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Aegeline inspired synthesis of novel β3-AR agonist improves insulin sensitivity *in vitro* and *in vivo* models of insulin resistance.

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Running title: Compound 10C revert insulin resistance by inducing browning

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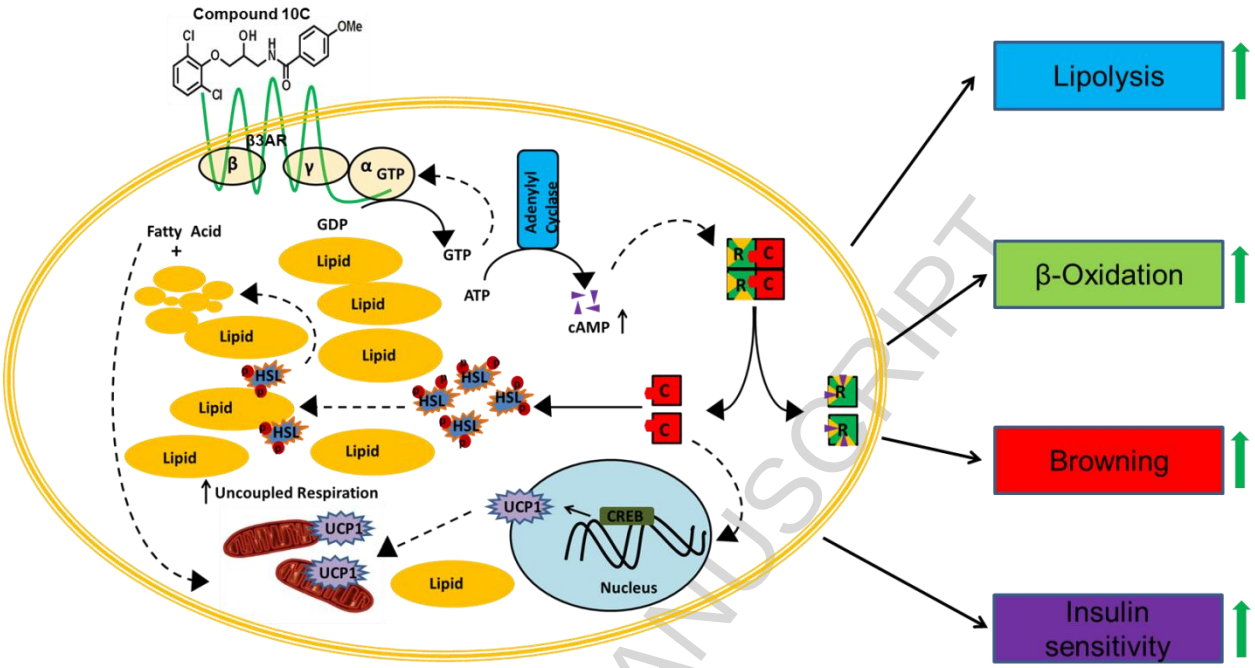
Graphical Abstract



ABSTRACT

BACKGROUND AND PURPOSE

In our drug discovery program of natural product, earlier we have reported Aegeline that is N-acylated-1-amino-2- alcohol, which was isolated from the leaves of *Aeglemarmelos* showed anti-hyperlipidemic activity for which the QSAR studies predicted the compound to be the β3-AR agonist, but the mechanism of its action was not elucidated. In our present study, we have evaluated the β3-AR activity of novel N-acyl-1-amino-3-arylopropanol synthetic mimics of aegeline and its beneficial effect in insulin resistance. In this study, we have proposed the novel pharmacophore model using reported molecules for antihyperlipidemic activity. The reported pharmacophore features were also compared with the newly developed pharmacophore model for the observed biological activity.

EXPERIMENTAL APPROACH

Based on 3D pharmacophore modeling of known β3AR agonist, we screened 20 synthetic derivatives of Aegeline from the literature. From these, the top scoring compound **10C** was used for further studies. The in-slico result was further validated in HEK293T cells co-trransfected with human β3-AR and CRE-Luciferase reporter plasmid for β3-AR activity.The most active compound was selected and β3-AR activity was further validated in white and brown adipocytes differentiated from human mesenchymal stem cells (hMSCs). Insulin resistance model developed in hMSC derived adipocytes was used to study the insulin sensitizing property. 8 week HFD fed C57BL6 mice was given 50mg/Kg of the selected compound and metabolic phenotyping was done to evaluate its anti-diabetic effect.

RESULTS

As predicted by in-silico 3D pharmacophore modeling, the compound **10C** was found to be the most active and specific β3-AR agonist with EC_{50} value of 447nM. The compound **10C** activated β3AR pathway, induced lipolysis, fatty acid oxidation and increased oxygen consumption rate (OCR) in human adipocytes. Compound **10C** induced expression of brown adipocytes specific markers and reverted chronic insulin induced insulin resistance in white adipocytes. The compound **10C** also improved insulin sensitivity and glucose tolerance in 8 week HFD fed C57BL6 mice.

CONCLUSION

This study enlightens the use of *in vitro* insulin resistance model close to human physiology to elucidates the insulin sensitizing activity of the compound **10C** and edifies the use of β3AR agonist as therapeutic interventions for insulin resistance and type 2 diabetes.

Key words:β3-AR agonist, Browning, hMSC derived adipocytes, Insulin resistance.

1. Introduction

Earlier as part of drug discovery program, we have identified Aegeline isolated from the leaves of *AegeloMarmelos* as a potent antidyslipidemic agent with predicted β3-AR activity in QSAR study[1]. We have further reportedthat synthetic derivatives of the compound Aegeline also show LDL oxidation and anti-oxidant property[2].Based on theseprevious studies, we hypothesize that Aegeline and its analogues may also induce browning phenomenon and improve insulin sensitivity. Brown adipose tissue (BAT) is primarily responsible for non-shivering thermogenesis. BAT is highly vascularized and is very sensitive to sympathetic hormones such as norepinephrine, which triggers gene expression to proliferate and activate non-shivering thermogenesis via UCP1[3-5]. Sympathetic nerve activity increases UCP1 mRNA levels mainly through the effects of gene transcription. This response is mediated predominantly by β-adrenergic receptors and cAMP and to a lesser extent by α1-AR[6-8]. Apart from cold exposure, non-shivering thermogenesis can also be induced using β3-AR agonists such as BRL-35135 and CL-316243[9, 10]. CL-316243 β3-AR agonist increases serum level of free fatty acids and increase energy expenditure and decrease food intake despite reduced leptin level in the animal model[11]. Unfortunately, in both humans and primates, these β3-AR agonists demonstrated limited efficacy[12, 13]. Class I β3-AR agonist were based on rodent β3-AR, which share only 79% similarity to humans[14]. The above difference in receptor homology have rendered class I β3-AR less effective on humans. Clinical trials using class I β3-AR agonist such as CL316243 and BRL37344 for obesity and diabetes were disappointing due to lack of selectivity, potency and poor pharmacokinetics.

In this study,we have synthesized series of moleculescontaining pharmacophore predicted to be responsible for anti-hyperlipidemic and LDL-Oxidation**6A-D** to **10A-D**based on the earlier

reported SAR activity. We used 3D pharmacophore modeling to predict the β3-AR activity of synthesized derivative of Aegeline.Among the derivatives, compound **10C**was found to be most active and was further studied in white and brown adipocytes differentiated from human mesenchymal stem cells (hMSC). These cells are of human origin, thus we were able to circumvent the problems of selectivity and receptor homology faced during the development of class-Iβ3-AR. In the present study, we found increased expression of brown adipocytes specific marker, β-oxidation and lipolysis in white adipocytes upon treatment with compound **10C**. The compound also increased mitochondrial biogenesis and uncoupled respiration in white adipocytes. As β3-AR agonists are reported to increase insulin sensitivity, we tested the compound in chronic insulin (CI) induced insulin resistance (IR) inhMSC derived adipocytes. Compound **10C** increased phosphorylation of AKT and reverted CI induced IR. The specificity study using β1-AR and β2-AR indicated the compound to be a selective β3-AR agonist.The *in vivo* study on HFD induced insulin resistant C57BL6 mice further validated the *in vitro* results.

