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Tschimganidine reduces lipid accumulation through AMPK activation and alleviates high-fat diet-induced metabolic diseases

Min-Seon Hwang[1,2,3,#], *Jung-Hwan Baek*[1,2,3,#], *Jun-Kyu Song*[1], *In Hye Lee*[4] *& Kyung-Hee Chun*[1,2,3,*]

[1]Department of Biochemistry & Molecular Biology, Yonsei University College of Medicine, Yonsei University, Seoul 03722, [2]Institute of Genetic Science, Yonsei University College of Medicine, Yonsei University, Seoul 03722, [3]Graduate School of Medical Science, Brain Korea 21 Project, Yonsei University College of Medicine, Yonsei University, Seoul 03722, [4]Department of Life Science, College of Natural Science, Ewha Womans University, Seoul 03760, Korea

Obesity increases the risk of mortality and morbidity because it results in hypertension, heart disease, and type 2 diabetes. Therefore, there is an urgent need for pharmacotherapeutic drugs to treat obesity. We performed a screening assay using natural products with anti-adipogenic properties in 3T3-L1 cells and determined that tschimganidine, a terpenoid from the Umbelliferae family, inhibited adipogenesis. To evaluate the anti-obesity effects of tschimganidine *in vivo*. Mice were fed either a normal chow diet (NFD) or a high-fat chow diet (HFD) with or without tschimganidine for 12 weeks. Treatment with tschimganidine decreased lipid accumulation and adipogenesis, accompanied by reduced expression of adipogenesis and lipid accumulation-related factors. Tschimganidine significantly increased the phosphorylation of AMP-activated protein kinase (AMPK) and decreased that of AKT. Depletion of AMPK relieved the reduction in lipid accumulation resulting from tschimganidine treatment. Moreover, tschimganidine administration drastically reduced the weight and size of both gonadal white adipose tissue (WAT) and blood glucose levels in high-fat diet-induced obese mice. We suggest that tschimganidine is a potent anti-obesity agent, which impedes adipogenesis and improves glucose homeostasis. Tschimganidine can then be evaluated for clinical application as a therapeutic agent. [BMB Reports 2023; 56(4): 246-251]

INTRODUCTION

The rise in obesity and obesity-related metabolic diseases is an epidemic worldwide (1). In the United States, over 78 million adults are obese and have comorbidities (2). In obese adipose tissue, chronic inflammation is accompanied and it also affects the function of other organs, such as the stomach, liver and heart (3). Therefore, it is necessary to research therapeutic agents for preventing and managing obesity and obesity-related metabolic diseases.

A screening analysis for natural compounds exhibiting anti-obesity effects has previously been performed (4). We found a terpenoid, tschimganidine, that inhibits lipid accumulation in adipocytes, 3T3-L1 cells. Its structure is shown in Fig. 1A. Tschimganidine is a member of the Umbelliferae family (5). Terpenoids have been reported to be selectively toxic against gram-positive bacteria (6) and to have anti-cancer effects (7). However, there are only two reports on the biological activity of tschimganidine, i.e., it can act as an agonist of oestrogen receptor-alpha (ERα) (8) and extend the lifespan of yeast (9). Tschimganidine can also act as a phytoestrogen (10). Phytoestrogens are natural phytochemicals that have a function similar to gonadal oestrogen hormones and are potential alternatives to hormone replacement therapy (5). Among phytoestrogens, terpenoids can exhibit both oestrogenic and anti-oestrogenic activities by targeting oestrogen receptors (11). In particular, tschimganidine, by functioning as an ERα agonist has oestrogen-mimicking characteristics (8). ERα agonists have been reported to exert anti-obesity effects by stimulating oestrogen receptors (12). However, research on these effects of terpenoids is lacking, so, it is worthwhile to conduct a study on how tschimganidine affects obesity.

Based on these points, we first hypothesized that tschimganidine, as an ERα agonist, would have a therapeutic effect on obesity and metabolic diseases. And we aimed to elucidate the mechanism of how tschimganidine reduces adipogenesis or lipid accumulation in adipocytes. Herein, We evaluated tschimganidine in adipocytes and an obese mouse model to determine whether tschimganidine has potential as a preventive or therapeutic agent for obesity in the future.

*Corresponding author. Tel: +82-2-2228-1699; Fax: +82-2-312-5041; E-mail: khchun@yuhs.ac
#These authors contributed equally to this work.

