Dioscin inhibits adipogenesis through the AMPK/MAPK pathway in 3T3-L1 cells and modulates fat accumulation in obese mice

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Abstract. Dioscin (DS) is a steroidal saponin present in a number of medicinal plants and has been shown to exert anticancer, antifungal and antiviral effects. The present study aimed to determine the effects of DS on the regulation of adipogenesis and to elucidate the underlying mechanisms. In vitro experiments were performed using differentiating 3T3-L1 cells treated with various concentrations (0-4 µM) of DS for 6 days. A cell viability assay was performed on differentiating cells following exposure to DS. Oil Red O staining and triglyceride content assay were performed to evaluate the lipid accumulation in the cells. We also carried out the following experiments: i) flow cytometry for cell cycle analysis, ii) quantitative reverse transcription polymerase chain reaction for measuring adipogenesis-related gene expression, and iii) western blot analysis to measure the expression of adipogenesis transcription factors and AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC) and mitogen-activated protein kinase (MAPK) phosphorylation. In vivo experiments were performed using mice with obesity induced by a high-fat diet (HFD) that were treated with or without DS for 7 weeks. DS suppressed lipid accumulation in the 3T3-L1 cells without affecting viability at a dose of up to 4 µM. It also delayed cell cycle progression 48 h after the initiation of adipogenesis. DS inhibited adipocyte differentiation by the downregulation of adipogenic transcription factors and attenuated the expression of adipogenesis-associated genes. In addition, it enhanced the phosphorylation of AMPK and its target molecule, ACC, during the differentiation of the cells. Moreover, the inhibition of adipogenesis by DS was mediated through the suppression of the phosphorylation of MAPKs, such as extracellular-regulated kinase 1/2 (ERK1/2) and p38, but not c-Jun-N-terminal kinase (JNK). DS significantly reduced weight gain in the mice with HFD-induced obesity; this was evident by the suppression of fat accumulation in the abdomen. The present study reveals an anti-adipogenic effect of DS in vitro and in vivo and highlights AMPK/MAPK signaling as targets for DS during adipogenesis.

Introduction

Obesity is the most common metabolic disease worldwide. It is characterized by an excessive storage of body fat, and is associated with a number of metabolic complications, including type 2 diabetes, hypertension and cardiovascular diseases (CVD) (1). Such complications could lead to higher mortality rates in obese as opposed to lean patients.

Adipogenesis contributes to excess fat deposition in adipocytes during the differentiation process from preadipocytes. The molecular and cellular mechanisms of adipogenesis have been extensively studied using the 3T3-L1 preadipocyte cell line as these cells differentiate into adipocytes upon stimulation; this process is similar to the development of obesity in humans (2-5). Adipogenesis involves concerted transcriptional and cellular events, including growth arrest, re-entry into the cell cycle for mitotic clonal expansion (MCE) and the start of transcription during differentiation (2). Numerous genes have been shown to be involved in the development of obesity. For instance, peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein (C/EBP)β, C/EBPα, C/EBPδ and sterol regulatory element binding protein-lc (SREBP-lc) (2,6). Furthermore, AMP-activated protein kinase (AMPK) has been suggested to function as a sensor of cellular energy status and, when activated, accelerates the ATP-producing catabolic pathways and simultaneously reduces the anabolic pathways that consume ATP (6,7). Several studies have identified AMPK signaling as the target for the treatment of obesity and diabetes (8-10). Moreover, mitogen-activated protein kinases (MAPKs), namely, extracellular regulated kinase 1/2 (ERK 1/2), p38 and c-Jun-N-terminal kinase (JNK) are known to play a

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crucial role in several cellular processes, including adipocyte differentiation (11).

