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# Antidiabetic features of AdipoAI, a novel AdipoR agonist.

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**Abstract**

Adiponectin is an antidiabetic endogenous adipokine that have protective role in unfavorable metabolic sequalae arising from obesity. Recent evidence suggests a sinister link between hypoadiponectinemia and development of insulin resistance/ type 2 diabetes (T2D). The insulin sensitizing property of adiponectin is through specific adipoq receptors R1 & R2, activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)α pathways. AdipoAI is a novel synthetic analogue of endogenous adiponectin with possibly similar pharmacological effects. Thus, there is a need of orally active small molecules that activate Adipoq subunits, and its downstream signaling could ameliorate obesity related type 2 diabetes. This study was aimed to access the effects of AdipoAI on obesity and T2D. Through *in-vitro* and *in-vivo* analyses, we investigated the anti-diabetic potentials of AdipoAI and compared it with AdipoRON, another orally active adiponectin receptors agonist. Our results showed that *In-vitro* treatment of AdipoAI (0-5μM) increased adiponectin receptor subunits AdipoR1/R2 with increase in AMPK and APPL1 protein expression in C2C12 myotubes. Similarly, *in-vivo*, oral administration of AdipoAI (25 mg/kg) observed similar effects as that of AdipoRON (50 mg/kg) with improved control of blood glucose and insulin sensitivity in diet-induced obesity (DIO) mice models. Further, AdipoAI significantly reduced epididymal fat content with decrease in inflammatory markers and increase in PPAR-a and AMPK levels and exhibited hepatoprotective effects in liver. Further, AdipoAI and AdipoRON also observed similar results in adipose tissue. Thus, our results suggest that low doses of orally active small molecule agonist of adiponectin AdipoAI can be a promising therapeutic target for obesity and T2D.

Antidiabetic features of AdipoAI, a novel AdipoR agonist.

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**Running title** : AdipoAI improves type 2 diabetes.

Abstract

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Adiponectin is an antidiabetic endogenous adipokine that have protective role in unfavorable metabolic sequalae arising from obesity. Recent evidence suggests a sinister link between hypoadiponectinemia and development of insulin resistance/ type 2 diabetes (T2D). The insulin sensitizing property of adiponectin is through specific adipoq receptors R1 & R2, activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)α pathways. AdipoAI is a novel synthetic analogue of endogenous adiponectin with possibly similar pharmacological effects. Thus, there is a need of orally active small molecules that activate Adipoq subunits, and its downstream signaling could ameliorate obesity related type 2 diabetes. This study was aimed to access the effects of AdipoAI on obesity and T2D. Through *in-vitro* and *in-vivo* analyses, we investigated the anti-diabetic potentials of AdipoAI and compared it with AdipoRON, another orally active adiponectin receptors agonist. Our results showed that *In-vitro* treatment of AdipoAI (0-5μM) increased adiponectin receptor subunits AdipoR1/R2 with increase in AMPK and APPL1 protein expression in C2C12 myotubes. Similarly, *in-vivo* , oral administration of AdipoAI (25 mg/kg) observed similar effects as that of AdipoRON (50 mg/kg) with improved control of blood glucose and insulin sensitivity in diet-induced obesity (DIO) mice models. Further, AdipoAI significantly reduced epididymal fat content with decrease in inflammatory markers and increase in PPAR-α and AMPK levels and exhibited hepatoprotective effects in liver. Further, AdipoAI and AdipoRON also observed similar results in adipose tissue. Thus, our results suggest that low doses of orally active small molecule agonist of adiponectin AdipoAI can be a promising therapeutic target for obesity and T2D.

**Keywords:** Adiponectin, AdipoAI, Obesity, Type 2 diabetes, Inflammation, Diet induced obesity

**Significance Statement**

Obesity is a concerning health issue, and has doubled in more than 70 countries, resulting in escalation of obesity-related disease like type 2 diabetes (T2D). The adiponectin receptors (AdipoR1 and AdipoR2) have emerged as important targets for controlling inflammation, obesity and T2D. To maximize the potential of adiponectin receptors in targeting the fundamental etiopathology of obesity and T2D, we have designed, synthesized, Adiponectin receptor agonist named AdipoAI (standing for anti-inflammation) which in our pre-clinical trials on high fat diet (HFD) observed reduced glucose resistance, insulin tolerance, promoted adiponectin signaling in liver and adipose tissues of obese mice, and resulted decrease liver inflammation. Thus, our trial has affirmed, AdipoAI as a potent agonist of adiponectin with anti-diabetic, hepatoprotective, anti-inflammatory features.

## Introduction

The exponential growth in the incidence of Type 2 diabetes (T2D) is largely due to sedentary lifestyles and changes in dietary preferences, which adversely affects metabolism, immune system etc.(Daryabor, Atashzar, Kabelitz, Meri, & Kalantar, 2020; Fahed, El-Hage-Sleiman, Farhat, & Nemer, 2012; Leroith & Accili, 2008). The duo viz. obesity and T2D are known for their unfavorable consequences on adipose tissue, muscle, liver, and pancreatic islets which further results in altered levels of chemokines and cytokines among others, leading to insulin resistance syndrome (Nikolajczyk, Jagannathan-Bogdan, Shin, & Gyurko, 2011; Tsalamandris et al., 2019). Hence, inflammatory conditions such as metabolic inflammation is linked to obesity and contributes downstream to the pathogenesis of T2D and its clusters of characteristics manifestations (Walker & Colledge, 2013).

Further, adiponectin (Adipoq), an antidiabetic adipokine (Karamian, Moossavi, & Hemmati, 2021) has been well documented for its possible therapeutic potentials (Choi, Doss, & Kim, 2020; Karamian et al., 2021; J. Y. Kim, Barua, Jeong, & Lee, 2020). Recent understanding of adiponectin receptors (AdipoR1 and AdipoR2) has paved the way forward in understanding the development of insulin resistance and progressive obesity linked diseases such as T2D (Whitehead, Richards, Hickman, Macdonald, & Prins, 2006). Moreover, reduction in adiponectin hormone secretion from adipose tissue is linked to insulin resistance in T2D as reported in animal trials where adiponectin improved insulin resistance (Okada-Iwabu et al., 2013). Interestingly, a recent orally active small molecule AdipoR agonist (AdipoRON) has exhibited favorable anti-diabetic features such as ability to bind AdipoR1/R2, activation of AMPK and PPAR-α pathways, decreasing insulin

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resistance and glucose tolerance in mice models in high fat diet (Okada-Iwabu et al., 2013).