2. Materials and Methods

2.1 Synthesis of compound 10C

To a stirred solution of phenol and K_2CO_3 in dry acetone epichlorohydrin was added and the reaction mixture then refluxed until phenol was consumed. The reaction mixture was filtered washed with acetone and dried under reduced pressure,diluted with water,and extracted with ethylacetate.The organic layer was dried over anhydrous sodium sulphate,solvent was evaporated under reduced pressure to obtain crude reaction product.This was further purified by column chromatography over silica gel(60-120 mesh) to get pure oxirane(6-10).A mixture of oxiranes(6-10) and Aromatic nitriles in dry DCM was stirred for 15 min at 0^0 c , then added BF_3*OEt_2 drop

wise maintaining temperature 0^0 c. Reaction mixture was stirred for15 min and ice was removed, reaction was allow to stir at room temperature. Completion of reaction was monitored by TLC and quenched with saturated sodium bicarbonate. The aqueous phase was extracted with ethylacetate(3*50 ml),and the combined organic layer were dried over Na_2SO_4,filtered and concentrated in vacuum. Column chromatography on silica gel(60-120 mesh) using ethylacetate and hexane as eluent provided the desired compounds(**6A-10D**).Detailed experimental data and procedure for the synthesis of all the compounds along with compound **10C**are given in the supporting information. The schematic diagram of synthesis of Compound 10C is given in figure 1E.

2.2 Screening of compounds in CREB-luciferase assay system

HEK293T cells were co-transfected with human β1AR/β2AR/β3-AR plasmids (β1 and β2-AR plasmids were purchased from Addgene and β3-ARwas purchased from University of science and technology Missouri).CREB luciferase plasmid was gifted by Dr. SabyashachiSanyal as described in figure legends. peGFPC1 plasmidDNA3 (purchased from Addgene) was used as a transfection control. After 48hr of transfection cells were given with compound treatment as mentioned in figure legend and lysate was prepared. Luciferase activity was measured by providing luciferin substrate to the lysate and reading was measured inPerkin Elmer Envision 2100 luminometer. Luciferase readings were normalized by GFP readings.

2.3 Common feature pharmacophore generation

The model was developed as per the protocol reported previously by Prathipati et al (1). Detailed procedure is provided in supplementary information.

2.4 hMSC culture and differentiation in to white and brown adipocyte

hMSCs were cultured in high-glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and antibiotics. hMSCs were differentiated into white and brown adipocytes as mentioned previously [15].Briefly, hMSCs were treated with differentiation cocktail containing 500µM IBMX, 5µg/ml insulin, 1µM dexamethasone and 200µM indomethacin (All purchased form Sigma Chemicals). Cells were maintained in differentiation cocktail containing media for 9 days and media was changed every third day. For differentiation of hMSC into brown adipocyte, above mentioned cocktail was used except indomethacin was replaced with 0.5µm rosiglitazone and 2nM of tri-iodothyronine.

2.5 Insulin resistance development

Insulin resistance model was developed as described previously [15]. Briefly, fully differentiated adipocytes were incubated with 500pM of insulin (Sigma, Cat No.I5500) for 72hrs. Medium was changed every 24 hrs to replenish the insulin level. After 72 hrs, cells were washed extensively using KRH buffer (121 mMNaCl, 4.9 mMKCl, 1.2 mM $MgSO_4$, 0.33 mM $CaCl_2$) supplemented with 5mM glucose and 0.5% BSA (Step down media) prior to acute insulin pulsing.

2.6 Oil Red O staining

Performed as described elsewhere[16, 17].

2.7 [3H] 2-Deoxyglucose uptake

[3H] 2-DOG glucose uptake was performed in differentiated adipocytes as described previously[15]. Glucose uptake was measured in triplicate and normalized with total protein

content. Radioactivity was measured in cell lysate by liquid scintillation counter (Beckman Coulter, LS 6500, Beckman Coulter Inc. USA) and represented as fold difference.

2.8 Real Time PCR

Total RNA was isolated from cells using TRIZOL reagent (Invitrogen CA, USA). cDNA was prepared using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real time PCR analysis of different genes was performed in Light Cycler 480 (Roche Diagnostics) using SYBR Green master mix (Roche diagnostics). Relative change in gene expression was calculated using (2-ΔΔCt) method and normalized with endogenous reference gene (18S rRNA). The primer sequences of the genes analyzed is provided in supplementary table 1.

2.9 JC-1 and TMRE staining

JC-1 (M34152) and TMRE (T669) dye were procured from Invitrogen Life Technologies and experiments were performed as manufacturer's protocol. Briefly, fully differentiated adipocytes were given with compound **10C** (1µM) treatment for 72hrsand stained with TMRE (500nm) and JC1 (2µM) for 30mins. The cells were washed with PBS and Images were takenwithLeica DFC450 C microscope.

2.10 Mitochondrial respiration study

To study mitochondrial respiration activity, hMSC were seeded in 8 well flux analyzer cell plate in at a density of 10,000 cells/well and differentiated into white adipocytes as mentioned above. OCR of white adipocytes with/without compound treatment was determined using XFp Extracellular Flux analyzer (Seahorse Bioscience) in presence of 1µM oligomycin, 1µM FCCP

and 0.5 µM Rotenone/Antimycin A mixture. To study metabolic pathway, we used inhibitors of enzyme mitochondrial pyruvate carrier (UK509, 2µM), Glutaminase (BPTES, 3µM) and carnitinepalmitoyltransferase (Etomoxir, 4µM). Readings were normalized with total protein concentration.

2.11 cAMP Assay

25µg of protein was taken for measurement of cAMP level in adipocytes with or without compound **10C** (1µM) treatment for 24hrs according to manufacturer's instruction manual (Cell Signaling Technologies).

2.12 Glycerol release Assay

Glycerol release assay was done using RANDOX kit (GLY 105) purchased from Randox Laboratories, Antrim, UK. Briefly, fully differentiated adipocytes were kept in serum free high glucose media for 2hrs before incubating with varying concentration of compound **10C** for another 2hrs as given in figure legend (Fig. 3C). The supernatant was collected and glycerol was measured as per manufacturer's instruction manual.

2.13 Glut4 translocation Assay

3T3-L1 mature adipocytes were transfected with HA-Glut4-GFP using Gene Pulser electro-poration system (Biorad) and plated onto a coverslip placed in 6 well plate. After 24hrs of electroporation, the media was changed and CI treatment was given with/without 1µM compound **10C** as mentioned in the figure legend. Cells were incubated in high glucose media for 2hrs and 1nM insulin stimulation was given for 20 min. Cells were fixed and incubated with anti HA primary antibody overnight at 4ºC. Cells were washed 3 to 4 times with PBS and

incubated with anti-mouse secondary antibody conjugated with Alexafluor-594 for 30mins. Images were taken on confocal microscope.

2.14 Western immunoblotting

Western blotting was performed as described previously (27340034). Antibodies pAKTSer 473, pAS160 Thr 642 (#8881), AKT protein (#4691), AS160 protein (#2670), pHSLSer 660 (#4126), pHSLSer563 (#4139), HSL protein (#18381), PGC1α (#2178), UCP1 (14670) were purchased from Cell Signaling Technology (Beverly, MA),PRDM16 (106410) from abcam, (Cambridge, USA). Anti-β-actinPKA-Cα (sc-903), and PKA-Cβ (sc-904) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Image J software (NIH) was used for densitometric quantification of protein bands.

2.15 Cell Viability Assay

MTT assay: 3T3-L1 cells were seeded in 96 well plate at a density of 1×10^4 cells per well. Cells were allowed to grow in complete media for 24hrs. Cells were then treated with various concentrations (1 µM to 1000 µM) of compound 10C. After 24hrs media containing compound 10C was removed and cells were incubated for 3hrs with media containing MTT at a concentration of 0.5µg/ml. The supernatant was aspirated and DMSO was added to each well. Absorbance was taken at 570 nm using multi-well plate reader. The result given is the representative of three independent experiments. Error bars in the graph represent mean ± SD.

2.16 Animal care and treatment

The study was conducted in C57BL6 male mice at the animal facility of CSIR- Central Drug Research Institute, Lucknow. All experimental procedures were approved by the institutional

animal ethics committee and were conducted in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEAs), India. The micewere housed at temperature of 23±2 °C; humidity 50–60%; light 300 Lux at floor level with regular 12h light cycle. 24 male mice were housed (six mice/cage) and given water and normal chow diet ad libitum. Compound **10C (**50mg/kg) was given orally to 8 week HFD fed mice. n= 6 mice/group were used in the study. Body weight and food intake of the mice were recorded weekly.

2.17 Organ collection

After 12 weeks of treatment as given in experimental design (Figure 6A) mice were fasted for 6hrs and euthanized using anesthetic ether. Epididymalwhite adipose tissue (eWAT) was excised from mice after acute pulsing with insulin (0.75U/kg). Tissues were processed as per the subjected techniques and/or stored at -80^{0}C till use.