Dioscin (DS) is a steroidal saponin present in a number of medicinal plants, such as *Dioscorea nipponica* Makino and *Dioscorea zingiberensis* Wright. Traditionally, saponins from *Dioscorea* plants are used to treat cardiovascular diseases, rheumatoid arthritis, asthma and hyperlipidemia (12). Several studies have demonstrated that DS exerts anticancer (13-15), antifungal (16) and antiviral effects (17). A previous study reported that rats fed a high-fat diet (HFD) containing 5% *Dioscorea nipponica* Makino presented with reduced weight and adipose tissue gain when compared to the control group (18). Similarly, mice fed an HFD containing the aqueous extract of the *Dioscorea* plant, Rhizoma Dioscoreae Tokoronis, showed lower body weight and adipose tissue in comparison to the control mice (19), suggesting that *Dioscorea* plants have anti-obesity effects. A recent study reported that a steroidal saponin, pseudoprotodioscin, present in the *Dioscorea* plant, inhibited adipogenesis in 3T3-L1 cells (12). However, the potential role of DS, an active compound in the *Dioscorea* plant, in adipogenesis and its underlying mechanisms of action have not yet been fully elucidated.

Materials and methods

**Materials and reagents.** DS (PubChem CID: 119245) (Fig. 1, adopted from http://www.ncbi.nlm.nih.gov/pccompound) was obtained from the Nanjing Zelang Medical Technology Co., Ltd., (Jiangsu, China) with 98% purity as determined by high performance liquid chromatography, and was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) for the experiments. Dexamethasone, insulin, propidium iodide, 3-isobutyl-1-methylxanthine (IBMX), RNase A, orlistat and Oil Red O staining solution were purchased from Sigma. Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were from HyClone (Logan, UT, USA), Zoletil 50 was obtained from Virbac Laboratories (06516 Carros, France), Triton X reagent and the SuperScript II kit were obtained from Invitrogen (Carlsbad, CA, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). Anti-AMPK, anti-p-AMPK, anti-ACC, anti-phosphorylated (p)-ACC, anti-PPARγ, anti-C/EBPα, anti-C/EBPβ, anti-C/EBPδ, anti-ERK, anti-p-ERK, anti-p38, anti-p-p38, anti-JNK and anti-p-JNK antibodies were purchased from Cell Signaling Biotechnology (Beverly, MA, USA). The protein assay kit (RIPA buffer), rabbit and mouse secondary antibodies, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Animals and diet.** C57BL/6J male mice (5 weeks old) were purchased from Samtako Inc. (Seoul, Korea). The animals were maintained in a room under the following conditions: 12-h light/dark cycles, a temperature of 22±2°C and a relative humidity of 50±5% during the whole experiment period. Mice were fed a normal diet for 2 weeks for adaptation. Subsequently, they were randomly divided into 5 groups (n=8). Two groups were fed either a normal diet (normal control, NC) or an HFD (kcal%, protein 20, carbohydrate 35, fat 45; D12451 Research Diets Inc., New Brunswick, NJ, USA). The other groups were fed an HFD and were intragastrically administered with DS or orlistat at the indicated concentrations dissolved in 0.5% carboxymethyl cellulose (CMC) in distilled water. This solution was freshly prepared each day prior to administration. The mice in the control group were administered 0.5% CMC. The mice were treated as described above for 7 weeks. Body weight was measured each week. All animal experiments were approved by the Institutional Animal Care and Use Committee at Chonbuk National University, Jeonju, Korea.

**Determination of body fat composition by microtomography or computed tomography (micro-CT) and abdominal fat isolation.** The mice were starved for 6 h and in vivo micro-CT images of the anesthetized mice [Zoletil 50, 2 mg/kg, intraperitoneally (i.p.)] were acquired using a Skyscan-1076 micro-CT scanner (Skyscan, Aartselaar, Belgium). CT was performed using the following parameters: pixel size, 18 μm; source voltage, 48 kVp; and source current, 200 μA. The X-ray detector comprised a 12-bit water-cooled charge-coupled device high-resolution (4,000x2,300-pixel) camera and an X-ray scintillator. The images were acquired in increments of 0.6 degrees. The exposure time for each view was 0.46 sec; a 0.5-mm aluminum energy filter was used. Following micro-CT scanning, the mice were sacrificed by exposure to diethyl-ether, abdominal fat was isolated and images were acquired using an Olympus SP-500UZ camera (Olympus, Center Valley, PA, USA).