Besides these, T2D also results in uncontrolled hepatic glucose production, which leads to hyperglycemia as noted in diabetic pathologies. Studies also revealed that activation of peroxisome proliferator-activated receptors (PPARs) involves cascading effects on various signaling pathways which results in suppressing hepatic glucose production (Awazawa et al., 2011).Moreover, reports on animal and human trials suggests that adiponectin can decrease hepatic gluconeogenesis and stimulation of fatty acid oxidation in liver (Choi et al., 2020; Howlader, Sultana, Akter, & Hossain, 2021). Furthermore, adiponectin signaling in liver is linked to AdipoR2 which activates peroxisome proliferator-activated alpha (PPARa) (Achari & Jain, 2017) suggesting its therapeutic role in T2D. Additionally, the insulin sensitizing properties of adiponectin is through increase in fatty-acid oxidation via activation of AMPK (AMP-activated protein kinase) and regulation of peroxisome proliferator-activated receptor (PPAR)-a (Tomas et al., 2002; Yamauchi et al., 2002).

We previously reported the anti-inflammatory properties of AdipoAI (Qiu et al., 2021; Wu et al., 2022). AdipoAI attenuated NF-ϰB (nuclear factor kappa B), MAPK (mytogen activated protein kinase) and c-MAF (Qiu et al., 2021) signaling pathways through activation of Adiponectin signaling subunits AdipoR1 and APPL1 in Lipopolysaccharide (LPS)- induced macrophages and decreased the production of cytokines. Furthermore, AdipoAI was found to inhibit osteoclastogenesis at lower dose and inhibited the expression of proinflammatory mediators in periodontal tissues (Wu et al., 2022) indicating its potential candidature towards activation of adiponectin receptor against development of T2D pathology.

In this investigation, we hypothesized that our chemically designed and characterized AdipoAI small molecule have potent anti-diabetic therapeutic potential similar to Adiponectin and thereby we evaluated its *in-vitro* and *in-vivo* effects in comparison to a known anti-diabetic small molecule agonist AdipoRON (Okada-Iwabu et al., 2013) for further scientific affirmation.

## Materials and Methods

### Animal experiment

All the animals reported were approved by the animal care committee of Tufts University, Boston, MA. Diet induced obese (DIO, C57BL/6) male mice at 17 weeks of age were purchased from Jackson's laboratory and housed control pathogen free conditions (22°C) 12h light-dark cycle. One week after arrival mice were divided into three groups (n=6 per group) as follows CMC-Na (Carvoxymethylcellulose-sodium salt, thermoscientific, A18105.36) 0.5% (HFD group), AdipoAI 25mg/kg (HFD along with AdipoAI), AdipoRON 50 mg/kg (HFD along with AdipoRON) were fed with high fed diet consisting of 58% fat, 25.6% carbohydrates and 16.4% protein with access to water for 4 weeks. Small molecule AdipoAI (25 mg/kg) and AdipoRON (50 mg/kg) were dissolved and administered in 0.5% CMC-Na (carboxymethyl cellulose) orally by 18G curved gavage as reported by others (Shi et al., 2013).Oral treatment was introduced at 18 weeks and continued for 4 weeks (2X/week) and mice weights were recorded carefully every week as illustrated in concept map Fig 3A. At the end of $3^{rd}$ and $4^{th}$ week oral glucose and insulin tolerance test were performed.

### Cell culture

C2C12 mouse myoblast cell lines were obtained from ATCC, CRL-1772) and were allowed to grow in 90% Dulbecco's modified high glucose Eagle's medium (DMEM) containing 10% v/v fetal bovine serum (FBS) in 10cm cell culture dish at 37°C in 5% $CO_2$ incubator until 80% confluent. The media was replaced to myogenic differentiation medium containing 98% DMEM and 2% v/v horse serum and was changed every other day for 7 days. The differentiated myotubes were then plated in 96, 6 and 12 wells plates for RT-PCR and immunoblot analysis.

### CCK8 assay

CCK-8 assay was performed as described previously (Qiu et al., 2021) briefly C2C12 cells ($0.05X10^4$ cells/well) were plated in 96 well plate overnight (day 0), following AdipoAI and AdipoRON treatment for 24hours. After 24hours treatment CCK-8 (cell counting kit) (Dojindo, Rockville, MD, USA) assay was

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performed according to manufacture instruction the media was discarded carefully and 10ul of CCK-8 was added to the plates and incubated for 2h in incubator. The absorbance was recorded at 450nmn and calculated with respect to nontreated control groups.

**Realtime PCR**

Real time PCR was carried out according to methods previously described (Ahuja, Kim, Sung, & Cho, 2020; Wu et al., 2022). DNA free RNA was extracted from the C2C12 myotubes using zymo quick RNA mini kit (R1055) from with DNase treatment and RNA from tissues were isolated using Trizol (Invitrogen) according to manufacture protocol. Briefly 1ug of total RNA was reverse transcribed using first strand cDNA synthesis kit (thermos fisher) real time PCR was performed in triplicate using Bio-Rad icycler following manufacture protocols. The relative gene expression of the target genes was calculated by using $2^{-[?][?]ct}$ method (Qiu et al., 2021), the values were normalized to the housekeeping gene GPADH. Primer sequence of the respected primers are mentioned in table no1.