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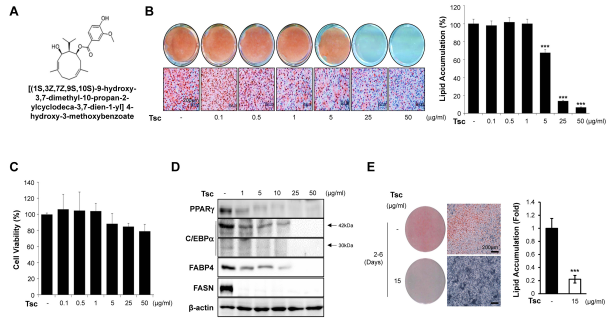

Fig. 1. Tschimganidine reduces lipid accumulation in 3T3-L1 cells without cytotoxicity (A) Structure of tschimganidine. (B) ORO staining of tschimganidine-treated 3T3-L1 cells. After MDI induction, 3T3-L1 cells were treated with tschimganidine on days 2 to 6. ORO staining was performed on day 6. Measurement of lipid accumulation. ORO was eluted with 100% isopropanol from the stained cells, and the absorbances were measured at 500 nm. ***P < 0.001, dimethyl sulfoxide vs. tschimganidine. (C) Cell viability assays. Confluent 3T3-L1 cells were treated with tschimganidine for 48 h. (D) Protein expression of PPARγ, C/EBPα, FABP4, and FASN was detected by western blotting. Protein expression was normalized to that of β-actin. (E) ORO staining of tschimganidine-treated 3T3-L1 cells. After MDI induction, 3T3-L1 cells were treated with tschimganidine on days 2 to 6. ORO staining was performed on day 6.

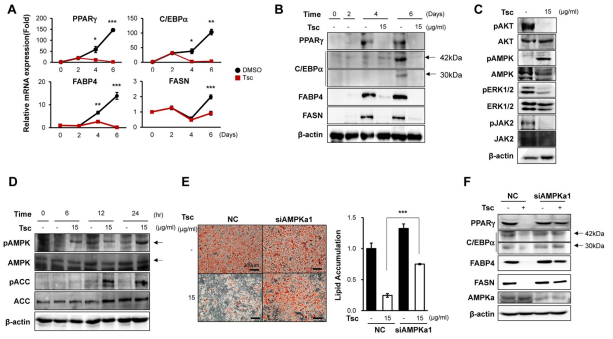

Fig. 2. Tschimganidine reduces the expression levels of adipogenesis-related factors after adipocyte differentiation through activation of AMPK. (A) mRNA expression of PPARγ, C/EBPα, FABP4, and FASN was detected using real-time polymerase chain reaction. RNA samples were prepared on days 0, 2, 4, and 6. 3T3-L1 cells were treated with tschimganidine on days 2 to 6. mRNA expression was normalized to that of β-actin. *P < 0.05, **P < 0.01, ***P < 0.001, DMSO vs. tschimganidine. (B) Protein expression of PPARγ, C/EBPα, FABP4, and FASN was detected by western blotting. Protein samples were prepared on days 0, 2, 4, and 6. 3T3-L1 cells were treated with tschimganidine on days 2 to 6. Protein expression was normalized to that of β-actin. (C) Western blotting of signal transduction-related proteins. 3T3-L1 cells were treated with tschimganidine on day 2 and incubated for 24 h. (D) The activity of signal transduction-related proteins, such as AMPK and ACC, was detected by western blotting. 3T3-L1 cells were treated with tschimganidine on day 2. (E) ORO staining and lipid accumulation of AMPKa1 knockdown-3T3-L1 cells with or without tschimganidine. After transfection of AMPKa1 small interfering RNA (siRNA), 3T3-L1 cells were differentiated using MDI. Then, 3T3-L1 cells were treated with tschimganidine on day 2. ORO staining was performed on day 6. ***P < 0.001, scRNA vs. siAMPK. (F) Protein expression of PPARγ, C/EBPα, FABP4, and FASN was detected by western blotting. Protein samples were prepared on day 4. Protein expression was normalized to that of β-actin.

