Original Research

Inhibition of adipocyte differentiation and adipogenesis by the traditional Chinese herb *Sibiraea angustata*

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Abstract

Obesity has become a major health concern due to its strong association with the metabolic syndrome. Inhibition of adipocyte differentiation represents a key strategy to inhibit obesity. *Sibiraea angustata* (SA), a traditional Chinese herb, has a wide range of pharmacological effects, such as improving digestive functions. Here, we report a novel antiadipogenic effect of SA. By using the SA water extract (SAW), SA acetic ether extract (SAA) and the 3T3-L1 model of adipocyte differentiation and adipogenesis, we showed that both SAW and SAA impaired the proliferation and adipo-differentiation of 3T3-L1 in a dose- and time-dependent manner. At the molecular level, treatment of 3T3-L1 cells with SAW or SAA inhibited the expression of the key adipocyte differentiation regulator CCAAT enhancer binding protein β (C/EBPβ), as well as peroxisome proliferator activated receptor γ, adipocyte protein-2, lipoprotein lipase and glucose transporter 4. Cell cycle analysis showed that both SAW and SAA blocked cell cycle at the G1–S transition phase, causing cells to remain in the preadipocyte state. The expression of CyclinA and cyclin-dependent kinase 2 was also inhibited by SAW and SAA. Treatment with SAW also prevented the localization of C/EBPβ to the centromeres. Taken together, our results show that SA has a potent antiadipogenic effect in 3T3-L1 cells due to the inhibition of adipocyte differentiation and adipogenesis. We propose that SA may be used as a safe and effective neutraceutical to manage obesity.

Keywords: *Sibiraea angustata*, 3T3-L1, adipocyte differentiation, adipogenesis, obesity

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Introduction

In recent years, obesity has become a major global health concern. An unhealthy lifestyle, eating behavior and cultural adaptation contribute to the rapid increase in obesity in both developed and developing countries. It has been estimated that 26% of Americans are overweight.[1] A recent survey in China showed that between 1992 and 2002, more than 60 million Chinese became obese. Increased adiposity is considered to be one of the most important risk factors for metabolic disorders, such as coronary heart disease, hypertension, type 2 diabetes mellitus, cancer, respiratory complications and osteoarthritis.[2]

Obesity occurs when energy intake by an individual exceeds the rate of energy expenditure.[3] At the cellular level, obesity was originally considered a hypertrophic disease resulting from an increase in fat cell number or the size of individual adipocytes.[4–6] New fat cells could arise from a pre-existing population of undifferentiated progenitor cells or through the dedifferentiation of adipocytes to preadipocytes, which then proliferate and redifferentiate into mature adipocytes. In both cases, the generation of new fat cells plays a key role in the development of obesity. The differentiation of adipocytes from preadipocytes has been extensively studied in preadipocyte cell lines such as 3T3-L1 and 3T3-F422A cells.[7–9] The adipogenic process consists of the preadipocyte proliferation and their differentiation into mature adipocytes, and this proliferation has proven to be a prerequisite for latter adipogenesis.[10,11] In the presence of a hormonal cocktail consisting of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX) and insulin, 3T3-L1 preadipocytes differentiate into adipocyte-like cells expressing adipocyte-specific genes and accumulating triacylglycerol-rich lipid droplets.[12] Upon receiving the appropriate combination of adipogenic signals, growth-arrested 3T3-L1 preadipocytes initiate mitotic clonal expansion (MCE) and re-enter the cell cycle progression.[13] MCE is a prerequisite for terminal differentiation.[14] The expression and activity of CCAAT enhancer binding protein β (C/EBPβ) are required for MCE.[15] Upon withdrawal from the cell cycle, preadipocytes initiate expression of adipocyte-specific genes,[16] such as peroxisome proliferator activated

receptor γ (PPARγ), CCAAT-enhancer binding protein (C/EBPs) family and adipocyte protein-2 (aP2).

Having recognized the health benefit of weight loss, there have been extensive efforts in developing pharmacotherapies that enhance weight loss when combined with interventions aimed at changing lifestyle. These include the use of traditional Chinese medicine (TCM), an important component of complementary and alternative medicine, in managing obesity. *Sibiraea angustata* (SA) is a TCM herb that grows at 3000–4000 m above sea level and is widely distributed in the Northwest of China. SA has been traditionally used for eliminating 'stomach heat' and improving digestive functions. There is also an interesting phenomenon that sheep have been observed to lose weight when they had SA accidently,[17] and we propose that SA may be used as a safe and effective neutraceutical to manage obesity. The goal of the present study is, therefore, to determine whether SA has a novel activity in inhibiting adipocyte differentiation and adipogenesis.