**Western blot analysis**

C2C12 myotubes ($1X10^6$) were incubated at different concentrations of AdipoAI and AdipoRON for 90 minutes. The cells were then washed with ice-cold PBS. The pellet was lysed using ice-cold 1X NUPAGE LDS lysis buffer (Invitrogen) approximately 200ul of the sample buffer were added to each sample the cells were vortex briefly and centrifuge at 8,000rpm for 5 mins, the supernatant was collected and heated at 70degC for 10min. The proteins were than loaded in 4.12% bis-tris gel using MES running buffer (Invitrogen) at 100V for 2 hours (Qiu et al., 2021; Wu et al., 2022), proteins were then transferred to 0.2 um polyvinylidene difluoride membrane using transfer buffer (Tris-base, glycine,10% SDS, methanol). Membrane was blocked using 3% bovine serum albumin (BSA) in TBST for 1 hour followed by incubation with specific antibody (p-AMPK, APPL1, PGAC-y) overnight. After primary antibody the membrane were than washed 3 times with TBST for 10minutes and incubated with HRB labeled secondary antibody containing 3% BSA for 1h at room temperature, after incubation the membrane were washed 3 times with TBST and were visualized using ECL reagent (Ahuja et al., 2020).

**Oral glucose and Insulin tolerance test**

DIO Mice were starved overnight (16h), followed by oral administration of glucose (1mg/kg) were given to each group (n=6). Blood glucose levels were evaluated prior to dosing, and carefully monitored every 15 minutes time interval for a period of 120 mins, following oral administration. Insulin tolerance test (0.75 IU insulin per kg body weight) was performed in treated DIO mice models after 6 hours of fasting. Blood glucose was monitored at specific time interval (0 -120 mins) by using a sensitive glucometer.

**Histology and Immunostaining of liver tissues**

Histology staining was performed as described previously with few modifications (Qiu et al., 2021). Briefly, Liver tissues from mouse were collected from all the treated groups after postmortem and fixed in ice cold 4% paraformaldehyde (PFA) overnight at 4degC. The following day samples the sections were paraffin embedded and were cut in 4um thickness following deparaffinization, the sections were stained with hematoxylin for (5-15 mins) washed and counter stained with eosin. The slides were quickly dehydrated and sealed using mineral oil. The images were documented at 20X magnification on Olympus BX53.

For immunostaining 4-um liver sections were cut and mounted on slides from all the groups the sections were deparaffinized with xylene and dehydrated in ethanol. The procedure was performed according to manufacture instructions (ab209101, Rabbit specific IHC polymer detection kit). The sections were immersed in 0.3% H2O2/methanol was used for 30 mins to reduce nonspecific staining to remove exogenous peroxidase avtivity, next the sections were incubated in primary antibody Phospho-AMPKa (Thr 172) (Rabbit mAb# 2535, cell signaling) overnight at 4degC in 3% BSA in PBS, followed by PBS washing, the slides were stained with anti-rabbit IgG. DAB was used for color rendering, and counter stained with hepatotoxicant, dehydrated, and sealed. Images were documented using Olympus BX53 at 20X magnification.

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**Statistical analysis**

All the data are presented at mean +- SD, the analysis was performed by using GraphPad version 9.4.1 software. Statistical analysis was performed using one-way ANOVA with multiple comparison between groups, the values of $p<0.05$ were considered as statistically significant.

**Results**

*In-vitro* **evaluation of Adipo-AI on adiponectin signaling.**

To screen the optimal concentration and to calculate the $IC_{50}$ values, we exposed C2C12 myotubes to different doses of AdipoAI (0-10 mM) in comparison to AdipoRON (0-10 mM), an adiponectin agonist, upon 24hr of incubation (Fig 1A). Our CCK-8 experiment results showed decrease in cell viability for both AdipoAI and AdipoRON treated groups with an increase in their respective concentrations. The $IC_{50}$ values of AdipoAI and AdipoRON obtained from CCK8 assay was 4.7mM and 6.22mM respectively (Fig.1A). Therefore, the approximate $IC_{50}$ mean value of 5 mM was chosen to be at the end of the increasing scale, apart from the aforementioned value mentioned earlier (Qiu et al., 2021); for further in-vitro experiments.

It has been reported earlier that activation of adiponectin receptor is through AdipoR1/R2 (R). Additionally, the anti-inflammatory feature of AdipoAI is also due to involvement of Adiponectin receptor APPL1(R). Investigation of AdipoAI-Adipoq signaling pathways through mRNA gene expressions of AdipoR1, AdipoR2 and pPargc1a at different doses of AdipoAI in comparison to AdipoRON in C2C12 myotubes revealed that accelerating dose of AdipoAI (0-5mM) observed significant increase in AdopoR1 and pPargc1a gene expression levels in comparison to AdipoRON (0-5uM) (Fig 1B, D). Furthermore, gene expression of AdipoR2 was also dose dependently increased in AdipoAI until 2.5mM in comparison to AdipoRON, whereas 5mM dose observed an increase in AdipoRON treated group (Fig 1C) correlating our assumption that AdipoAI is a potential adiponectin agonist.

As activity of adiponectin is through AMP-activated protein kinase (AMPK),peroxisome-activated receptor (PPAR)-a (Gesta, Tseng, & Kahn, 2007; Yamauchi, Kamon, Waki, et al., 2003) & APPL1, a downstream subunit, involved in adiponectin signaling (Mao et al., 2006), we designed the experimental plan as per our earlier findings, where AdipoAI graded doses increased the gene expression profiles of AdipoR1/R2 and Ppargc1-a. We treated C2C12 myotubes with graded doses of AdipoAI and AdipoRON for 90 mins followed by immunoblotting assay that confirmed the ability of AdipoAI to activate AMPK and was subsequently compared to that of AdipoRON (Fig 1E). As shown in Fig 1E administration of AdipoAI induced dose dependent phosphorylation of AMPK and APPL1 in comparison to AdipoRON, however PPARg produced no statistically significant results. Over all these data affirms our prediction that lower doses of AdipoAI could act as a potent activator of AMPK and APPL1.

**AdipoAI administration improved insulin resistance and glucose tolerance in DIO mice models**

The DIO mouse models were subjected to glucose and insulin tolerance test in accordance with our perceived plan of interventional strategy for developing T2D pathology (Fig 2A, B). OGTT showed time dependent decrease in serum glucose profile for AdipoAI and AdipoRON groups when compared to those of CMC (Carboxymethylcellulose) groups (Fig 2A). Further, insulin tolerance test resulted in gradual reduction in the levels of serum glucose in AdipoAI and AdipoRON groups when compared to that of CMC group suggesting that AdipoAI have comparable beneficial features as that of AdipoRON (Fig 2B).