RESULTS

Tschimganidine diminishes lipid accumulation in 3T3-L1 cells without cytotoxicity

Tschimganidine was treated at various concentrations 2 days after inducing the cell differentiation of 3T3-L1 preadipocytes. Adipogenesis, the process by which adipocyte precursors develop into mature adipocytes, was investigated by ORO staining of the lipid droplets on day 6 (Fig. 1B). ORO staining showed that tschimganidine reduced lipid accumulation dose-dependently. Tschimganidine treatment at 5 μg/ml showed a reducing effect on lipid accumulation. The tschimganidine doses which were most effective for suppressing adipogenesis and lipid accumulation were 25 and 50 μg/ml, respectively (Fig. 1B). A cell viability assay was conducted to determine whether tschimganidine exerted this effect by inducing cell cytotoxicity. We confirmed that treatment with tschimganidine caused no significant difference in cell viability of adipocytes. (Fig. 1C). The expression of adipogenesis-related proteins, namely PPARγ, and its downstream factors, C/EBPα, FABP4, and FASN, decreased after tschimganidine treatment dose-dependently (Fig. 1D). Tschimganidine (15 μg/ml) was observed via ORO staining to have a dose-dependent inhibitory effect on lipid accumulation (Fig. 1E). These results suggest that tschimganidine inhibits lipid accumulation by downregulating the expression of adipogenesis-associated proteins.

Tschimganidine reduces the expression of adipogenesis-related factors after adipocyte differentiation

We examined how tschimganidine affects adipogenesis-related factors during adipocyte differentiation using whole cell lysates. Two days after adipogenic stimulation, 3T3-L1 cells were treated with tschimganidine, and cell lysates were obtained at each time point. Tschimganidine significantly suppressed the gene (Fig. 2A) and protein expression (Fig. 2B) of PPARγ, C/EBPα, FABP4, and FASN. We further investigated whether the effect of tschimganidine on adipogenesis also occurs during the early or late stages of adipogenesis. On day 0, adipocytes were treated with tschimganidine simultaneously with the induction of adipocyte differentiation by methylisobutylxanthine-dexamethasone-insulin (MDI). Treatment with tschimganidine at the early phase inhibited adipogenesis and lipid accumulation (Supplementary Fig. 1). The inhibitory effect was also observed when tschimganidine was treated on adipocytes at the post-adipocyte differentiation period. Tschimganidine treatment decreased lipid droplet size of differentiated 3T3-L1 cells compared to control cells.

According to the ORO staining results, tschimganidine retarded adipogenesis in the entire stages of adipocyte differentiation, including the early period (days 0 to 2) and the late period (days 6 to 10). These data indicate that tschimganidine represses adipogenesis and reduces lipid accumulation in the adipocytes.

Tschimganidine induces phosphorylation of AMP-activated kinase (AMPK) and Acetyl-CoA carboxylase (ACC)

Cell signalling molecules, such as AKT, AMPK, ERK, and JAK2, participate in adipogenesis and lipid accumulation (13). To investigate the molecular mechanisms of tschimganidine on adipogenesis and lipid accumulation, we observed the changes in the activation of these signalling molecules 24 h after treatment with tschimganidine on day 2 of adipocyte differentiation (Fig. 2C). The expression levels of AKT, AMPK, ERK 1/2, and JAK2 were unaffected by tschimganidine treatment. However, levels of phosphorylated AKT, ERK 1/2, and JAK2 were significantly decreased after tschimganidine treatment. Tschimganidine also increased AMPK phosphorylation. These results indicate that tschimganidine lowered the activation of adipogenesis-related signalling molecules during adipocyte differentiation. We also found that tschimganidine treatment increased AMPK and ACC phosphorylation time-dependently (Fig. 2D). Multiple studies have shown that AMPK is phosphorylated for activation and phosphorylated AMPK suppresses adipocyte differentiation (14-16).

We examined whether tschimganidine inhibits lipid accumulation via AMPK activation. The suppression effect of tschimganidine on lipid accumulation was attenuated when AMPK expression was knocked down using AMPK siRNAs. ORO staining, lipid droplet size, and lipid accumulation in tschimganidine-treated 3T3-L1 cells after AMPK-knockdown were lower than those of the control cells (Fig. 2E). Moreover, AMPK knockdown mitigated the inhibitory effect of tschimganidine on the expression of adipogenesis-and lipid accumulation-associated proteins (Fig. 2F). Therefore, tschimganidine inhibits adipogenesis and lipid accumulation via AMPK activation.