**Cell culture and differentiation.** 3T3-L1 preadipocytes were obtained from the American Type Culture Collection and maintained in DMEM containing 10% FCS and a relative atmosphere of 5% CO2 at 37°C. The differentiation of the preadipocytes was induced 2 days post-confluence (day 0) by the addition 0.5 mM IBMX, 1 μM dexamethasone and 10 μg/ml insulin [multiple daily insulin (MDI)] for 2 days. Subsequently, the culture medium was changed to DMEM/10% FCS containing insulin. After 2 days, the medium was replaced with DMEM/10% FCS and the cells were incubated for a further 2 days. DS was added on day 0 during differentiation until the cells were harvested for the experiments described below.

**Cell viability.** The cells were treated with MDI and various concentrations of DS for the indicated periods of time, and cell viability was measured using a CCK-8 kit according to
the manufacturer's instructions. Absorbance was measured at 450 nm on a microplate reader (Biochrom Anthos Zenyth 200; Biochrom Ltd., Cambridge, UK).

**Oil Red O staining.** The differentiation of the cells was induced as described above. On day 6, the cells were stained with Oil Red O, according to the manufacturer's instructions to visualize lipid accumulation in the cells. The intracellular lipid content was measured by extracting Oil Red O with isopropanol, and the absorbance at 520 nm was recorded using a spectrophotometer.

**Triglyceride assay.** Cellular triglyceride contents were measured using a commercial triglyceride assay kit (Triglyzyme test; Wako Pure Chemical Industries Ltd., Saitama, Japan), according to the instructions provided by the manufacturer. Briefly, the cells were washed twice with phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer. Following centrifugation at 3,000 x g for 5 min, the supernatants were assayed for the triglyceride and protein content. The triglyceride content normalized to the protein concentration determined using bovine serum albumin as the standard.

**Western blot analysis.** The cells were lysed in ice-cold RIPA buffer for 20 min and centrifuged (15,000 x g) for 20 min at 4°C. The protein concentration was measured using a bicinchoninic acid method. Lysates (30 µg) were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Subsequently, blocking was performed with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The membranes were probed with primary antibodies as indicated at 4°C overnight, washed with TBST 4 times, and subsequently incubated with horseradish peroxidase-conjugated secondary antibody for 45 min. The membranes were washed again 3 times with TBST and the proteins were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech Inc.).

**Isolation of total RNA and quantitative reverse transcription (RT-qPCR).** Total RNA was extracted using TRIzol reagent, according to the manufacturer’s recommendations. Isolated RNA (1 µg/µl) was used for cDNA synthesis using the SuperScript II kit. Aliquots of cDNA were amplified on the an ABI Real-Time PCR system from Applied Biosystems Inc. (Forster City, CA, USA) using the SYBR-Green Master Mix from Applied Biosystems. GAPDH was used as the invariant control. Primers specific for the genes examined are listed in Table I. The results were presented as levels of expression relative to that of the control.

**Cell cycle analysis.** The cells were harvested at the indicated time points following MDI stimulation with or without DS. Subsequently, the cells were fixed overnight with 70% ethanol at -20°C, washed twice with PBS, and stained with 50 µg/ml propidium iodide (IP) solution containing 25 µg/ml RNase A for 30 min at 37°C. Cell cycle analysis was performed using the FACS-Calibur flow cytometry system (BD Biosciences, San Diego, CA, USA), and data analysis was performed using FlowJo v10 software (TreeStar, Inc., Ashland, OR, USA).

**Statistical analysis.** All values are presented as the means ± SEM. Statistical significance was determined using the Student’s t-test. P-values <0.05 were considered to indicate statistically significant differences.