**Fat distribution and body weights of mouse models exposed to AdipoAI**

Further, comparative monitoring, from a period of 4 weeks, in DIO mice models, revealed that the AdipoAI group had decrease weight at 20 and 21 weeks compared to that of AdipoRON and CMC groups (Fig 3B). However, these observations were statistically insignificant. Interestingly, the weights of iWAT (inguinal white adipose tissue) (Fig 3C) and eWAT (epididymal white adipose tissue) (Fig 3D) were decreased for both AdipoAI and AdipoRON group but AdipoAI mouse demonstrated the most weight reduction for eWAT among all the groups significantly (Fig 3D). The weight of liver also correlated to our assumption (Fig 3E).

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**AdipoAI and AdipoRON activates different adiponectin subunits in different tissues.**

Reports indicates that obesity may be related with liver dysfunction, (Marchesini, Moscatiello, Di Domizio, & Forlani, 2008) further, literature also suggests that metabolic or physiological status of liver are altered in obesity and in T2D pathology (De Silva et al., 2019). Therefore, considering all these factors the hepatic screening of the DIO mice models was performed which interestingly revealed that the AdipoAI group increased gene expression profiles for Ppargc1a, AdipoR2 and Ppara (Fig 4 A, B & C) with simultaneously decreased the expression of IL-6, INF-b, TNF-a (Fig F, G & H) a potent inflammatory biomarker. We further tested weather AdipoAI could result in phosphorylation of AMPK by performing immunoblotting and IHC staining of liver tissue treated with AdipoAI, AdipoRON and CMC (Fig 4 D & E). Immunostaining and protein expression against AMPK known to improves insulin sensitivity and glucose homeostasis (Coughlan, Valentine, Ruderman, & Saha, 2014) we observed increased and prominent Phosphorylation of AMPK in both AdipoAI and AdipoRON liver tissue (Fig 4 D&E). Further, H&E-stained images of AdipoAI group showed evenly populated hepatocytes with mostly normal cytoplasmic contents and architecture (Fig 4I). The CMC and AdipoRON group exhibited sporadic infiltration with disarranged hepatocytes (arrowhead) and diffuse fatty changes. The overall results suggest that AdipoAI can results in phosphorylation of AMPK and activation of adiponectin subunits with decrease in cytokine levels in liver.

**AdipoAI increases AdipoR1-PGC1-a expression in adipose tissues.**

As PGC1a (pPARGC1a) regulation is involved in lipid metabolism in muscle adipocytes (Bogacka, Xie, Bray, & Smith, 2005; Liang & Ward, 2006; Lin et al., 2002; Patti et al., 2003). We assume that AdipoAI and AdipoRON oral administration regulates adiponectin and PGC1a expression levels in white adipose tissue (WAT). In WAT AdipoAI and AdipoRON both increase AdipoR1, pPARGC1a, PPARA gene expression levels in comparison to DIO mice (Fig 5A, C & D). Higher gene expression of all the three tested gene was observed in AdipoAI treated group, suggesting that AdipoAI could activate adiponectin signaling pathways through AdipoR1 and lower doses of AdipoAI may influence the lipid metabolism in WAT which could be a potential target.

**Discussion**

Diabetes mellitus is a chronic metabolic disease that is increasing in prevalence globally (Cheng & Fantus, 2005), affecting more than 6% of the US population with reports of ˜ 25% in some geriatric group, and manifesting impaired glucose tolerance (IGT) (Inzucchi, 2002) and associated complications. Hyperglycemia i.e. elevated glucose concentrations/glucotoxicity from Diabetes has unfavorable foreseeable effects on multiple organs, where it results in dysfunction in insulin secretion from pancreas and faulty insulin gene expression through interfering various pathways that ultimately leads to low glucose sensitization and beta-cell stress(Hasnain et al., 2014), eventually resulting in irreversible beta-cell failure. Low-grade inflammation, another metabolic forerunner of diabetes, is being constantly associated with insulin resistance and obesity (Shoelson, Herrero, & Naaz, 2007; Shoelson, Lee, & Goldfine, 2006). Therefore, designing molecules that are efficient in targeting hyperglycemic and inflammatory pathways could be the most important components in the overall strategy in preventing and controlling diabetes and its sequelae.

Latest scientific evidence indicates that adiponectin could result in nullification of cases in obesity and metabolic diseases such as T2D (Balasubramanian et al., 2022; Y. Kim et al., 2018). In view of all these recent developments, adiponectin receptors (AdipoR1 and AdipoR2) and its scaffold proteins APPL1 and APPL2 have emerged as important target for controlling excess inflammation, a condition frequently associated with T2D. While AdipoRON, the first-generation agonist of adiponectin receptors, has been shown to prevent diet-induced inflammation and insulin resistance in mice (Iwabu et al., 2010; Okada-Iwabu et al., 2013), To maximize the potential of adiponectin receptors in targeting the one of the cascading effects required for pathogenesis of T2D fundamental etiopathology of T2D, we have designed, synthesized, and characterized a synthetic and orally active small molecule agonist of adiponectin AdipoAI and compared our results with AdipoRON *invitro* and *invivo* . In the present study, we demonstrated that AdipoAI could behave as an antidiabetic adipokine and is analogous to AdipoRON (Akimoto, Maruyama, Kawabata, Tajima, &

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Takenaga, 2018), supporting our notion that AdipoAI could be used as an anti-diabetic therapeutic agent and the active properties of which were noted to be similar and/or marginally superior to AdipoRON. Confirming our prediction, that showed the exposure of AdipoAI initiated signaling pathways of adiponectin and AMPK activation in C2C12 myotube cells, with improved serum, gene expression and histopathological profiles in DIO (diet induced obesity) mouse study models respectively in comparison to AdipoRON.