Tschimganidine affects body weight, insulin tolerance, and glucose tolerance in high-fat diet-fed mice

The anti-obesity effects of tschimganidine were evaluated *in vivo*. Mice were fed an NFD or HFD (containing 60% of the total kcal as fat) for 12 weeks, and intraperitoneal injection of tschimganidine was started 5 weeks after the HFD. Tschimganidine treatment reduced body size in HFD-fed obese mice, whereas there was no difference in body size in NFD-fed mice, regardless of tschimganidine treatment (Fig. 3A). The weight loss effect of tschimganidine was notably shown in HFD-fed mice (Fig. 3B). In particular, the mice treated with 5 μg/kg tschimganidine had a lower body weight than the control group. However, no significant differences in food intake were noted between the NFD and HFD groups (Fig. 3C).

The effect of tschimganidine on metabolic parameters, such as alanine aminotransferase (ALT), glucose, and triglyceride (TG)

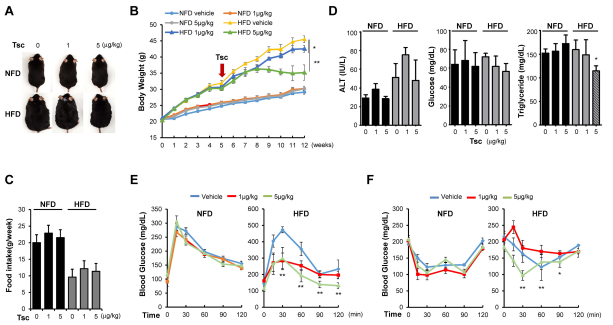

Fig. 3. Tschimganidine reduces body weight gain and improves insulin and glucose tolerances in high-fat diet-fed mice. (A, B) Decreased body size and weight of HFD-fed mice treated with tschimganidine. Tschimganidine was administered to both HFD-fed and NFD-fed mice. Body weight was recorded every two days. (C) Food intake was measured by weighing the remaining chow. (D) Measurement of ALT, glucose, and triglyceride levels. Blood was drawn and analysed from the NFD- or HFD-fed mice with or without tschimganidine treatment. (E, F) Glucose tolerance test and insulin tolerance test of. Vehicle (DMSO) or tschimganidine (1 or 5 μg/kg) treated mice. Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min. *P < 0.05, **P < 0.01; (n = 6 per each group of NFD-fed mice and n = 5 per each group of HFD-fed mice). vehicle vs. 1 and 5 μg/kg tschimganidine treatment.

levels, were evaluated in the sera of experimental mice. Tschimganidine moderated the levels of ALT, glucose, and TG in the HFD-fed mice (Fig. 3D). We also tested whether tschimganidine improves glucose homeostasis *in vivo*. Tschimganidine treatment significantly improved glucose tolerance compared to control conditions in HFD-fed mice (Fig. 3E). In the ITT results, no differences were noted in blood glucose levels between the tschimganidine-treated and control NFD-fed mice; however, the tschimganidine-treated HFD-fed mice recorded lower blood glucose levels than in the control HFD-fed mice (Fig. 3F). These data suggest that tschimganidine delays the increase in body weight and ameliorates metabolic parameters such as ALT, blood glucose, and TG levels; insulin sensitivity; and glucose tolerance in HFD-fed mice.

Tschimganidine reduces adipocyte size and adipogenesis-related factors in the adipose tissues of HFD-fed mice

The amount of gWAT and iWAT in NFD- and HFD-fed mice with or without tschimganidine treatment was evaluated. The amounts of gWAT and iWAT were reduced in tschimganidine-treated HFD-fed obese mice (Fig. 4A). The tschimganidine-treated HFD-fed mice had lower gWAT and iWAT weights than the control mice (Fig. 4B). H&E staining of paraffin-embedded gWAT samples was performed for histological analysis. Significantly more adipocytes exhibited reduced size in the gWAT of the tschimganidine-treated NFD-fed and HFD-fed mice (Fig. 4C). Similarly, the tschimganidine-treated mice in both NFD-fed and HFD-fed groups had smaller adipocytes in