**Results**

**Effect of DS on adipocyte viability.** Several studies have demonstrated the cytotoxic effects of DS in a number of cell lines (14,15). This prompted us to examine the possibility that the use of DS may result in cytotoxicity to 3T3-L1 cells. To examine this possibility, we examined the effects of DS on the viability of 3T3-L1 cells by CCK-8 assay. DS (0-4 µM) showed no significant cytotoxicity towards the differentiating preadipocytes. However, 8 µM DS inhibited the viability of the cells by approximately 50% at 24 h (Fig. 2A and data not shown). Therefore, we selected the maximum dose of DS (4 µM) for

<table>
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<tr>
<th>Gene</th>
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<th>Reverse primer</th>
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<td>SREBP1c</td>
<td>GGGTTTGAAAGCATCGAAGA</td>
<td>CGGGAAATCAGTACTTGTGTT</td>
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<td>AGCCTTTTCTCATGGAAGA</td>
<td>TTGTGCAACAGCCACTC</td>
</tr>
<tr>
<td>FAS</td>
<td>TGATGTTGACAACAGGAGG</td>
<td>GGCTGTTGACTCTTAGTGATAA</td>
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<td>GLUT4</td>
<td>TGGGCCCCTTCTTAGATGGG</td>
<td>CTTAAGAGCTCGGGCCCAA</td>
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<tr>
<td>Adiponectin</td>
<td>GCAGAGAGTGGCCTCTGGGA</td>
<td>CCCCCCAGCTCTGGTCAACCTC</td>
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<tr>
<td>Leptin</td>
<td>TCCAGAAAAGTCCAGAGCTAAC</td>
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<tr>
<td>β-actin</td>
<td>CCTAAGGCAACCGTGAAA</td>
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| SREBP1c, sterol regulatory element binding protein-1c; aP2, activating protein 2; GLUT4, glucose transporter 4; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase. |
further experiments on the effects of DS on adipogenesis to rule out the possibility that the inhibition of adipogenesis by DS may result from its cytotoxic effects on 3T3-L1 cells.

**DS inhibits lipid accumulation in 3T3-L1 adipocytes.** We examined the effects of DS on the differentiation of 3T3-L1 preadipocytes to adipocytes. The cells were stimulated with MDI to initiate differentiation. The culture medium was supplemented with various doses (0-4 µM) of DS for 6 days. DS dose-dependently decreased intracellular fat accumulation compared to the control as demonstrated by morphologic and quantitative analysis of intracellular lipids by Oil Red O staining (Fig. 2B and C). Consistent with these results, the triglyceride content in the 3T3-L1 cells treated with the indicated doses of DS was lower than that in the control cells differentiated for 6 days (Fig. 2D).

**DS suppresses MCE during the early phase of adipogenesis.** During adipogenesis, multiple rounds of cell cycle progression contribute to the MCE process. Therefore, we sought to examine the effects of DS on the cell cycle progression of 3T3-L1 cells during the MCE process. The results from flow cytometry revealed that the DS-treated cells showed a delayed cell cycle progression 48 h following stimulation with MDI (Fig. 3). The percentage of preadipocytes in the G₀/G₁ phase was approximately 58%, while 45% of the untreated adipocytes and 65% of the DS-treated adipocytes were in the G₀/G₁ phase. These observations suggested that DS inhibits clonal expansion of the cells by inducing G₀/G₁ phase arrest.

**DS inhibits the protein expression of adipogenic transcription factors.** The differentiation of preadipocytes into adipocytes involves the sequential activation of several pro-adipogenic transcription factors, such as, C/EBPα/β/δ and PPARγ. Thus, we examined whether the reduced fat accumulation in the adipocytes was due to the downregulation of the aforementioned adipogenic transcription factors. As shown in Fig. 4, DS...
significantly inhibited the protein expression of C/EBPα/β/δ and PPARγ, suggesting that DS inhibits adipogenesis by suppressing the expression of adipogenic transcription factors.