We previously reported the anti-inflammatory properties of AdipoAI that was found to be dependent on adiponectin receptor subunits viz. AdipoR1 and APPL1 suggesting that AdipoAI could mimic effects of adiponectin (Qiu et al., 2021). It was also claimed that decrease in adiponectin levels is accompanied by decrease in AMPK and PPARa activation, resulting in increased susceptibility for diabetes through decrease in fatty acid synthesis and increase in fatty acid oxidation (Handa et al., 2014). AMPK and PPAR activation play an important role in cell survival and energy production (Jager, Handschin, St-Pierre, & Spiegelman, 2007; Yamauchi et al., 2002). Further, in skeletal muscle cells, adiponectin pathway activation is known to be associated with AMPK, p38MAPK and PPARa activity (Yamauchi, Kamon, Waki, et al., 2003). Additionally, APPL1 interacts with adiponectin receptors in mammalian cells suggesting that APPL1, AMPK and adiponectin are interconnected (Mao et al., 2006). Our results collectively suggest that lower doses of AdipoAI significantly increased the mRNA expression levels of peroxisome proliferator-activated receptor Ppargc1a, AdipoR1/R1, and increase protein expression of AMPK and APPL1 in C2C12 myotubes when compared with AdipoRON (Yamauchi et al., 2007).

Latest scientific evidence indicates that adiponectin could result in nullification of cases in obesity and metabolic diseases such as T2D (Kawano & Arora, 2009; Nigro et al., 2014). Advantageously, contribution of adiponectin in decreasing triglyceride, suppressed inflammation, decrease glucose intolerance and insulin resistance through activation of AMPK are acknowledged (Tomas et al., 2002) are beneficial in T2D cases (Kasper, 2015). We observed the similar pattern in our DIO mice models where biweekly administration of AdipoAI (25mg/kg) decreased inguinal and epididymal fat content. We noted the mRNA levels of isolated WAT that were found to be higher in AdipoR1 and compliment similar studies with increased expression of PGC-1a & Ppara genes (Rasmussen et al., 2006). There could be several possible mechanisms for the reduced fat accumulation in response to AdipoAI exposure. One of the possibilities might be AdipoAI induced increased fat oxidation (Weyer et al., 2000) with another possibility of reduction in triglyceride uptake and its subsequent storage in adipose tissue (Browning et al., 2011). Further, to the discussion PGC-1a is a key molecule in mitochondrial biogenesis and is associated with insulin resistance and obesity (Shen et al., 2022). Specific overexpression of PGC-1 a expression in adipocyte improved metabolic disfunction in HFD mice and improved insulin sensitivity (Shen et al., 2022).

Further AdipoAI decreased blood glucose levels during oral glucose and insulin tolerance test, which was similar to AdipoRON groups, suggesting that lower AdipoAI (25mg/kg) administration was potent enough in exerting similar effects to that of mean doses of AdipoRON (50 mg/kg). The interpretation of the obtained results could be extrapolated from similar trail with another adiponectin agonist where the assessment of whole-body insulin sensitivity of DIO mice models reveled the involvement of hepatic fibroblast growth factor 21 (FGF21), that is linked to short-term adiponectin agonist-induced hepatic PPARα activation (Wang et al., 2022).

The pathological features/manifestations of T2D and thereby we proceeded with the screening of respective parameters for specific feature/manifestations in DIO mouse study models. We planned liver evaluation as adiponectin and its mechanism of action, which acts mainly through AdipoR2 (Yamauchi, Kamon, Ito, et al., 2003), are correspondingly found to be concentrated in the aforementioned organ (Ruan & Dong, 2016). As noted in our study favorable effects were exhibited by AdipoAI on livers of DIO mouse models which increase in gene expression of adiponectin subunit AdipoR2 in AdipoAI and AdipoRON, interestingly AdipoAI increased pPargc1a, pPara mRNA expression levels with reduced pro-inflammatory markers (TNF-a, IL1-b, IL-6) in liver tissues these overall results suggest hepato-protective features AdipoAI in lower doses compared to AdipoRON. These data were further corroborated by immunoblotting and immunohistochemistry staining which revealed increased expression of phosphorylation of AMPK in both the treated groups,

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suggesting that lower dose of AdipoAI could phosphorylate AMPK which in turn inhibits inflammation, ER and oxidative stress, (Ruderman, Carling, Prentki, & Cacicedo, 2013).

Apart from its sporadic or zonal steatosis pathology, that have strong association with diabetes, insulin resistance, and obesity, all contributing towards further lipid accumulation in hepatic parenchymal cells leading to hepatic steatosis, one of the most common liver diseases reported globally. As speculated, the H&E staining of DIO mouse liver tissue exposed to AdipoAI group showed relatively normal hepatic architecture organized mostly in loose rows and separated by sinusoids. However, CMC and AdipoRON group showed disarranged hepatocytes with appearance of diffused fat vacuoles with frank Mallory-Denk bodies (Liu et al., 2020). indicating towards development of microvesicular steatosis (David, Boyer, Wright, & Manns, 2006). The histologic features resembling steatohepatitis found in control groups could be most probably be due to insulin resistance associated with obesity (Gwaltney-Brant, 2021). Our data suggest that AdipoAI likely have an influence on the increase in adiponectin secretion, which subsequently lead to one mechanism of AMPK activation through LKB1 as reported in a similar trial (Santamarina et al., 2015).

In conclusion, our trial has affirmed the anti-diabetic features viz. hepatoprotective, normoglycemic, anti-inflammatory and weight reduction effects of AdipoAI in DIO mice models at significantly lower doses when compared to AdipoRON. The results will contribute towards the interpretation and prediction of possible clinically relevant effects in future studies and devising interventional strategies of T2D and its characteristics manifestations.

**Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author Contributions**

AA and EZ contributed equally to the manuscript by planning and performing the experiments. AA did the data acquisition, analysis, and interpretation. AA, XZ, QT, JK contributed to design and critically revised the manuscript, AA, SD, QT, XZ and JK drafted and critically revised the manuscript All authors critically discussed, corrected, and reviewed the manuscript.