2.18 Intraperitoneal Glucose tolerance test (IPGTT)

IPGTT was performed as described previously [15]. Briefly, mice were fasted for 8hrs. Basal fasting glucose (0th minutes) was measured with the help of Accu-Check Active glucometer by tail cut method. For glucose tolerance test, glucose (2g/kg) was administered intra-peritoneal, and blood glucose was measured at 15, 30, 60, 90, and 120 minutes.

2.19 Insulin tolerance test (ITT)

ITT was performed in 5hrs fasted mice. Regular Insulin (0.75U/kg, Sigma Aldrich, USA) was injected intra-peritoneal, and glucose was measured at 0, 15, 30, 60 and 90 minutes from tail cut method.

2.20 Statistical analysis

Results are expressed as mean ± SD of at least three to five separate experiments. Densitometry of western blots is expressed as mean ± SD of three independent experiments. Statistical significance was determined by Student's t-test and one way ANOVA for real time results.$EC_{50}$was calculated by non-linear regression curve, where actual concentrations are converted to logarithmic value and plotted in log agonist verse response curve(GraphPad Prism version 5). p values < 0.05 were considered significant.

3. Results

3.1 Chemistry:

The synthesis of N-acylated 1- amino 3- aryloxy 2- propanols by known protocol i.e.Ritter reaction. The oxirane were prepared with the substituted phenol with epichlorohydrin in acetone in presence of base followed by ring opening with different benzonitriles. The ring opening reaction of 2-(phenoxymethyl) oxiranes(**6-10**) in benzonitriles in presence of BF_3*OEt_2,led to synthesis of N-(2 hydroxy-3-phenoxypropyl)benzamides (**6A-6D**; **7A-7D**; **8A-8D**; **9A-9D** and **10A-10D**) in good yield.

3.2 Pharmacophore modeling: β3-AR 3D pharmacophore generation

In this work we redeveloped a Pharmacophore model reported previously for β3-AR agonist using the reported dataset as well as parameters. The developed Pharmacophore model has good corroboration between the activity reported and predicted for a set of molecules under study. The redeveloped Pharmacophore model was used as query for predicting the test set of compounds including the series under study for β3-AR. previously. The model was used to predict the

compound 10C which was predicted as active in the reproduced model. The compound 10C was used as a ligand for the Pharmacophore query of β3-AR. The mapping resulted in the predicted activity of 312.411 nM. These results are very good in terms of activity so we preceded our compound for further biological evaluation starting from *invitro* analysis.

3.3 Screening of human β3-AR agonist using CREB luciferase assay system

HEK 293T cells transfected with human β3-AR and CREB-Luciferase (cyclic AMP response element binding protein) plasmids,were treated with series of 20 compounds (500nM) containing common class II β3-ARpharmacophore for 6hrs. Three compounds **6B**, **8D** and **10C** showed a significant increase in luciferase activity, among which compound **10C** had highest luciferase activity, which is represented as fold difference (Figure 2A). The EC_{50} value of Aegelinewas found to be 1.298e-005 (Figure 2B). The compound **10C** treatment for 6 and 12 hrs increased luciferase activity in concentration-dependent manner(Figure 2C and D).Treatment of compound **10C** on HEK 293T cells transfected with human β3-AR increased intracellular cAMP level in concentration dependent manner (Figure 2E). The EC_{50} value of the compound **10C** was found to be 447nM ± 8.00445e-07 and the activity got saturated above 1μm concentration (Figure 2B and C). The compound **10C** showed better human β3-ARactivity compared to class I β3-ARagonist, BRL37344 (Figure 2F). The compound **10C** showed no cytotoxicity on white adipocytes differentiated from hMSCs even at the concentration as high as 100μM (Figure 2G). To check the activity of compound **10C** on other isoforms of β-AR, HEK 293T cells co-transfected with human β1-AR, β2-AR and β3-AR along with CREB luciferasewere treated witheither isoproterenol (10μM) or compound **10C**(1μM) for 6hrs and luciferase assay was performed. Isoproterenol was used as a positive control as it is a non-selective β-AR agonist.

Compound **10C** showed β3-AR specific activity (Figure 2H) which was further validated at various concentrations (Figure 2I).

3.4 Compound 10Cinduces lipolysis and activates β-oxidation.

Expression and phosphorylation of proteins involved in theβ3-AR pathway were found to be increased 15mins onwards incompound **10C**treated white adipocytes.In order to have clear window for biological alterations, we have used 1µM of compound **10C** for treatment in general unless otherwise specified. The EC_{50} value was found to be 447nM and its activity saturated at concentration above 1µM as observed in figure (1B and 1C). We observed increased expression of protein kinase A (PKA) catalytic α and β subunit on treatment with compound **10C**inwhite adipocytes. Phosphorylation of HSL at both Ser 563 and 660 was increased on treatment with compound **10C**. UCP1 expression was also found to be increasedon treatment with compound **10C** (Figure 3A& Supplementary Figure 2A). We found the concentration dependent increasein the expression and phosphorylation of the above mentioned proteins involved in theβ3-AR pathway on treatment with compound **10C** in white adipocytes differentiated from hMSCs. Concentration dependent activation of the β3-AR pathway on treatment with the compound **10C** was more prominent in brown adipocytes compare to white adipocytes (Figure 3B&Supplementary Figure 2B). This might be due to higher expression of β3-AR in brown adipocytes compared to white adipocytes[18]. The compound **10C** showed concentration dependent increase in lipolysis as evident from the glycerol release assay (Figure 3C). To confirm whether the compound **10C** induces lipolysis, white adipocytes were treated with increasing concentration of compound **10C** (100nm to 100µM) for 72hrs. Oil red O staining of white adipocytes treated with the compound **10C** indicate decreased lipid content with increasing concentration of compound **10C,** as evident from the image (Figure 3D) and Oil red O

absorbance (Figure 3E). White adipocyte on treatment with the compound **10C** for 20mins (acute) showed increased maximal respiration and spare respiratory capacity in presence of proton gradient uncoupler FCCP (Figure 3F). Oxygen consumption rate was measured in the compound **10C** treated white adipocytes in presence of different metabolic pathway inhibitors such as UK509 (Mitochondrial pyruvate carrier inhibitor) for the glycolytic pathway, BPTES (Glutaminase inhibitor) for the Glutamate pathway andEtoxomir (Carnitinepalmitoyltransferase 1A inhibitor) for the β-oxidation (Figure3G). The compound **10C** treated white adipocytes showed significant reduction in OCR in presence of CPT1A inhibitor, which in turn indicates that these cells utilize more fatty acid as fuel for β oxidation compared to untreated adipocytes. The above results thusconfirm that compound **10C** increases lipolysis and β oxidation.

3.5 Chronic treatment of compound 10C induces transdifferentiation from white to brown adipocyte:

Above experimental results of mitochondrial respiration study and mechanistic study of the compound **10C** showed induction of brown adipocyte properties. β3-ARagonist are known to induce non-shivering thermogenesis and browning phenomenon. As anticipated white adipocytes treated with compound **10C** for 72hrs time period showed increased expression of transcription factors associated with brown adipocytes such as PRDM16, PGC1α, NRF1, DIO2, CIDEA, and EVA1 (Figure4A). The compound **10C** treatment also led to the increased expression of genes related to mitochondria such as UCP1, COX, CytC, ATP5G1 and fatty acid oxidation related genes such as CPT1A, MCAD FATP1 (Figure 4B and C). One of the strikingobservation is increased expression of Carnitinepalmitoyltransferase 1A (CPT1A) enzyme in compound**10C** treated white adipocytes. Compound **10C** increased expression of brown adipocyte specific markers such as PRDM16, UCP1 and PGC1α expression at protein level in both cases **a**) hMSC

differentiated to white adipocytes in presence of compound **10C**(1μM) . **b**) Fully mature white adipocytes given 72hr treatment of compound **10C** (1μM) (Figure 4D). The above experiment suggests that the compound **10C**not only induces browning phenomenon in white adipocytes but is also capable of differentiating hMSC to brown-like adipocytes in absence of browning inducing agent like troglitazone. White adipocytes on 72hrs (chronic) treatment with compound **10C** showed increased basal OCR, maximal respiration as well as increased spare respiratory capacity (Figure4E). This indicates compound **10C** increase mitochondrial respiration either by increasing expression of the UCP-1 protein (Fig 4 C &D) or by increasing mitochondrial biogenesis. We wanted to assess whether the increase in basal OCR incompound **10C** treated white adipocytes is due to increase in mitochondrial biogenesis or not. For the same, we performedTMRE and JC1 staining of white adipocytes treated with 1μM of compound **10C** for 72hrs. We observed increased mitochondrial biogenesis and membrane potential in compound **10C** treated adipocytes (Figure 4F and G). This further confirmsahigher number of functionally active mitochondria,a hallmark feature of brown adipocyte in white adipocytes treated with compound **10C**. These studies confirm white to brown trans-differentiation potential of the compound**10C**.