**DS inhibits adipogenesis-related gene expression in 3T3-L1 cells.** As the adipogenic transcription factors were downregulated by DS, we further examined the expression of other adipogenesis-related genes involved in lipogenic and fatty acid oxidation and glucose homeostasis pathways. As shown in Fig. 5A, DS (4 µM) significantly inhibited the mRNA expression of SREBP-1c, activating protein 2 (aP2), fatty acid synthase (FAS), glucose transporter 4 (GLUT4) and leptin. However, there was no significant difference in the mRNA levels of hormone-sensitive lipase (HSL), lipoprotein lipase (LPL) and adiponectin when compared to the controls (Fig. 5B).

**DS regulates the AMPK pathway during adipogenesis.** AMPK and its target, acetyl-CoA carboxylase (ACC), are the key regulators of preadipocyte differentiation and adipogenesis. Our results revealed that DS enhanced the phosphorylation of AMPK and ACC, phosphorylated and total, was analysed by western blot analysis, as described in the ‘Materials and methods’. (B) The graphs show the band intensity ratio of the phosphorylated form by the total protein expression of AMPK and ACC. Data represent means ± SEM (P<0.05), and the experiments were performed at least 3 times with similar results.

**Figure 6. Dioscin (DS) enhances the phosphorylation of AMP-kinase (AMPK) and its target, acetyl-CoA carboxylase (ACC).** 3T3-L1 cells were treated with the indicated concentrations of DS for 48 h. (A) Subsequently, the protein expression of AMPK and ACC, phosphorylated and total, was analysed by western blot analysis, as described in the ‘Materials and methods’. (B) The expression of the lipolytic genes, lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL), was also analysed as described above. Data are presented as the means ± SEM (P<0.05). The experiment was performed in triplicate.
our results in comparison to the controls. Moreover, treatment with DS (100 mg/kg) markedly inhibited body weight gain in comparison to the controls from the first weeks of treatment (Fig. 8A). This decrease in body weight was due to a significant decrease in fat accumulation as established by micro-CT scanning and macroscopy of abdominal fat (Fig. 8B).

Discussion

Obesity is a chronic, socially stigmatized and costly disease that has become an epidemic worldwide. The drugs currently available for the treatment of obesity have raised safety concerns and have demonstrated poor efficacy. For instance, orlistat is known to cause fetal fat loss and gastrointestinal symptoms. Thus, it is necessary to identify new medicinal products that are safe and effective in treating obesity (21,22).

The increased growth and proliferation of adipocyte precursor cells lead to increased adipose mass, which represents the size and number of adipocytes. Our results revealed that DS at a dose of up to 4 µM did not affect the viability of differentiating 3T3-L1 cells. This finding prompted us to eliminate the possibility that DS-induced cytotoxicity accounts for the inhibition of adipogenesis. Moreover, DS delayed the cell cycle progression in differentiating 3T3-L1 cells during the early phase of adipogenesis upon MDI stimulation. Further experiments demonstrated that DS dose-dependently reduced adipogenesis in 3T3-L1 cells, accompanied by a decreased triglyceride content.

PPARs are ligand-activated transcription factors that have 3 homologues: PPARα/β/γ, and have been reported to play a role in glucose and protein metabolism. They also regulate the proliferation and differentiation of preadipocytes (23-25). C/EBPs are crucial proteins involved in preadipocyte differentiation. During the early steps of adipocyte differentiation steps, the activation of C/EBPB and δ is known to result in the activation of C/EBPα and PPARγ, which in turn control adipogenesis and insulin sensitivity in adipocytes (26-28). Moreover, the insulin-dependent glucose uptake requires the translocation of GLUT4, whose expression is regulated by C/EBPs from intracellular storage sites to the cell surface (7,11,29). Another transcription factor, SREBP-1c, has been implicated in preadipocyte differentiation and fatty acid metabolism (6). C/EBPα, PPARγ and SREBP-1c activation is known to regulate the adipocyte differentiation markers and genes associated with lipid metabolism, such as aP2, fatty acid synthase (FAS), LPL and HSL (30,31).