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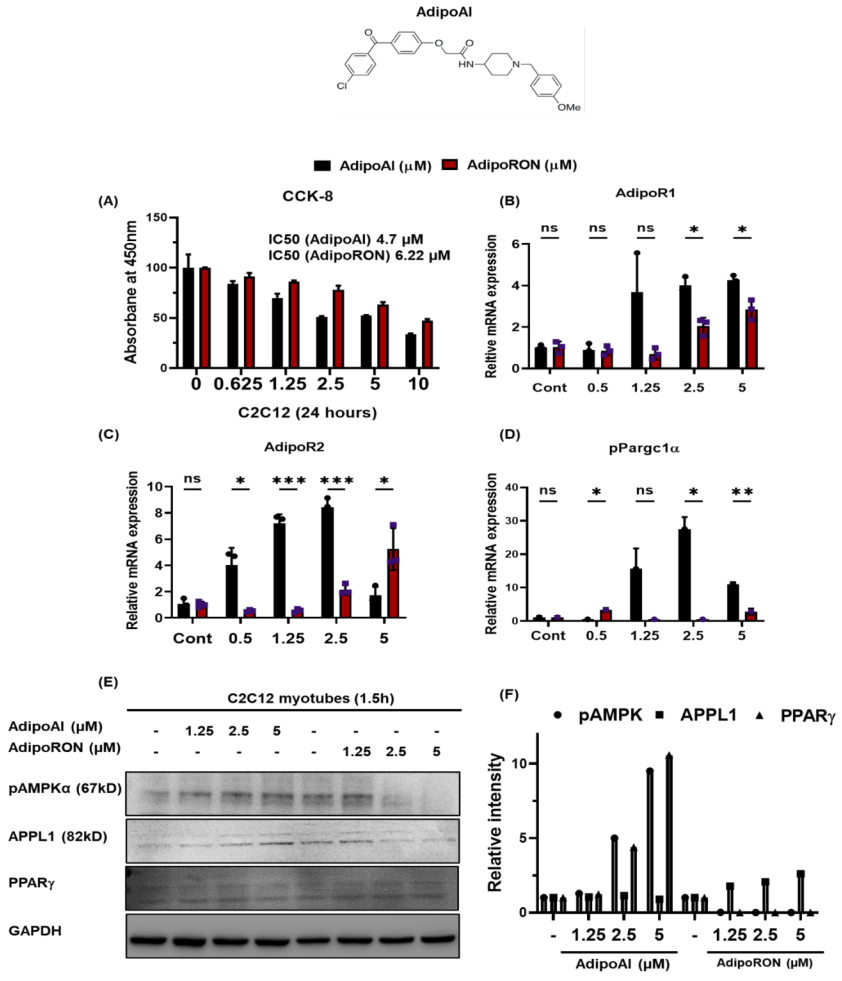




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## Figures

Figure 1: Comparative *in vitro* studies of AdipoAI and AdipoRON to determine cellular toxicity and mRNA gene expression levels: (A) Cellular toxicity and behavioral patterns of C2C12 myotubes treated with AdipoAI and AdipoRON (0-10 mM) after 24 hrs incubation by using CCK8 assay; determined at 450 nm absorbance spectra. Cell cytotoxicity test reveals $IC_{50}$ values of AdipoAI and AdipoRON. Quantitative gene expression analysis of (B) AdipoR1 (C) AdipoR2 (D) Ppargc1a in C2C12 myotubes treated with AdipoAI and AdipoRON (0-5 uM) after 1.5 hours of treatment. (E) Adiponectin signaling in differentiated C2C12 myotubes treated with different concentrations of AdipoAI and AdipoRON for 1.5 hours with respect to controls (untreated). . Statistical significance was calculated based on ordinary one-way analysis of variance (ANOVA) with multiple comparison using GraphPad Prism 9.4.1 software and all the values are presented as mean ± SD with three independent experiments sets with *p<0.05; **p<0.001.Figure 2. Oral Glucose tolerance (OGTT) and Insulin tolerance test (ITT) test at 20 and 21 weeks in diet induced obesity (DIO) mice models for accessing AdipoAI efficacy. (A) Oral glucose tolerance test tested at 20 weeks in DIO mice treated with CMC (0.5%), AdipoAI (25 mg/kg) and AdipoRON (50 mg/kg). (B) Insulin tolerance test tested at 21 weeks in DIO mice treated with CMC (0.5%), AdipoAI (25 mg/kg) and AdipoRON (50 mg/kg). Data are represented as mean ± SD of 6 mice per group.; *p<0.05, **p<0.001 compared to CMC vehicle.