3.6 Compound 10C reverses chronic insulin induced insulin resistance in white adipocytes differentiated from hMSC.

Chronic insulin induced IR adipocytes were treated withcompound **10C**(1μM) for 24hrs and glucose uptake was measured after stimulation with 10nm ofinsulin for 20mins as given in (Figure 5A). Compound **10C** treated CI induced IR adipocyte showed increased glucose uptake upon insulin stimulation compared to CI induced IR adipocytes. The glucose uptake in compound **10C** treated CI induced IR adipocytes upon insulin stimulation were similar to control

adipocytes (Figure 5A). We also observedasignificant increase in phosphorylation of AKT and AS160 at residue Ser473 and Thr642 respectively, upon insulin stimulation in 24hrscompound **10C** treated IR adipocytes compared to that of untreatedIR adipocytes. The phosphorylation levels were similar to control adipocytes (Figure 5B). We also performed Glut4 translocation assay in the CI induced IR by transfecting with HA-GLUT4-GFP. The CI induced IR adipocytes were given 1μM compound **10C** treatment for 2hrs before stimulation with 10nM insulin and fixed for Glut4 translocation assay.Anti-HA tagged with Alexa flour-594 was used to detect membrane bound Glut4, as the Glut4 binds to membrane the HA tag is exposed to outer surface which binds to anti-HA tagged with Alexa-594. Glut4 translocation assay further confirmed that on 0.5nM insulin stimulation compound **10C** treated CI induced IR adipocytes showed increased Glut4 translocation compared to IR adipocytes, which was evident on microscopic observation as well as quantification of red/green intensity provided by the image (Figure 5C & D).

3.7 Compound 10Ctreatment improved lipid profile and reversed HFD induced insulin resistance in C57BL/6 mice

A schematic representation of experiment design indicating compound treatment in 8 weeks HFD fed mice (Figure 6A). The compound **10C** treatment to 8 weeks HFD fed mice did not alter food intake but reduced whole body weight (Figure 6B and C).The compound **10C** treated mice had decreased eWAT weight compared to untreated HFD fed mice (Figure 6D). Morphological analysis by HE staining of eWAT indicated decreased adipocytes hypertrophy in compound **10C** treated mice (Figure 6E-G). Compound **10C** ameliorated HFD induced dyslipidemia in terms of significantly decreased serum triglyceride and increased serum HDL level (Figure 6H & K). We also found decrease pattern but not significant in serum total cholesterol and LDL after

compound **10C** treatment (Figure 6I & J). Compound **10C** treatment led to increased glucose tolerance and insulin sensitivity as evident by ITT and IPGTT (Figure 6L-O).

We did serum analysis of recommended hormones. In this cohort, compound 10C attenuated HFD induced hyperinsulinemia and hyperlpetinemia(Supplementary Figure 3C). Results were also matched with reduced adipocyte hypertrophy as observed in H&E staining. To check the molecular basis of increased insulin sensitivity, we probed proteins involved in insulin signaling. We found increased phosphorylation of AKT and AS160 in eWAT of the compound **10C** treated mice compared to untreated mice (Figure 6P).In line with *in vitro* findings, we checked brown adipocyte specific markers in the compound **10C** treated mice. We found increased expression of Cpt1A, Ucp1, Pgc1α, which are known to induce browning characteristics (Figure 6Q).Similar to *in vitro* results, the compound **10C** activated β3-AR pathway as evident by increased phosphorylation of HSL at Ser 563 and increased expression PKA-Cα in eWAT of HFD fed mice (Figure 6R). From the above results, it can be concluded that the compound **10C** increases insulin sensitivity and improve lipid profile by inducing browning phenomenon in eWAT of HFD fed mice.

4. Discussion

A larger percentage of therapeutic drugs available today are inspired from natural products and their synthetic moieties. In an endeavor to identify novel natural products or its analogues capable of trans-differentiating white adipocyte to brown, we screened synthetic analogues of Aegeline previously reported for antidyslipidemic activity and predicted to be β3-AR agonist[1]. The compound **10C** identified in screening, induced browning of white adipocyte *in vitro*, activated β3-AR pathways leading to increased lipolysis and β-oxidation and increased OCR by

increasing mitochondrial biogenesis and functionality. Compound**10C**also improved lipid profile and insulin sensitivity in HFD fed mice.

Since the discovery of brown adipose tissue in adult humans in late 1990[19], the scientific community has considered activating brown adipose tissue as a mean to utilize excess stored fat[20]. Cloning ofhuman β3-AR receptor has further revitalized above idea as the chronic treatment of β3-AR agonist can improve the BAT content in adult humans[21]. The anti-obesity effect of β3-AR agonist corresponds to the activation of thermogenesis in BAT by UCP1 and activation of lipolysis in WAT[22, 23]. Chronic stimulation of β3-AR in obese animals reduced adiposity and increased expression of the UCP1 protein in adipose tissue[24]Given the background, chronic treatment of compound **10C** increased lipolysis assessed in terms of glycerol release and increased UCP1 expression. As anticipatedthe compound increased fatty acid oxidation as evident by increased expression of genes involved in fatty acid oxidation such as CPT1A, FATP1 and mCAD. CPT1A is the main carrier of fatty acid from cytosol to mitochondria where fatty acid is oxidized[25]. By measuring the decrease in OCR in presence of CPT1A inhibitor on compound treated adipocytes, we were able to calculate the fatty acid oxidation rate on compound treatment.

In the past decade, many novel β3-AR agonists have been developed. SWR-03425A, a novel human β3-AR agonist significantly decreased blood glucose and serum insulin level in KK-Ay mice[26]. Similarly, Mirabegronanother human β3-AR agonist currently approved by FDA to treat overactive bladder syndrome has shown to activate brown adipose tissue in human and increase resting metabolic rate[27]. Chronic treatment of Mirabegronalso improved glucose disposal rate and insulin sensitivity in HFD fed mice[27]. The above studies prove beyond any doubt that β3-ARagonists are capable of reversing insulin resistance and increase insulin

sensitivity. The lipolytic action of compound **10C** is partly mediated by adenylatecyclase activity, which in turn increases intracellular cAMP level and partly by activation of HSL by activated PKA[28]. We further used this opportunity to test the compound **10C** in CI induced insulin resistance model developed by us in white adipocytes differentiated from hMSCs [15]. The compound **10C** reverted CI induced insulin resistance as evident from increased phosphorylation of protein involved in insulin signaling, glucose uptake and GLUT4 translocation. Our *in vivo* results validates that compound **10C**increases insulin sensitivity by activating β3-AR pathway and induces browning like characteristics in eWAT as evident from increased expression of UCP1, PGC1α, PKA-Cα and increased phosphorylation of HSL at Ser 563 residues.Although, we have already observed elevated energy expenditure related genes in eWAT of compound 10C treated mice (Figure 6Q). Therefore, to address this query, we did relative gene expression analysis in subcutaneous white adipose tissue (SAT) also. SAT is predominantly reported for browning and thermogenesis over visceral adipose tissue (e.g. eWAT)[29, 30]. In SAT of compound 10C treated mice, we found significantly elevated expression of *Pgc1α, Ucp1, Elvol3,* and *cpt1* genes as compared to untreated HFD fed group(Supplementary Figure 3A). Morphological analysis by H&E staining showed beige phenotype in the SAT of compound 10C treated mice(Supplementary Figure 3B). Taken together, these results suggested that compound 10C promoted energy expenditure and browning in SAT and therefore, exerted anti-obesity effects. Given the account, compound **10C** demonstrated overall benefit in altered metabolic status in HFD fed C57BL/6 mice in terms of improved dyslipidemia and insulin sensitivity.

In summary, Aegeline inspired synthesis led to identification of chemical compound**10C** capable of specifically activatingβ3-AR in nanomolarEC_{50} range. Compound **10C**is capable of inducing

browning in white adipocyte and has trans-differentiating potential. Given the bioactivity and mechanistic profile of the compound, compound **10C** has an overall beneficial effect in metabolic disorders in terms of increased lipolysis, increase thermogenesis, decreased dyslipidemia and betterment in glucose and insulin tolerance in HFD fed animal models. Although more insights and further developments are warranted, nevertheless, Aegeline inspired synthetic compound **10C** can be lead compound for trans-differentiating white to brown adipocytes.