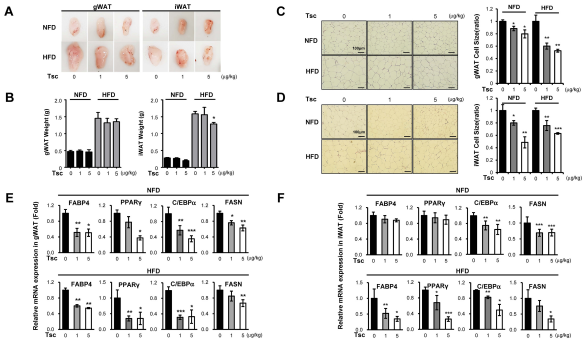

Fig. 4. Tschimganidine reduces adipocyte size and adipogenesis-related factors in fat tissues of high-fat diet-fed mice. (A) White adipose tissues of NFD- or HFD-fed mice administered vehicle (DMSO) or tschimganidine. (B) Gonadal and inguinal WAT weight was measured after the 12-week diet period. (C) The adipocyte size in gonadal WAT sections was determined by staining with H&E. Size measurements were performed using ImageJ software. (D) The adipocyte size of inguinal WAT sections was determined by staining with H&E, and the size measurements were performed as above. (E, F) Gonadal and inguinal WAT were collected, and tissue lysates were prepared. The mRNA expression of FABP4, PPARγ, C/EBPα, and FASN was detected by real-time PCR analysis. β-actin was used as a normalization control. Data are presented as the mean ± SD; *P < 0.05; **P < 0.01; and ***P < 0.001 for vehicle vs. 1 and 5 μg/kg tschimganidine treatment.

the iWAT compared to the untreated control mice (Fig. 4D).

The gene expression of FABP4, PPARγ, C/EBPα, and FASN, which are markers of adipogenesis and lipid accumulation in the gWAT and iWAT, were evaluated. The mRNA expression levels of FABP4, PPARγ, C/EBPα, and FASN decreased in the gWAT of the tschimganidine-treated NFD-fed and HFD-fed mice (Fig. 4E). Similarly, tschimganidine inhibited the mRNA expression of FABP4, PPARγ, C/EBPα, and FASN in the iWAT of the HFD-fed mice (Fig. 4F). Tschimganidine also decreased C/EBPα and FASN levels in the iWAT of the NFD-fed mice. Tschimganidine treatment also diminished adipocyte size in the brown adipose tissue of both NFD-fed and HFD-fed mice, as revealed by histological analysis (Supplementary Fig. 2). Taken together, tschimganidine served to reduce the weight and adipocyte size of both gWAT and iWAT by lowering adipogenesis-related gene expression.

Tschimganidine reduces steatosis in the liver tissues of HFD-fed mice

Finally, we assessed if tschimganidine also affects hepatic lipid accumulation of mice. The liver size was diminished in the tschimganidine-treated HFD-fed obese mice (Supplementary Fig. 3A). Administration of tschimganidine lowered the liver weight than that of control mice in the HFD-fed group (Supplementary Fig. 3B). Afterwards, H&E staining of paraffin-embedded liver tissues was carried out. Lipid accumulation in liver tissues was significantly reduced in tschimganidine-treated HFD-fed mice (Supplementary Fig. 3C). Measured hepatic TG and free fatty acid (FFA) levels were significantly lower in the tschimganidine-treated mice than in the control mice (Supplementary Fig. 3D, E). Additionally, mRNA levels of lipid accumulation-related factors, such as FABP4, PPARγ, and FASN, were lessened in the livers of tschimganidine-treated NFD-fed mice (Supplementary Fig. 3F). Similar to that in NFD-fed mice, tschimganidine also repressed the gene expression of FABP4, PPARγ, and FASN in the livers of HFD-fed mice (Supplementary Fig. 3G). Taken together, tschimganidine alleviated hepatic steatosis on HFD-fed mice.

DISCUSSION

Natural compound-derived therapeutics consist of bioactive phytochemicals with beneficial health effects and may be beneficial in treating obesity and metabolic diseases (5). These therapeutics are based on substances extracted from natural products, such as animals, plants, and minerals; therefore, they are popular as sources of medicine and alternative treatments for prescription drugs because of the experience of using natural products for a long time and the expectations for their effectiveness, despite controversy over their safety (17-19). In fact, about 25% of medicines sold today are derived from plants (18). Natural products have several key advantages; they are biologically active and are composed of proteins naturally selected to interact with other biological molecules (20). Natural products can be derived from secondary metabolites made to help living things survive; thus, they can exhibit low mammalian toxicity (21). Herbal secondary metabolites have also been found to contain phytochemicals with beneficial therapeutic effects, such as alkaloids, terpenoids, and phenolics (18). Therefore, they are used in various ways to prepare nutritional supplements, dietary supplements, and genetically modified foods (5). Additionally, several natural product-based drugs have been developed to enhance human health against cancer, cardiovascular disease, and osteoporosis (5).