Materials and methods

Drugs, chemical reagents and other materials

MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), propidium iodide (PI), ribonuclease (RNase), oil red O, IBMX, DEX, insulin, troglitazone (Tro), GW9662 and lactate dehydrogenase (LDH) leakage kit were purchased from Sigma (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Trizol, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotic mixture (penicillin–streptomycin) were purchased from Gibco-BRL (Grand Island, NY, USA). BCA-100 protein quantitative analysis kit was from Pierce (Rockford, IL, USA). PPARγ goat polyclonal antibody, C/EBPβ rabbit polyclonal antibody, fatty acid synthetase (FAS) rabbit polyclonal antibody, CyclinA rabbit polyclonal antibody, cyclin-dependent kinase 2 (CDK2) rabbit polyclonal antibody, β-actin rabbit polyclonal antibody and all secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein molecular weight markers were obtained from Pharmacia (Saclay, France). Polyvinyldifluoride (PVDF) membrane for Western blotting was purchased from Millipore (Bedford, MA, USA).

Preparation of SA water extract and acetic ether extract

SA was purchased from Herbs and Medical Supplies (Xi'an, China). Dry herbs were minced with a Cuisinart blender (model CBT-500W; Cuisinart, East Windsor, NJ, USA). For the preparation of the aqueous extracts, 50 g of minced dry herbs was boiled in 200 mL of endotoxin-free injectable water (NDC 03380013-04; Baxter, McGaw Park, IL, USA) for 40 min. The supernatants were transferred to 50-mL conical tubes and spun at 3000 rpm for 30 min at room temperature (RT). The supernatants were collected in new tubes and stored at -20°C until use. For acetic ether extract preparation, 50 g of dry herbs was mixed with 75 mL of acetic ether and rocked for 24 h at RT. The extracts were then spun at 3000 rpm for 30 min at RT, and the supernatants were collected and stored at -20°C until use.

Cultured cells

3T3-L1 cells were maintained in DMEM containing 10% FBS. To induce differentiation, two-day postconfluent preadipocytes (designated as day 0) were cultured in DMEM containing 10% FBS, 10 μg/mL insulin, 0.5 μmol/L DEX and 0.5 mmol/L IBMX for two days. Cells were then cultured in DMEM supplemented with 10% FBS and 10 μg/mL insulin for another two days, after which cells were refed every other day with DMEM containing 10% FBS at 37°C and 5% CO_2. SA water extract (SAW) and SA acetic ether extract (SAA) were diluted in DMEM when necessary before adding to the medium.

Proliferation assay

Two days after confluence, cells were treated with differentiation medium (DM) containing different concentrations of SAW or SAA. At each time point, 50 μg/mL MTT reagent was added to the cells and incubated for four hours. Treatment medium was removed and the absorbance was measured at 570 nm using a microplate reader from VersaMax Molecular Device (Sunnyvale, CA, USA).

LDH leakage assay

3T3-L1 preadipocytes were grown in a 48-well plate and incubated with either vehicle or 50, 100 and 200 mg/mL of SAW or SAA. At 24 h, LDH activity in the culture supernatants was measured by a colorimetric cytotoxicity assay kit from Sigma. The amount of LDH leakage was expressed as a percentage of total activity.

Adipocyte differentiation and oil red O staining

Two days after confluence, designated as day 0 (D0), 3T3-L1 cells were treated for two days with a DM containing 10 μg/mL insulin, 0.5 μmol/L DEX and 0.5 mmol/L IBMX. The medium was then replaced with DMEM containing insulin and the cells continued to differentiate until day 7 (D7). Test compounds were administered during the adipogenic process. For the oil red O staining, cells were washed three times with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde for one hour. Oil red O (0.5% in isopropanol) was diluted with water (3:2), filtered twice with a 0.45 μm filter, and added to fixed cells for 15 min at RT. Cells were washed with 70% ethanol and water before being visualized by light microscopy and photographed. In order to determine the extent of adipose conversion, 1 mL of isopropyl alcohol was added to the stained culture dish, the extracted dye was immediately removed by gentle pipetting and its absorbance monitored spectrophotometrically at 510 nm.[18]