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We are grateful to Dr. SabyasachiSanyal for gifting CREB-Luciferase reporter plasmid for development of β3-AR agonist screening platform. We are also thankful to staff of sophisticated analytical instrumentation facility (SAIF) for providing access to intravital microscopy and spectral data. We acknowledge the help provided by Prof. Nuzhat Husain (Director) Dr. Ram ManoharLohia Institute of Medical Sciencefor approval from stem cell ethics committee. We also acknowledge the help and support provided by staff of Sushrut Institute of Plastic Surgery (SIPS) Hospital for collection of liposuction sample.

6. Authors Contribution

Sujith Rajan: Wrote the manuscript and performed in-vitro experiments**. SabbuSatish:** Synthesized the compounds and wrote the manuscript. **Kripa Shankar:** Performed in vivo studies and checked the manuscript. **Sukanya Pandeti:** Synthesized compound and checked the manuscript. **Salil Varshney:** Checked the manuscript and performed statistical analysis.**Ankita Srivastava:** Performed experiment and check the manuscript. **Durgesh Kumar:** Performed in vivo studies and checked the manuscript. **Abhishek Gupta:** Checked and formatted the

manuscript.**Sanchita Gupta:**Performed experiment and checked the manuscript. **Rakhi Choudhary:**Performed in-silico screening and developed model.**Vishal M. Balaramnavar:** Performed in-silico analysis and checked the manuscript. **Tadigoppula Narender:** Conceptualized the idea and checked the manuscript. **Dr. Anil N Gaikwad:** Conceptualized the idea and corrected the manuscript.

7. Funding

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Figure Legend

1. Structure of compounds

The structure of compounds, Aegeline (A), CL316243 (B), BRL37344 (C) and Compound 10C (D). Synthesis of Compound 10 C (E).x

2. Screening of human β3-AR agonist using CREB luciferase assay system

HEK 293T cells transfected with human β3-AR and CREB- luciferase were treated with 500nM of each compound for 6 hrsand luciferase activity was measured using Perkin Elmer Envision 2100 luminometer All Luciferase activity was normalized with GFP.The increase in luciferase activity is represented as fold over control. n=3, error bars represent SD, *P<0.05, **p<0.01, ***P<0.001 as compared by paired Student *t* test (**A**). Dose response curve of Aegeline: HEK 293T cells transfected with β3-AR and CREB luciferase were treated with log concentration (1nm, 3nm, 10nm, 30nm, 100nm, 300nm, 1μM, 3μM and 10μM) of compound Aegeline for 6hr time interval.EC_{50} was calculated using Graphpad non-linear regression curve. n=3, error bars represent SD (**B**). Dose response curve of compound **10C**: HEK 293T cells transfected with β3-AR and CREB luciferase were treated with log concentration (1nm, 3nm, 10nm, 30nm, 100nm, 300nm, 1μM, 3μM and 10μM) of compound **10C** for 6 and 12hr time interval. EC_{50} was calculated using Graphpad non-linear regression curve.n=3, error bars represent SD (**C** and **D**). HEK 293T cells were transfected with β3-AR and treated with log concentration of compound **10C** for 6hrs. The cells were lysed and intracellular cAMP level was measured using kit. n=3(**E**). HEK 293T cells transfected with β3-AR and CREB- luciferase were treated with 1μM of compound BRL37344 or**10C** at for 6hrs. The luciferase activity was normalized with GFP and is represented as fold over control. n=3, error bars represent SD. *p<0.05, **p<0.01,***p<0.001 as

tested by Student *t* test (**F**). White adipocytes differentiated fromhMSC was treated with log concentration of compound **10C** for 24hrs and MTT activity was performed Absorbance was measured at 540nM. n=3, error bars represent SD (**G**). HEK 293T cells transfected with β1, β2 and β3-AR along with CREB luciferase were given compound **10C** (1μM) or isoproterenol (10μM) treatment for 6hr as shown in figure G. n=3, error bars represent SD. *p<0.05, **p<0.01,***p<0.001 as tested by Student *t* test (**H**). Similar to above experiments, human β1-AR, β2-AR and β3-AR along with CREB luciferase reporter transduced HEK cells were given compound **10C** treatment(Log concentration) for 6hr interval and luciferase reading was taken. (**I).** All luciferase readings were normalized with GFP. n=3, error bars represent SD.

3. Compound 10C induces lipolysis and activates β-oxidation.

White adipocytes differentiated from hMSC were treated with 1μM compound **10C**. Protein was isolated at different time intervals as given in figure and subjected to western blot analysis for following proteins, PKA-Cα, PKA-Cβ, pHSL (Ser 563 and 660 residue), HSL protein, UCP-1 and actin (**A**). White and brown adipocytes differentiated from hMSC were treated with increasing concentration of compound **10C** for 30mins as given in figureB. Protein was isolated and subjected to western blot analysis for following proteins PKA-Cα, PKA-Cβ, pHSL (Ser 563 and 660 residue), HSL protein, UCP-1 and actin (**B**). Glycerol release assay: white adipocytes were treated with varying concentration of compound **10Cfor 24hrs**as shown in figure and glycerol release was measured as per manufactures protocol. n=3, error bars represent SD (**C**). Fully mature white adipocytes were treated with varying concentrations of compound **10C** for 72hrs. White adipocytes were stained with ORO and images were acquired on Leica DFC450 C microscope at 10 and 40X (upper left corner) magnification (**D**). Accumulated ORO was extracted and absorbance was measured at 490 nm (**E**). n=3, error bar indicate SD, ***P<0.001,

P<0.01 and *P<0.05 as tested by Student *t* test. Mitochondrial respiration study: white adipocytes were treated with 1µM compound **10C for 20mins before measuring OCR in presence of ATP synthase inhibitor (1µM oligomycin), proton uncoupler (1µM FCCP) and electron transport chain inhibitors (0.5µMRotenone/Antimycin mix) was measured with Seahorse Bioscience XFp Extracellular Flux analyzer, n=3, error bars represent SD, **P<0.005, ***P<0.001 as tested by Student t test (**F**). OCR of white adipocytes with and without **10C** treatment for 72hrs were measured in presence of UK509 (2µM) (Mitochondrial pyruvate carrier inhibitor), BPTES (3µM) (Glutaminase inhibitor), Etoxomir (4µM) (Carnitinepalmitoyltransferase 1A inhibitor) (**G**). The below graph shows OCR calculated in presence of above mentioned inhibitors with/without compound treatment. The decrease in OCR in presence of various inhibitors as shown in graph corresponds to metabolic pathways glycolysis, glutamate pathway and β-oxidation pathway. n=3, error bars represent SD, ***P<0.001 as compared by paired Student *t* test (**G**).

4. Chronic treatment of compound10C induces browning of white adipocytes.

Expression profile of transcription factors in white adipocytes treated with compound **10C** at 1µM concentration for 72hrs. n=3, error bars represent SD, ***P<0.001 as tested one way ANOVA and Bonferroni's post-test analysis (**A**). Real-Time PCR analysis of genes related to mitochondrial biogenesis and fatty acid oxidation in white adipocytes treated with compound **10C** at 1µM concentration for 72hrs. n=3, error bars represent SD, ***P<0.001 as tested two way ANOVA and Bonferroni's post-test analysis (**B** and **C**). Western blot analysis of PRDM16, PGC1α and UCP1 in white and brown adipocytes treated with compound **10C** at 1µM concentration for 72hrs and hMSC differentiated to white adipocytes in presence of compound **10C** (1µM) which is denoted by symbol * in the figure. Densitometry of representative blots

normalized with actin. n=3, error bar indicate SD, ***P<0.001, **P<0.01 and *P<0.05 as tested by Student t test (**D**).White adipocytes were treated with compound **10C** for 72hrs and OCR was measured at basal and in the presence of ATP synthase inhibitor (1µM oligomycin), proton uncoupler (1µM FCCP) and electron transport chain inhibitors (0.5µMRotenone/Antimycin mix) was measured with Seahorse Bioscience XFp Extracellular Flux analyser, n=3, error bars represent SD, ***P<0.001 as tested by Student t test (**E**).White adipocytes were treated with 1µM **10C** for72hrs and stained with TMRE dye. Cells were excited at 405nM and image was taken at 20X magnification using Leica DFC450 C microscope. The graph besides the image shows the relative quantification of mean red intensity normalized with DAPI. n=3, error bars represent SD, ***P<0.001 as tested by Student t test (**F**). Similar to above experiment white adipocytes were treated with 1µM **10C** for72hrs prior staining with JC-1 dye. Cells were excited with 405 or 488nM and Image was taken at 20X magnification using Leica DFC450 C microscope. Images were analyzed using image J software. The red fluorescence show high membrane potential mitochondria (J aggregates) and green florescence show low membrane potential mitochondria (L monomers). The graph on the right hand side is the relative quantification of red to green ratio normalized with nuclear stain DAPI. n=3, the error bars denote SD, **P<0.001 as tested by Student t test (**G**).