We hypothesized that tschimganidine has potential as a natural product-derived therapy in the future by inhibiting adipogenesis and lipid accumulation and conducted research on this. In this study, we aimed to uncover the molecular mechanisms of how tschimganidine regulates lipogenesis. First, we determined that treatment with tschimganidine drastically decreased the expression of adipogenesis-associated genes, such as PPARγ, C/EBPα, FASN, and FABP4, as well as adipogenesis in 3T3-L1 cells. These results were confirmed using tschimganidine at a concentration that maintains cell viability, and it was proposed that the anti-adipogenic effect of tschimganidine is due to the reduced expression of these genes. We determined the optimal concentration of tschimganidine to suppress adipogenesis. Cells treated with 15 μg/ml tschimganidine displayed inhibited differentiation and reduced lipid accumulation based on ORO staining. We confirmed that the expression of factors involved in adipogenesis was significantly reduced. We also observed this effect *in vivo*; tschimganidine admin-

istration reduced obesity in mice fed a high-fat diet. The expression of lipid accumulation- and adipogenesis-associated genes was significantly lessened in the WATs of these mice. Here, we hypothesised that tschimganidine, a phytoestrogen, inhibits the expression of factors involved in adipogenesis.

We further detected a significant increase in the phosphorylation of AMPK following tschimganidine treatment. AMPK is a heterotrimeric enzyme involved in maintaining energy homeostasis in multiple tissues (22). Particularly in adipose tissue, AMPK has been reported to participate in the regulation of glucose and lipid metabolism in adipocytes (23). When AMPK is activated, it induces the phosphorylation of downstream signalling targets either directly or indirectly. This induction decreases the synthesis of cholesterol and fatty acids (24). Regulation of lipid metabolism is a well-known function of AMPK. AMPK also functions to regulate the rates of glycogen synthesis and breakdown (25). AMPK is activated when Adenosine triphosphate (ATP) is depleted due to ATP hydrolysis, increasing the binding of Adenosine monophosphate (AMP) and Adenosine diphosphate (ADP) to specific regulatory sites on AMPK (23). AMPK is activated by an increase in the relative AMP: ATP ratio and serves to activate fatty acid oxidation. It has been reported that activation of ERα induces AMPK phosphorylation, which increases beiging in white adipocytes (26). Therefore, we investigated whether tschimganidine induces AMPK phosphorylation and found that activation of AMPK by tschimganidine increases adipocyte lipolysis, despite controversy over whether AMPK may have a role in regulating lipolysis (27, 28). Treatment with tschimganidine increased phosphorylation of AMPK, accompanied by phosphorylation of ACC. However, the effect of tschimganidine treatment on the inhibition of lipid accumulation was diminished by the knockdown of AMPK gene expression. This indicates that the effect of tschimganidine on lipid accumulation was based on the activation of AMPK, which is the predominant mechanism by which tschimganidine affects adipogenesis and lipid accumulation as an ERα agonist.

Furthermore, Tschimganidine ameliorated glucose tolerance and insulin sensitivity in HFD-fed mice. Our data support that tschimganidine increases AMPK activation and induces blood glucose uptake and lipolysis in adipose tissues. Lipid contents were also significantly lowered in liver tissues from the HFD-fed mice. Tschimganidine treatment reduced hepatic TG and free fatty acid levels and decreased the expression of lipid accumulation-associated factors in liver tissues, suggesting that tschimganidine may be beneficial in treating fatty liver and related metabolic diseases. The results of this study show that tschimganidine may act as an effective therapeutic agent for obesity and metabolic diseases by inhibiting adipogenesis through the activation of AMPK. Despite these findings, further research is needed to determine the specific mechanism about which proteins tschimganidine targets and how they modulate AMPK activity.

In summary, tschimganidine, a terpenoid from the Umbelliferae family, reduces adipogenesis, lipid accumulation, and blood glucose levels via the AMPK activation. Tschimganidine is a potential therapeutic agent for obesity and metabolic diseases, and further mechanistic studies are needed to evaluate its clinical application.

MATERIALS AND METHODS

Materials and methods are described in Supplementary Information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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