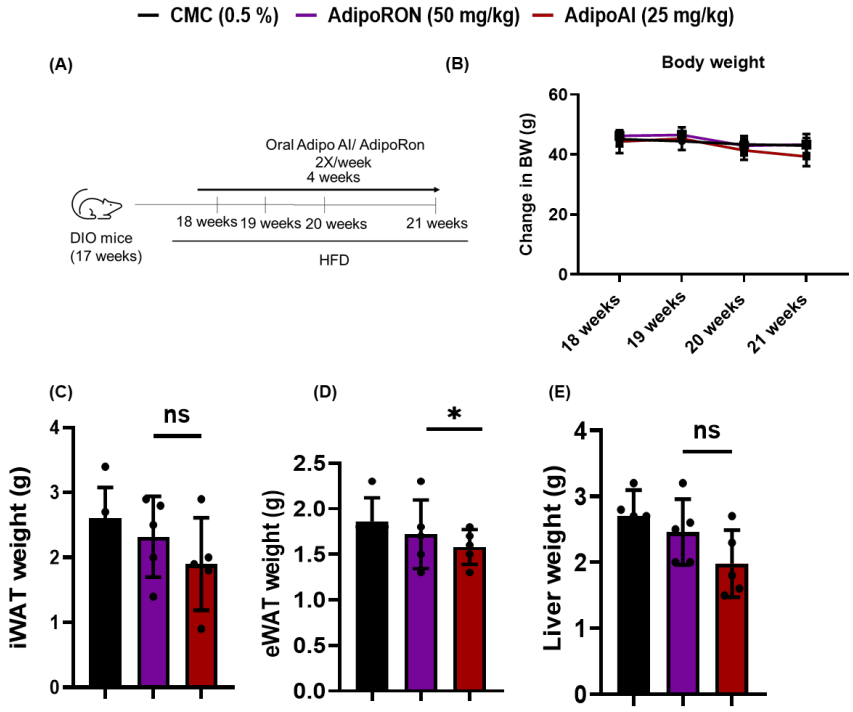


**Figure 2.** Body and adipocyte weight of DIO mice treated with CMC, AdipoAI and AdipoRON for 4 weeks (A) Concept strategy of *in vivo* trial (B) Comparative Body weights measured from 18-21 weeks in DIO mice treated with CMC (0.5%), AdipoAI (25 mg/kg) and AdipoRON (50 mg/kg) (C & D) changes in fat Inguinal and epididymal fat contents for gauging comparative adipose tissue biology in DIO mice models (E) liver weights of DIO mice with respective treated groups recorded at 21 weeks. Data were analyzed by using ordinary 1-way followed by Šidák's post hoc test. all the values are presented as mean ± SD with three independent experiments sets with *p<0.05; **p<0.01.

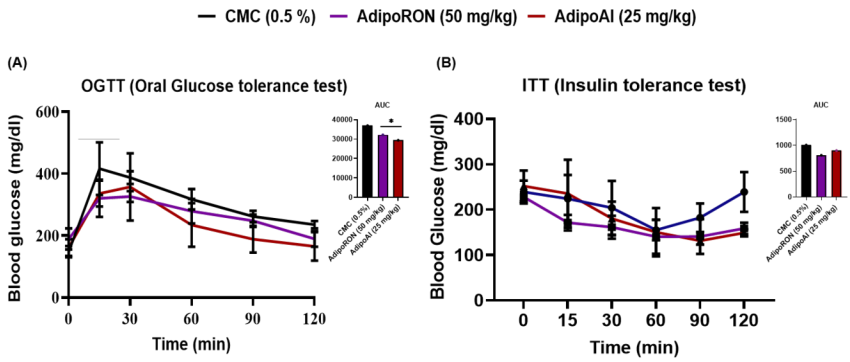


**Figure 3.** Oral Glucose tolerance (OGTT) and Insulin tolerance test (ITT) test at 20 and 21 weeks in diet induced obesity (DIO) mice models for accessing AdipoAI efficacy (A) Mice were starved overnight, and oral administration of glucose (1mg/kg) were given blood glucose levels were evaluated prior to dosing (0-15 min), and carefully monitored every 15 minutes time interval for a period of 120 mins, following oral administration. The AdipoAI (25 mg/kg) exposed mice models revealed a decreased in blood glucose levels and have similar profile of OGTT with respect to another established bioactive molecule such as AdipoRon (50 mg/kg). (B) Insulin tolerance test (0.75 IU insulin per kg body weight) was performed in AdipoAI and AdipoRON treated DIO mice models after 6 hours of fasting. Blood glucose was monitored at specific time interval (0 -120 mins) by using a sensitive glucometer depicting comparable curves of AdipoAI and AdipoRON. Data are represented as mean ± SD of 6 mice per group.; *p<0.05, **p<0.001 compared to CMC vehicle.

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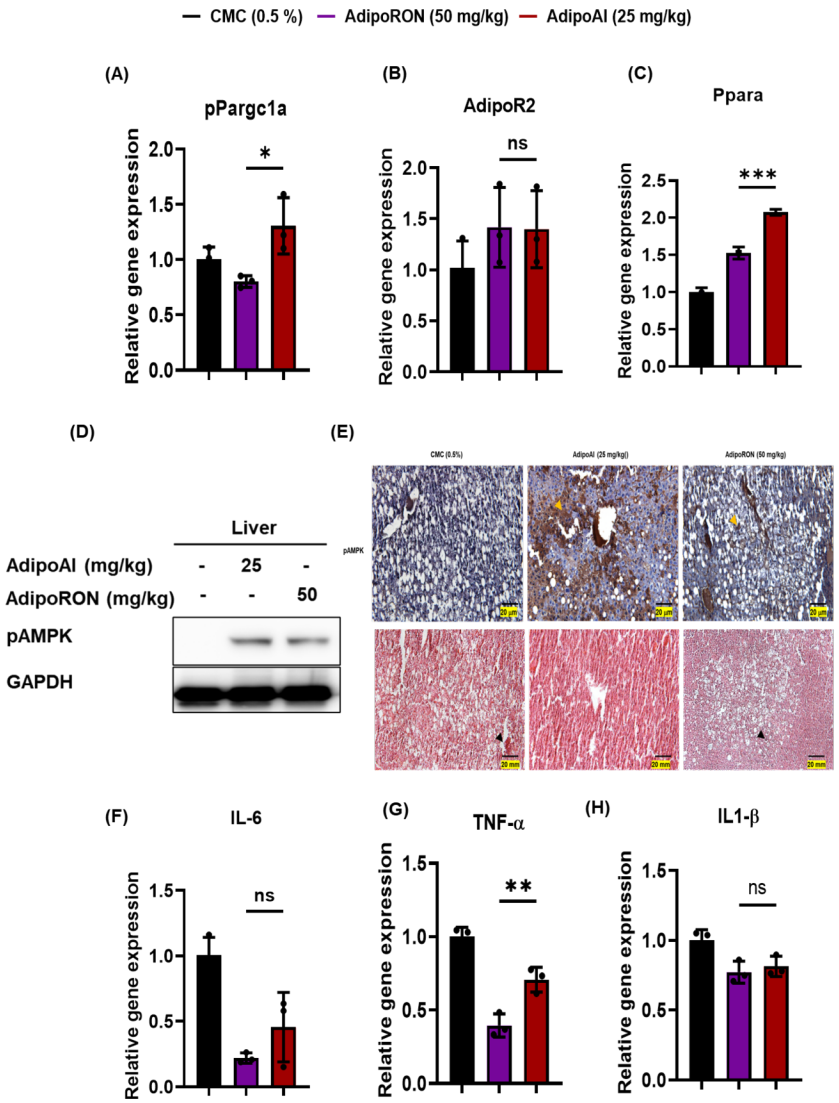