5.Compound 10C increases AKT phosphorylation and reverses chronic insulin induced insulin resistance in white adipocytes differentiated from hMSC.

Control and CI treated adipocytes were incubated with or without 1µM compound **10C** for last 24hrs of CI treatment. 10nM insulin stimulation was given as indicated in the figure. Glucose uptake reading was normalized with total protein concentration. n=3, the error bars indicate SD, ***P<0.001 as tested by one way ANOVA and Bonferroni post-test analysis (**A**). Similar to

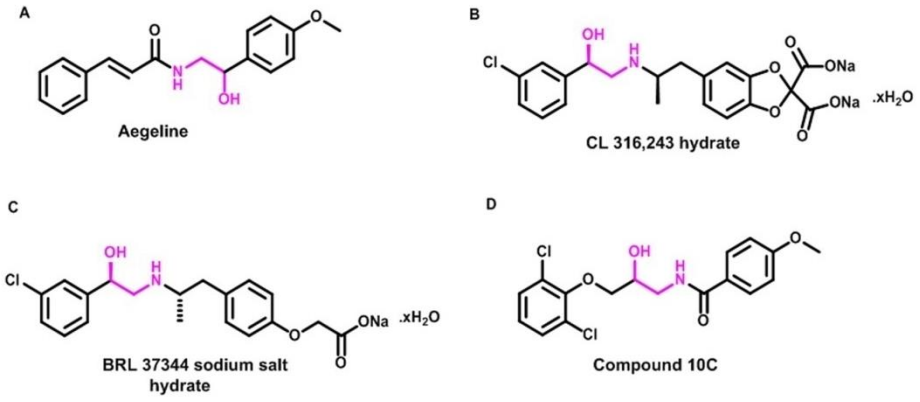
above experiment,CI induced IR adipocyteswere incubated with or without compound **10C** (1μM) for last 24hr of CI treatment. After 10nM insulin stimulation for 20min as shown in figure, protein was isolated and subjected to western blot analysis of pAKT (Ser 473), pAS160 (Thr 642) and their respective proteins. The graph adjcentto the blots show the densitometry of representative blots normalized with respective proteins. n=3, error bar indicate SD, ***P<0.001, **P<0.01 and *P<0.05 as tested by Student t test (**B**). Glut4 translocation assay using white adipcoytes transfected with HA-Glut4-GFP. Left panel shows the nucleus staining with DAPI. The second left panel show the GFP fluorescence and middle panel are the Alexafluor-594 fluorescence. Right panel shows the merged images which indicated complete overlap of three probes in CI induced IR adipocytes with or without **10C** treatment. Insulin stimulation was given as shown in figure. The images were taken at 20x magnification using intravital microscope. The graph above the image is the relative quantification of red to green ratio normalized with nuclear stain DAPI. n=3, the error bars denote SD, **P<0.001 as tested by Student t test(**C & D**).

6. Compound 10C reverse HFD induced insulin resistance in C57BL6 mice

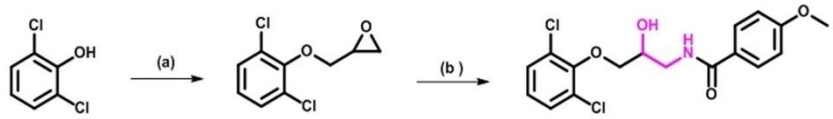
A schematic representation of *in vivo*experimental design in C57BL6 mice. (A). Food intake of HFD fed group with or without compound **10C** and chow fed animals for the 12 week time period (B). Body weight in percentage of chow and HFD fed mice treated with or without compound **10C**. (C).eWAT weight/Body weight of chow and HFD fed mice treated with or without compound **10C**, n=6, **P<0.01 as tested by one way ANOVA and Bonferroni posttest analysis (D).H & E staining of eWAT taken at 40X, scale bar represents 100μM. Adipocytes area was calculated by using FijiJ software and analyzed by column distribution analysis, n=6, error bars represent SEM, *P<0.05 as tested by student t test (E-F). Lipid profile of chow and HFD fed mice treated with or without compound **10C**, Serum triglyceride (H), Serum cholesterol

(I), Serum LDL (J), Serum HDL (K). Error bars represent SD, *P<0.05, **P<0.01 & ***P<0.001as tested by one way ANOVA and Bonferroni posttest analysis. IPGTT and its area under curve (AUC) of chow and HFD fed mice treated with or without compound **10C**. Error bars represent SD, **P<0.01 & ***P<0.001as tested by one way ANOVA and Bonferroni posttest analysis (L & M). ITT and its area under curve (AUC) of chow and HFD fed mice treated with or without compound **10C**. Error bars represent SD, **P<0.01 & ***P<0.001as tested by one way ANOVA and Bonferroni posttest analysis (N & O). Protein isolated after insulin pulsing from eWAT of chow and HFD fed mice treated with or without compound **10C**were subjected to western blot analysis. Western blot analysis of pAKT Ser473, Thr308, pAS160 Thr642 and their respective proteins, n=3 mice/group (P). Quantitative gene expression analysis of Cpt1A, Ucp1 and Pgc1α in eWAT of chow and HFD fed mice treated with or without compound **10C**,Error bars represent SD, **P<0.01 & ***P<0.001as tested by one way ANOVA and Bonferroni posttest analysis (Q). Western blot analysis of PKA-Cα, pHSL Ser563, HSL protein, UCP1 and actin in eWAT of chow and HFD fed mice with or without compound **10C** treatment, n=3 mice/group (R). In this experiment n=6 mice/group were used or otherwise indicate.

Figure 1



E Synthesis of Compound 10C :



Reagents and Conditions: (a) Epichlorohydrin, K_2CO_3, acetone, reflux, 8 h ; (b) 4-methoxy benzonitrile, $BF_3.OEt_2$, DCM, rt-70 °C, 4 h

