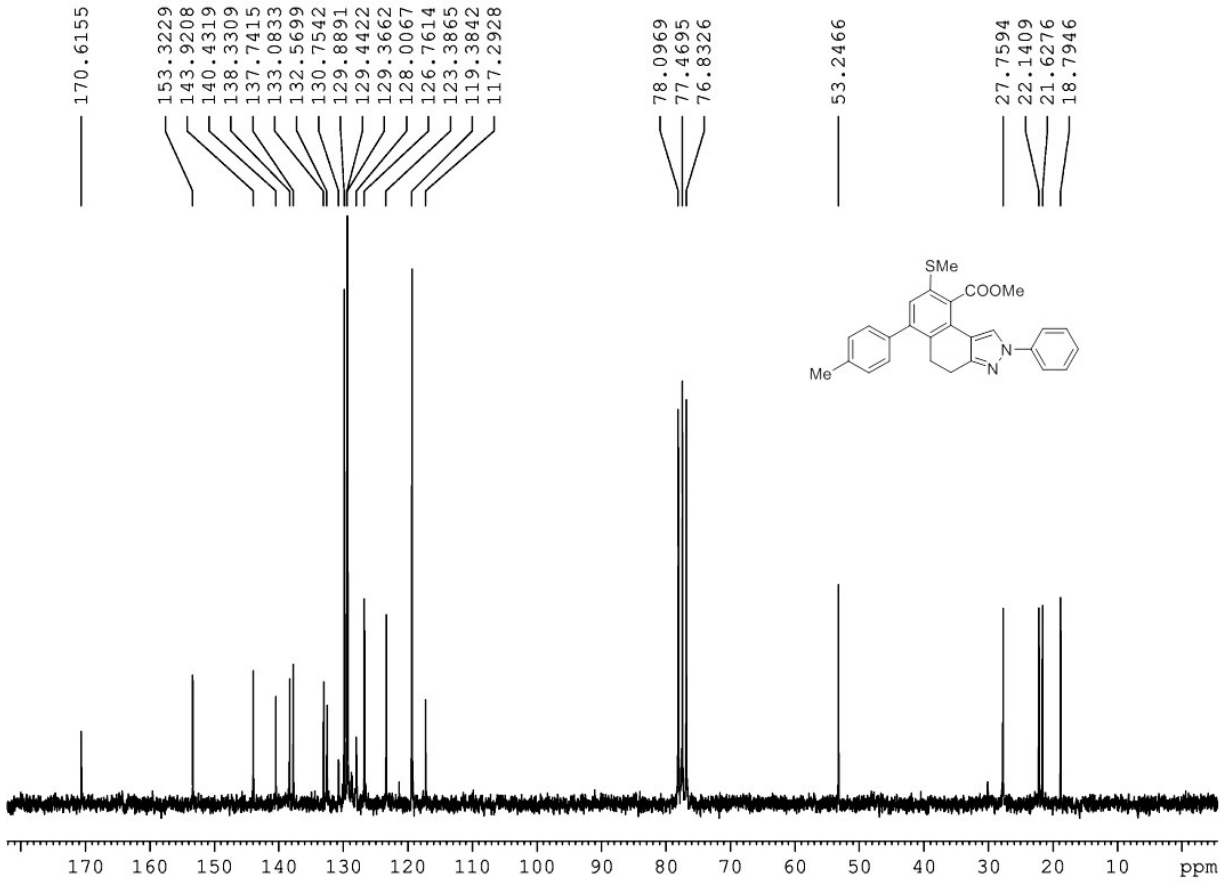


Figure S4. ^{1}H NMR and ^{13}C NMR of 5e.

HPLC Chromatograms

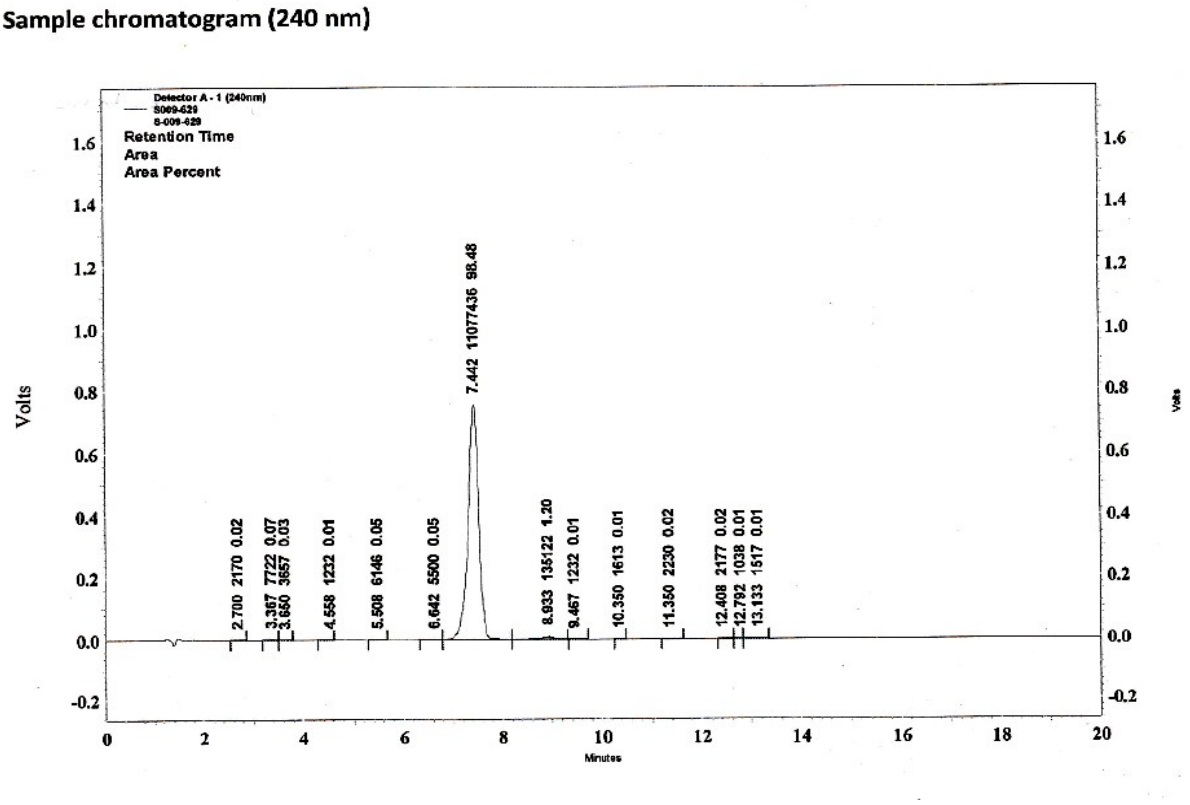


Figure S5. HPLC Chromatogram of **5e**

Table S1. PTB-1B inhibitory activity of compound 5e.

Compound	% Inhibition[a]	IC_{50}(μM)
5e	83.8	7.12
Suramin (Standard)	60.2	9.5

[a]Compounds were evaluated against PTP-1B at 100μM concentration in presence of 0.01% of Triton X-100; Values are mean ± S.E. of three independent experiments.