Transfection and reporter gene assay

3T3-L1 cells in 24-well plates were transiently transfected with 1.25 μg of total DNA (900 ng of PPARγ expression vector, 300 ng of luciferase reporter gene containing a PPAR-response element [tk-PPRE-Luc], and 50 ng of pRL-tk), using SuperFect Transfection Reagent from Qiagen (Valencia, CA, USA). Twelve hours after transfection, cells were treated with troglitazone (10 μmol/L, a PPARγ activator) or GW9662 (5 μmol/L, a PPARγ inhibitor) in the absence or presence of SAW or SAA, and incubated for 24 h. The firefly luciferase luminescence was normalized with the renilla using the Dual Luciferase Assay System from Promega (Madison, WI, USA). Transfection experiments were performed in triplicate and repeated independently at least three times.

Determination of cell numbers

Cell numbers were determined using a hemocytometer.

Cell cycle determination

Postconfluent 3T3-L1 preadipocytes were treated with DM in the presence or absence of SAW or SAA for 14 or 18 h. Then, cells were harvested and fixed with 75% ethanol at 4°C for 48 h. After removing ethanol, cells were stained with PI from Sigma and solution containing RNase (20 μg/mL) for 30 min. Fluorescence-activated cell sorting (FACS) analysis was performed with a Becton-Dickinson FACScantoII instrument and data analysis with FACS Diva software from Becton–Dickinson (San Jose, CA, USA).

Semi-quantitative reverse transcriptase polymerase chain reaction analysis

Total RNA was isolated using Trizol reagent (GIBCO-BRL) as suggested by the manufacturer. RNA concentration was determined using Gene Quant (Amersham Pharmacia Biotech, Uppsala, Sweden). Two micrograms of RNA were used for reverse transcription polymerase chain reaction (RT-PCR). The primers used in the experiments are shown in Table 1.

Table 1 Primers used for semi-quantitative RT-PCR

Gene	Sequence (5′–3′)
PPARγ	Forward: 5′-ACCACTCGCATTCCTTTGAC-3′ Reverse: 5′-TCAGCGGGAAGGACTTTATG-3′
C/EBPβ	Forward: 5′-ACAAGCTGAGCGACGAGTAC-3′ Reverse: 5′-ACAGCTGCTCCACCTTCTTC-3′
LPL	Forward: 5′-TGAGAACATTCCCTTCACCCT-3′ Reverse: 5′-CAGCGGAAGTAGGAGTCGTT-3′
aP2	Forward: 5′-TGGAAGCTTGTCTCCAGTGA-3′ Reverse: 5′-ATTTCCATCCAGGCCTCTT-3′
Glut4	Forward: 5′-AACGAGCTGGACGACGGACA-3′ Reverse: 5′-TTGCCCCTCAGTCATTCTCA-3′
β-Actin	Forward: 5′-TGACGGGGTCACCCACAC-3′ Reverse: 5′-CTAGAAGCATTTGCGGTG-3′

RT-PCR, reverse transcription polymerase chain reaction; PPARγ, peroxisome proliferator activated receptor γ; C/EBPβ, CCAAT enhancer binding protein β; LPL, lipoprotein lipase; aP2, adipocyte protein-2; Glut4, glucose transporter 4

Quantitative realtime PCR analysis

Cells were collected and total RNA was extracted with a spin column from Qiagen (Hilden, Germany). RNA was treated with DNaseI at RT for 15 min to remove genomic DNA contamination. The first strand cDNA was synthesized using a cDNA synthesis kit from Promega. The gene expression levels were analyzed by quantitative realtime RT-PCR using the ABI 7500 Real Time PCR system from Applied Biosystems (Foster City, CA, USA). The primers used in the experiments are shown in Table 2. After an initial incubation for two minutes at 50°C, the cDNA was denatured at 95°C for 10 min followed by 40 cycles of PCR (95°C, 15 s; 60°C, 60 s). All results were obtained from at least three independent experiments. The mRNA levels of all genes were normalized using β-actin as the internal control.