Figure 2

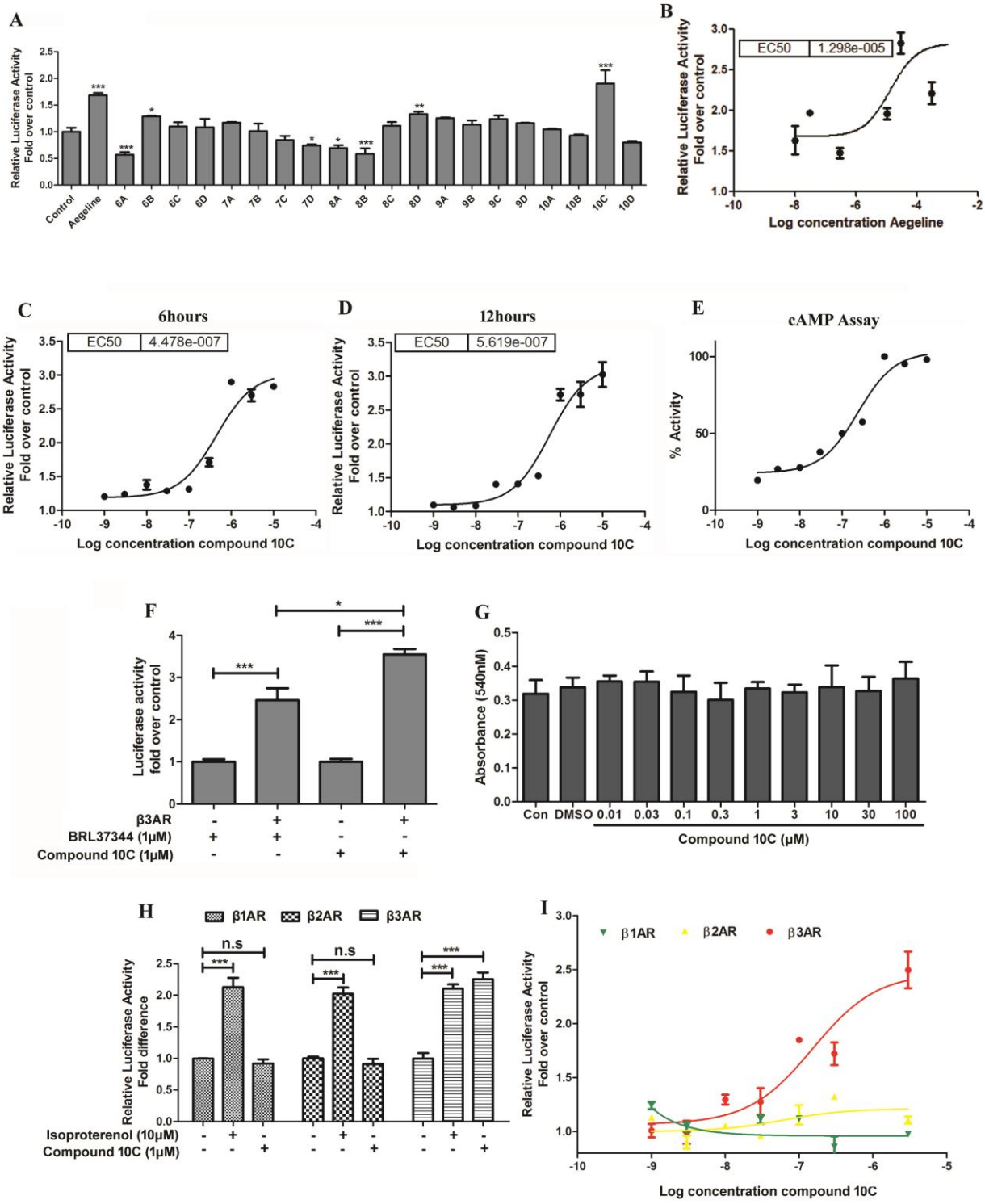


Figure 3

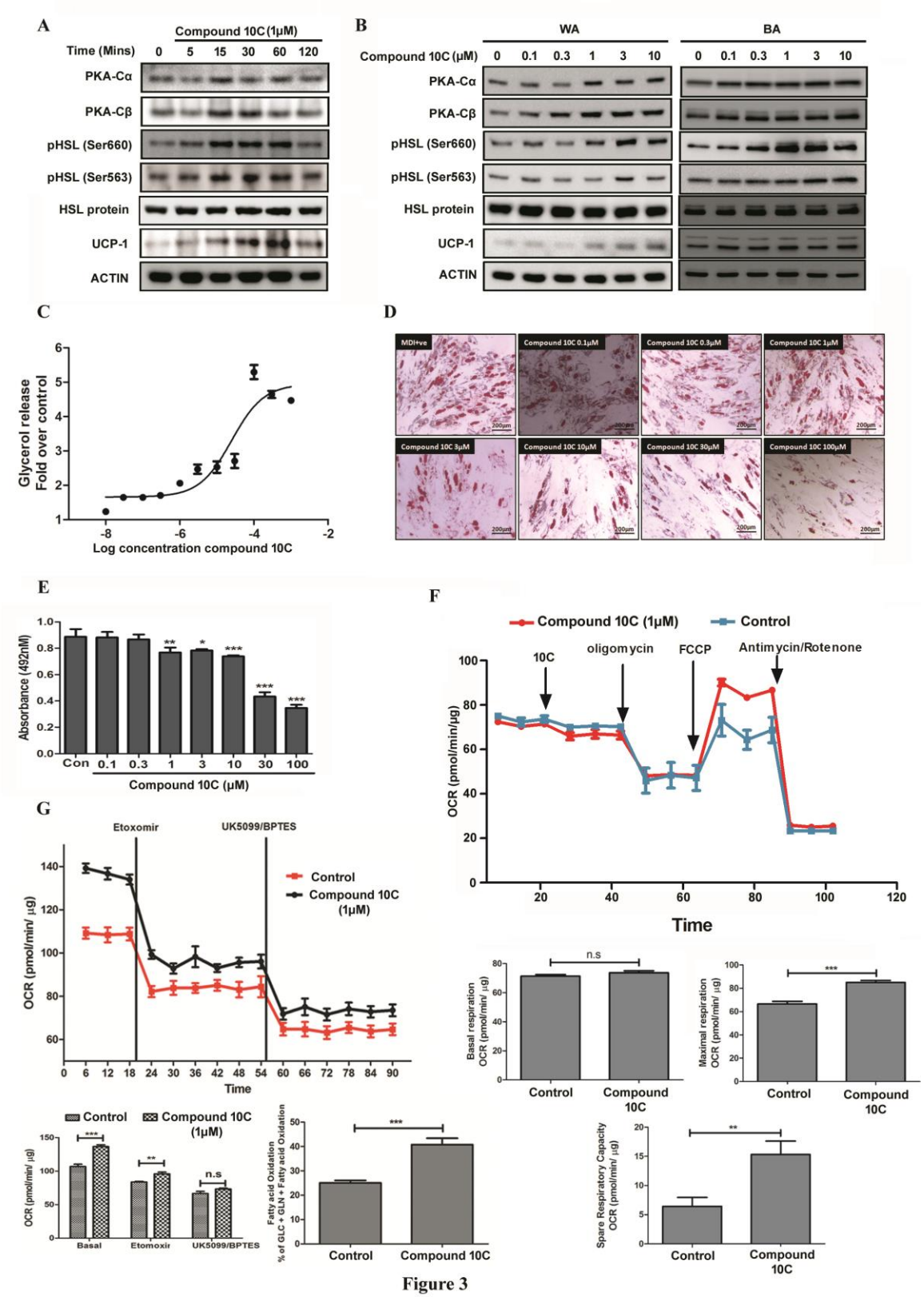


Figure 3

Figure 4

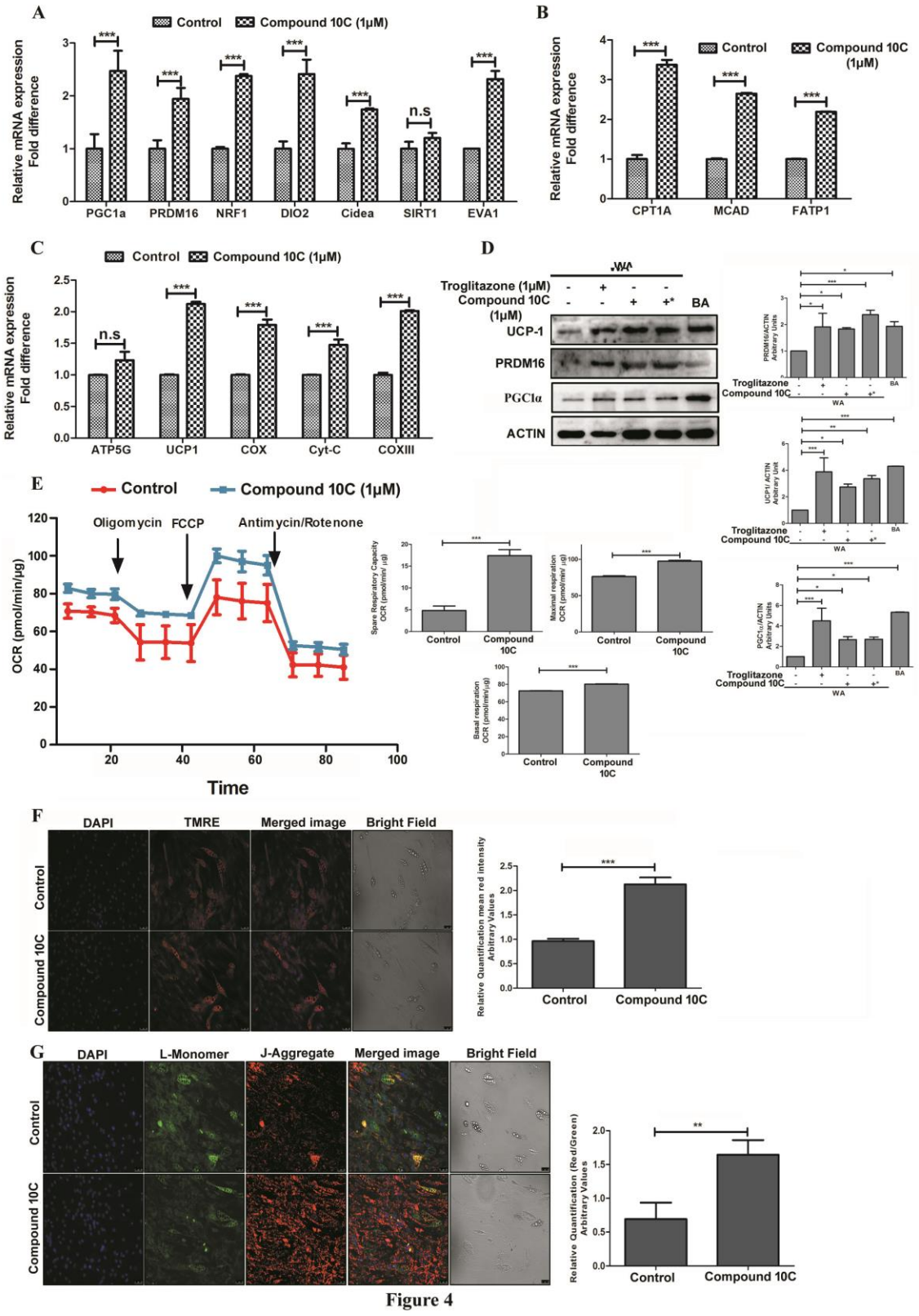


Figure 4

Figure 5

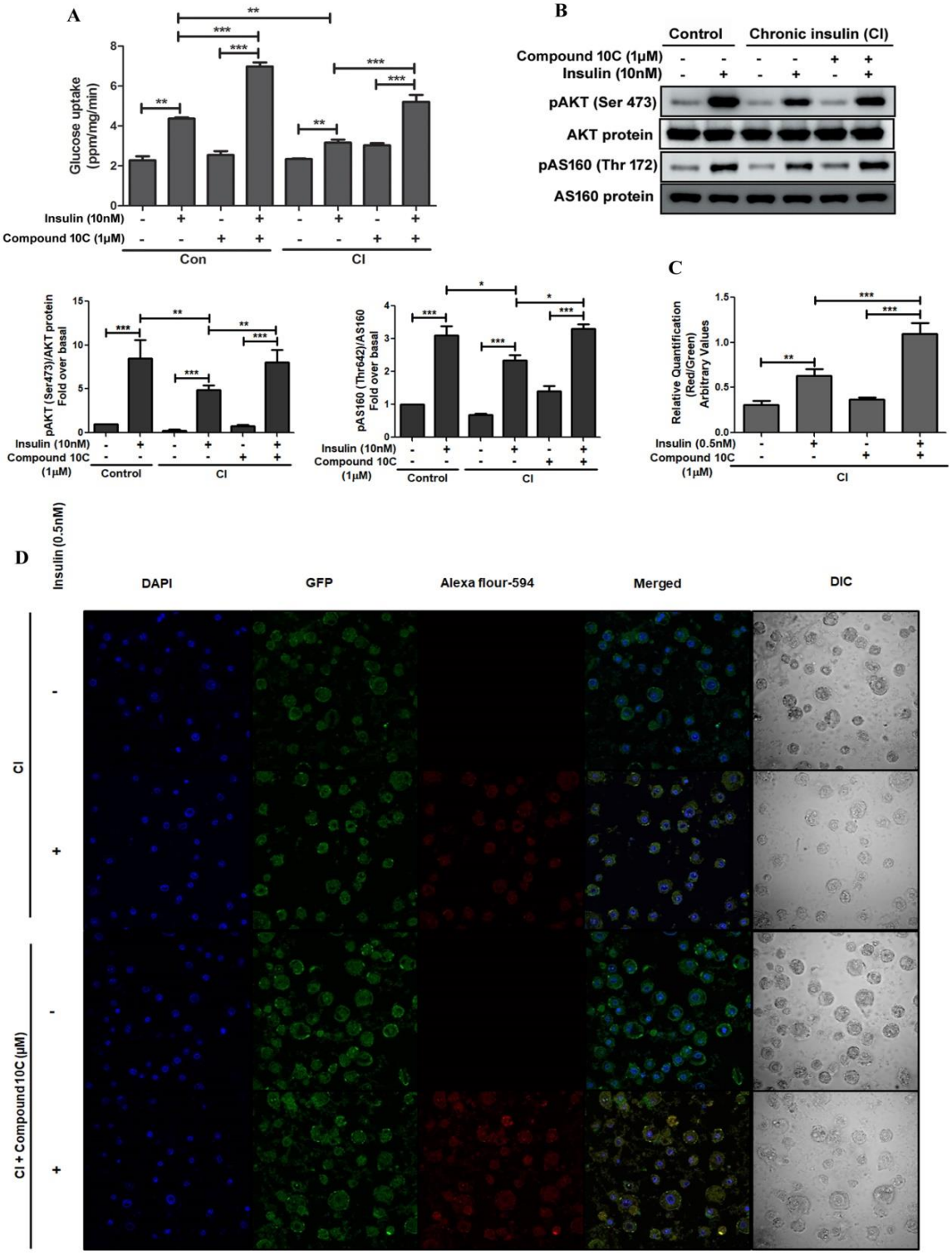
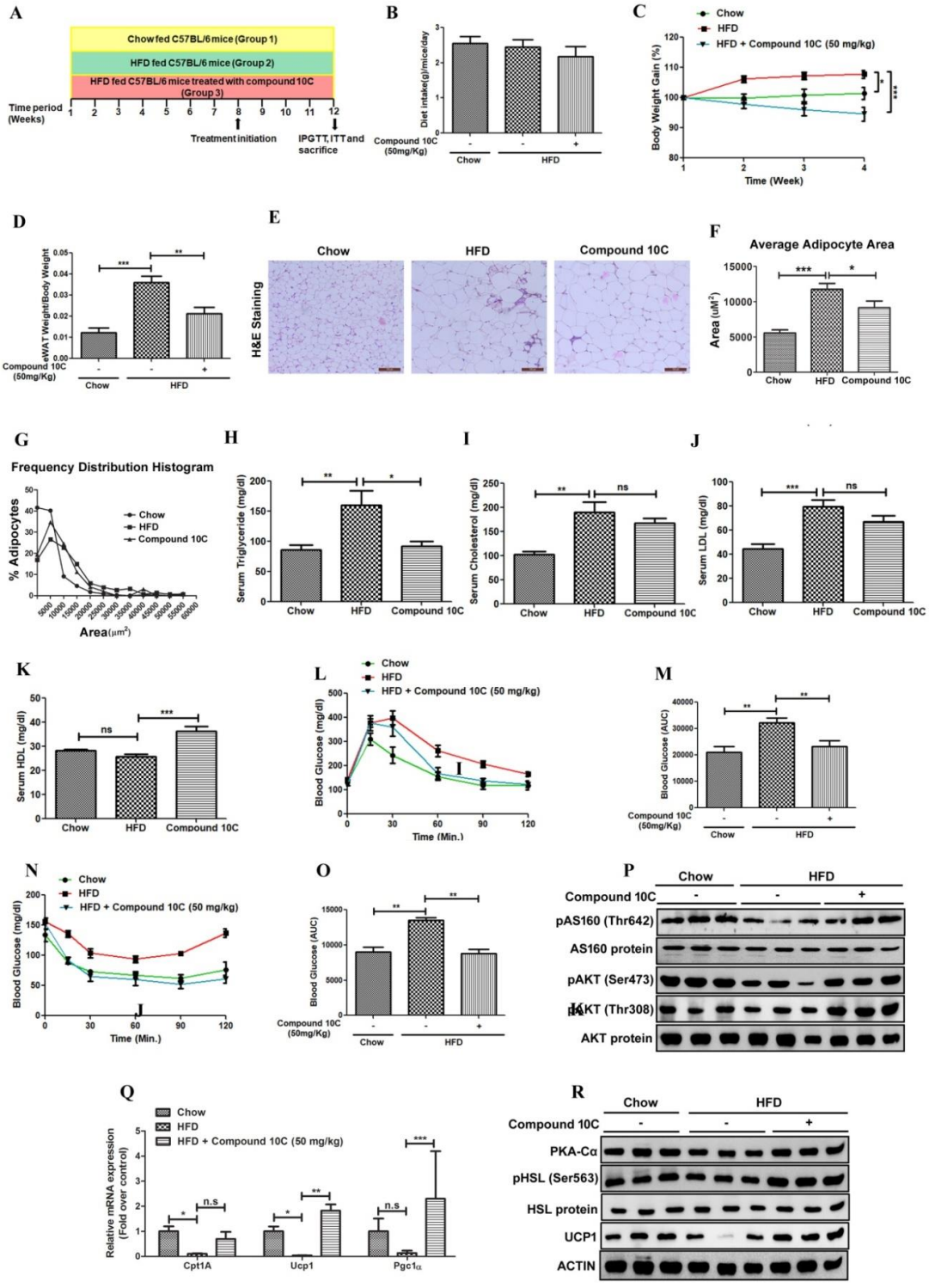


Figure 6



Highlights

- **Compound 10C is a synthetic molecule inspired from natural compound Aegeline exhibiting β3-AR agonistic activity (EC_{50}-447nM).**
- **Compound 10C induces browning in human mesenchymal stem cell derived white adipocytes.**
- **Compound 10C improves insulin sensitivity in both *in vitro* and *in vivo* models of insulin resistance**
- **Our studies indicate salutary potential of this novel β3-AR agonist inspired from Aegeline for reversing insulin resistance.**