**Figure 4.** Effect of AdipoAI treatment on adiponectin subunits expression and hepatic microarchitecture in liver tissues. (A, B & C) mRNA Gene expression profiles of Ppargc1a, AdipoR2 in liver of 21 weeks old DIO mice treated with CMC (0.5%), AdipoAI (25 mg/kg) and AdipoRON (50 mg/kg) administered biweekly respectively (D) Western blot analysis phosphorylation of AMPK in liver samples treated with indicated amount of AdipoAI and AdipoRON (E) Immunohistochemistry staining profiles of p-AMPK levels in liver tissues from mice study models showing increase AMPK localization in AdipoAI treated groups (E, F and G) mRNA expression of inflammatory cytokines IL-6, TNF-a, IL-1B in liver tissues treated with CMC, AdipoAI, AdipoRON biweekly for four weeks (H) Hematoxylin and eosin staining depict improved hepatic details compared to control. Data were analyzed by using ordinary 1-way followed by multiple comparison between groups. all the values are presented as mean ± SD with three independent experiments sets with *p<0.05; **p<0.01, ***p<0.001.

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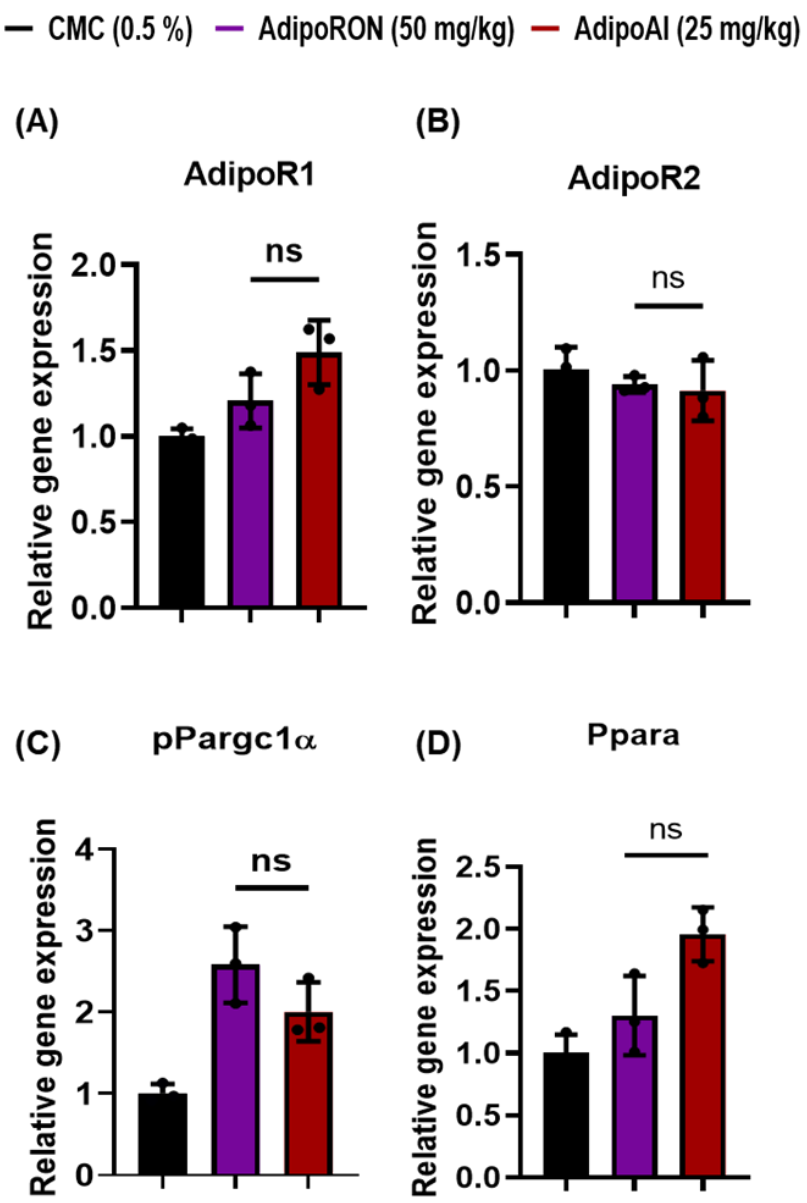


**Figure 5.** AdipoAI activates adiponectin receptor subunit AdipoR1. (A, B, C & D) A tissue distribution of AdipoR1, AdipoR2, Ppargc1a, Ppara was assessed by mRNA expression in WAT adipose tissue of DIO mice treated with CMC (0.5%), AdipoAI (25mg/kg) and AdipoRON (50 mg/kg). Data were analyzed by using ordinary 1-way followed by multiple comparison between groups. all the values are presented as mean ± SD with three independent experiments sets with *p<0.05; **p<0.01, ***p<0.001.

Table 1: RT-PCR primers sequence

| Gene | Primer sequence |
|---|---|
| AdipoR1 | TCAGGGATTGCTCTACTGATTATG<br>AGACGATGGAGAGGTAGATGAG |

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| Gene | Primer sequence |
|---|---|
| AdipoR2 | AGGCTGGCTAATGCTTATGG |
| | GATGTGGAAGAGCTGATGAGAG |
| pPARGC1a | CGACAGCTATGAAGCCTATGAG |
| | CTTCTGCCTCTCTCTCTGTTTG |
| IL6 F | GTTGCCTTCTTCTTGGGACTGATG |
| | CTGGCTTTGTCTTTCTTGTTATC |
| TNF | GGTGCCTATGTCTCAGCCTCTT |
| | GCCATAGAACTGATGAGAGGGAG |
| IL1b | ACATCAGCACCTCACAAGCA |
| | TTAGAAACAGTCCAGCCCCATG |
| pPARA | ACGATGCTGTCCTCCTTGATG |
| | GCGTCTGACTCGGTCTTCTTG |
| GAPDH | AGGTCGGTGTGAACGGATTTG |
| | TGTAGACCATGTAGTTGAGGTCA |