Western blotting analysis

The protein expression of PPARγ, C/EBPβ, CyclinA and CDK2 expression was determined by Western blotting. Preadipocytes and adipocytes were harvested from the culture plates with cell lysis buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate [SDS]) containing freshly added protease inhibitor cocktail (Sigma). Fifty micrograms of protein per lane and known molecular weight markers were separated by SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred onto PVDF membranes and incubated overnight at 4°C with blocking solution (8% non-fat milk in TBS). The blocked membranes were incubated with anti-C/EBPβ, anti-PPARγ, anti-CyclinA and anti-CDK2 antibodies (1:1000) for one hour at 37°C and then with secondary antibody (1:1000) for one hour at 37°C and then washed three times with TBS buffer containing 0.1% Tween 20 for 15 min at RT with shaking.

Immunofluorescence microscopy

Preadipocytes were plated on glass coverslips in 35 mm dishes and then induced to differentiate as described above. After induction, cells were washed with ice-cold PBS three times and fixed with 4% neutral formaldehyde for 30 min at RT. Cells were permeabilized with 0.5% Triton X-100 in bovine serum albumin (BSA) (2 mg/mL) for 30 min and blocked with BSA (2 mg/mL) for one hour at RT. Cells were incubated with anti-C/EBPβ antibody

Table 2 Primers used for quantitative realtime RT-PCR

Gene	Sequence (5′–3′)
FAS	Forward: 5′-GCTGCGGAAACTTCAGGAAAT-3′ Reverse: 5′-AGAGACGTGTCACTCCTGGACT-3′
PPARγ	Forward: 5′-CAAGAATACCAAAGTGCGATCAA-3′ Reverse: 5′-GAGCTGGGTCTTTTCAGAATAATAAG-3′
β-Actin	Forward: 5′-ATGGGTCAGAAGGACTCCTACG-3′ Reverse: 5′-AGTGGTACGACCAGAGGCATAC-3′

RT-PCR, reverse transcription polymerase chain reaction; PPARγ, peroxisome proliferator activated receptor γ

(1:200 dilution) in BSA (2 mg/mL) for one hour and then incubated for one hour with fluorescein isothiocyanate-labeled secondary antibody (1:50 dilution) and 10 min with 40,6-diamidino-2-phenylindole (DAPI), and fluorescence was visualized using a Zeiss LSM 510 Meta microscope.

Statistical analysis

The data were presented as means $\pm$ SEM and statistically analyzed using one-way analysis of variance when their variances were heterogeneous and unpaired *t*-test. Differences were considered significant at $P < 0.05$.

Results

Effects of SAW and SAA on adipocyte differentiation

The adipogenic process includes both preadipocyte proliferation and adipocyte differentiation. SAW and SAA were first evaluated for their effect on preadipocyte proliferation. DM-induced mitosis of 3T3-L1 cells was significantly inhibited by SAW and SAA treatment in a time- (Figure 1a) and dose-dependent (Figure 1b) manner. The growth inhibitory effect of SAW and SAA was not due to cytotoxicity, as determined by the measurement of LDH activity (Figure 1c). To test whether SAW and SAA inhibited adipocyte differentiation, SAW and SAA were added to the DM beginning at day 1 and continuing every two days until the completion of the differentiation program at day 7. At the dose of 200 mg/mL, both SAW and SAA markedly inhibited the differentiation of 3T3-L1 cells as confirmed by oil red O staining (Figure 1d) and measurement of intracellular lipid contents (Figure 1e).

Effects of SAW and SAA on the expression of key transcriptional regulators of adipocyte differentiation

In order to determine whether SAW and SAA inhibited adipocyte differentiation by negatively regulating the expression of adipogenic transcriptional regulators, we examined the effects of SAW and SAA on the protein expression of PPARγ and C/EBPβ during adipocyte differentiation. Whole-cell extracts were prepared day 1 postinduction for the measurement of C/EBPβ and day 7 postinduction for the measurement of PPARγ. Consistent with the decrease in lipid content, the expression of PPARγ and C/EBPβ was reduced by SAW and SAA treatment in a dose-dependent manner (Figure 2a). The expression of known PPARγ target genes lipoprotein lipase (LPL), aP2 and glucose transporter 4 (Glut4) was also decreased in a dose-dependent (Figure 2b) and time-dependent (Figure 2c) manner. The mRNA expression of PPARγ and FAS was also decreased in a dose-dependent manner as determined by realtime PCR analysis (Figure 2d).