Our findings revealed that DS significantly downregulated C/EBPα/β/δ, PPARγ, SREBP-1c and GLUT4 expression, which is essential for adipogenesis. Consistent with our results, previous studies have shown that ursolic acid and resveratrol-amplified grape skin extracts inhibit adipogenesis in 3T3-L1 cells through the downregulation of PPARγ and C/EBP isoforms (6,31). In accordance with our results, a previous study reported that 4-hydroxyderricin and xanthoangelol, plant-derived anti-obesity compounds, inhibited GLUT4 expression during the differentiation of 3T3-L1 cells (11). We also observed reduced mRNA levels of aP2 and FAS in adipocytes treated with DS, in accordance with a previous study showing the anti-adipogenic effects of resveratrol-amplified grape skin extracts on adipocytes (31). On the other hand,
leptin is secreted by adipocytes, suppresses food intake and promotes energy expenditure. Thus, leptin levels are increased with adipogenesis and obesity (32,33). In this study, decreased leptin levels in 3T3-L1 cells treated with DS indicated that DS exerted anti-adipogenic effects. Adiponectin, a vital hormone secreted by adipose tissue has been suggested to exert anti-diabetic, anti-inflammatory and anti-atherogenic effects (32). In our study, the increased adiponectin expression in DS-treated 3T3-L1 cells was possibly the result of the anti-inflammatory effects of DS on adipocytes. A previous study suggested that high adiponectin production due to the presence of niacin is the result of its direct anti-inflammatory properties (34). However, further studies are required to demonstrate the direct anti-inflammatory properties of DS on adipocytes.

AMPK is a major protein that regulates cellular energy homeostasis and regulates a number of biological pathways, such as carbohydrate and lipid metabolism. Hence, it is one of the most important targets for treating diabetes and obesity (8-10,36). ACC is important for the synthesis and consumption of fatty acids and is a target of AMPK (11). In the present study, DS increased the phosphorylation of AMPK and ACC during preadipocyte differentiation. These results indicate that DS inhibits adipogenesis through the AMPK pathway. In accordance with our results, several compounds, such as chitin, ginsenosides, epigallocatechin gallate and aspigenin have been reported to target AMPK for the inhibition of adipocyte differentiation (6).

Previous studies have indicated that the MAPKs, ERK1/2, JNK and p38, are involved in adipocyte differentiation (11). The downregulation of ERK1/2 or p38 by their inhibitors has been shown to result in reduced adipocyte differentiation (20,37,38), suggesting that ERK1/2 and p38 are essential for adipogenesis. Consistent with this result, our data demonstrated that DS suppressed the phosphorylation of ERK1/2 and p38. However, we did not observe any effect on JNK activation upon DS treatment during adipocyte differentiation. There is limited evidence on the role of JNK in adipocyte differentiation (20). However, contrary to our findings, a previous study demonstrated that JNK phosphorylation is important for adipocyte differentiation and that JNK inhibitor (SP600125) reduced the lipid accumulation in adipocytes (11).

In the present study, we investigated the potential role of DS in regulating the adipogenesis of 3T3-L1 preadipocytes. We demonstrate that DS inhibits adipogenesis without exerting any cytotoxic effects on differentiating preadipocytes. This anti-adipogenic effect targets the MCE phase, where DS retards cell cycle progression and decreases the expression of pro-adipogenic transcription factors. The DS-associated blockage of the MCE phase is accompanied by an inhibition of the phosphorylation of the MAPKS, ERK1/2 and p38. Moreover, DS induced the phosphorylation of AMPK. In our in vivo mouse model of obesity, DS significantly suppressed body weight gain and abdominal fat accumulation. This finding was consistent with previously reported results showing that treatment with Dioscorea plant extract reduced body weight and fat accumulation in mice with HFD-induced obesity (18,19).

Taken together, our data demonstrate that DS is a natural anti-adipogenic molecule that targets the AMPK/MAPK pathway, inhibits the MCE phase and decreases the expression of adipogenic transcription factors during the process of adipogenesis in 3T3-L1 cells; it also modulates body weight and fat accumulation in mice with HFD-induced obesity.

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References