SA inhibited the transcriptional activity of PPARγ in 3T3-L1 cells

Activation of PPARγ is known to promote adipocyte differentiation. Having demonstrated the effects of SAW and SAA on adipocyte differentiation, we went on to determine whether SAW and SAA can inhibit the transcriptional activity of PPARγ. In this experiment, 3T3-L1 cells were transiently transfected with PPARγ and the PPARγ-responsive tk-PPRE-Luc reporter gene before the treatment of SAW/SAA in the presence or absence of troglitazone, a synthetic PPARγ agonist. As shown in Figure 3, SAW and SAA inhibited the basal and troglitazone-inducible activity of PPARγ in a dose-dependent manner.

Treatment with SA inhibited MCE and the expression of CyclinA and CDK2 and the effect of SAW on the nuclear localization of C/EBPβ

In order to determine whether SAW and SAA affect MCE, the effects of SAW and SAA on DM-induced cell proliferation were investigated. Cell numbers were quantified by counting 3T3-L1 cells during differentiation. As shown in Figure 4a, significant inhibition of MCE by SAW and SAA was observed in days 3–7 of the differentiation. We next examined the effects of SAW and SAA on cell cycle distribution using flow cytometry. At 14 h after induction, 49.54% of DM-treated preadipocytes entered the S phase, while only 17.44% of SAW (100 mg/mL) and 31.56% of SAA (100 mg/mL)-treated preadiocytes entered the S phase. At 18 h after induction, 58.22% of DM-treated preadipocytes entered the S phase, while only 18.43% (100 mg/mL) and 11.16% (200 mg/mL) of SAW and 33.22% (100 mg/mL) and 29.71% (200 mg/mL) of SAA-treated preadiocytes entered the S phase (Figure 4b). These results suggested that it was possible that SA inhibited the differentiation of preadipocytes by preventing preadipocytes from traversing the G1–S checkpoint of the cell cycle. When cell cycle proteins were analyzed, we found that the differentiation induction of CyclinA and CDK2 protein expression was inhibited in SAW- and SAA-treated cells in a dose-dependent manner (Figure 4c). C/EBPβ is a key adipogenic transcription factor. The effect of SAW on the localization of C/EBPβ to the centromeres was examined by immunofluorescence microscopy. At eight hours after induction, C/EBPβ in DM-treated preadipocytes exhibited a diffused nuclear immunostaining (Figure 4d), indicative of its presence in the nuclei. At 24 h after induction, C/EBPβ in DM-treated preadipocytes exhibited punctuate staining, indicative of association of C/EBPβ with centromeres.[15] Moreover, C/EBPβ staining co-localized with DAPI staining, which stained centromeric satellite DNA.[19] In contrast, treatment with SAW disrupted the subcellular distribution of C/EBPβ in that at 24 h postinduction, the nuclear staining of C/EBPβ remained diffuse (Figure 4d).

Discussion

Antiobesity strategies are classified into four categories: reducing food intake, blocking nutrient absorption, increasing thermogenesis, and modulating fat metabolism and storage.[20] Blockade of adipocyte differentiation is one of the antiobesity strategies falling under the category of modulating fat storage. Antiadipogenic compounds from natural

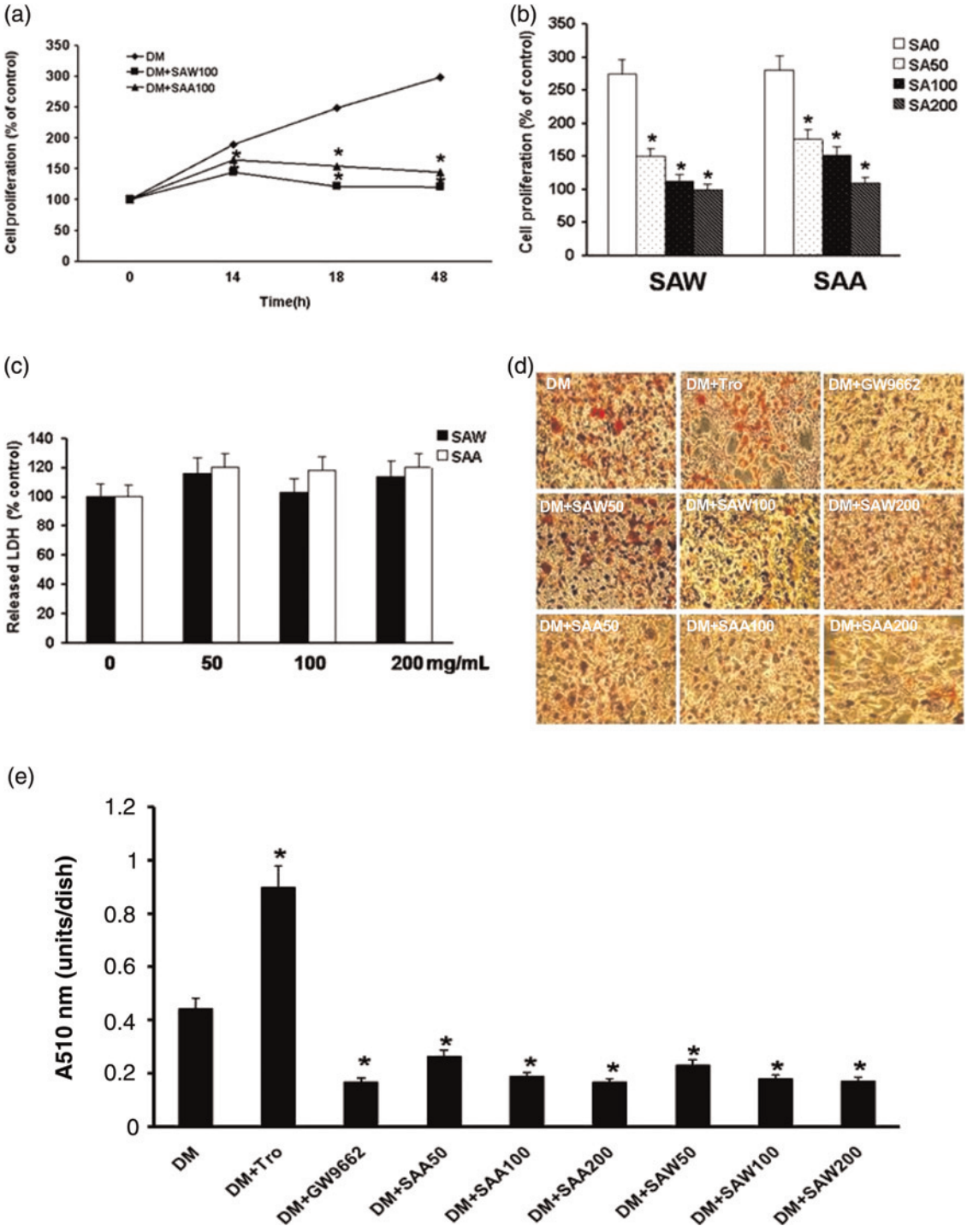

Figure 1 Inhibition of 3T3-L1 adipocyte differentiation by *Sibiraea angustata* water extract (SAW) and *Sibiraea angustata* acetic ether extract (SAA). (a) Postconfluent 3T3-L1 cells were induced to differentiate with differentiation medium (DM) plus SAW (100 mg/mL) or SAA (100 mg/mL). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed at 14, 18 and 48 h. (b) Postconfluent 3T3-L1 cells were induced to differentiate with DM plus different concentrations of SAW or SAA. MTT assay was performed at 18 h. (c) Cytotoxicity assay. Lactate dehydrogenase (LDH) content released into culture supernatant was measured. (d) 3T3-L1 preadipocytes were induced to differentiate in the presence of SAW or SAA (50, 100 and 200 mg/mL). On day 7, cytoplasmic triacylglycerol was stained with oil red O. Troglitizone (Tro) was used as a peroxisome proliferator activated receptor γ (PPARγ) activator; GW9662 was used as a PPARγ inhibitor. (e) On D7, accumulated lipids were stained and measured. The stained lipid content was quantified by measuring absorbance. Statistical significance: $^{*}P < 0.05$ (A color version of this figure is available in the online journal)

products have been proposed to be useful in the prevention of obesity or excess body fat with less side-effects. The goal of this study was to determine whether and how SA, a TCM herb, inhibits 3T3-L1 adipocyte differentiation and adipogenesis.

Under the induction of DM, 3T3-L1 preadipocytes proceed through one or two rounds of cell division as MCE and then initiate the differentiation program. A blockade of MCE will abort the differentiation of 3T3-L1 cells. We showed that SAW and SAA inhibited DM-induced mitosis of 3T3-L1

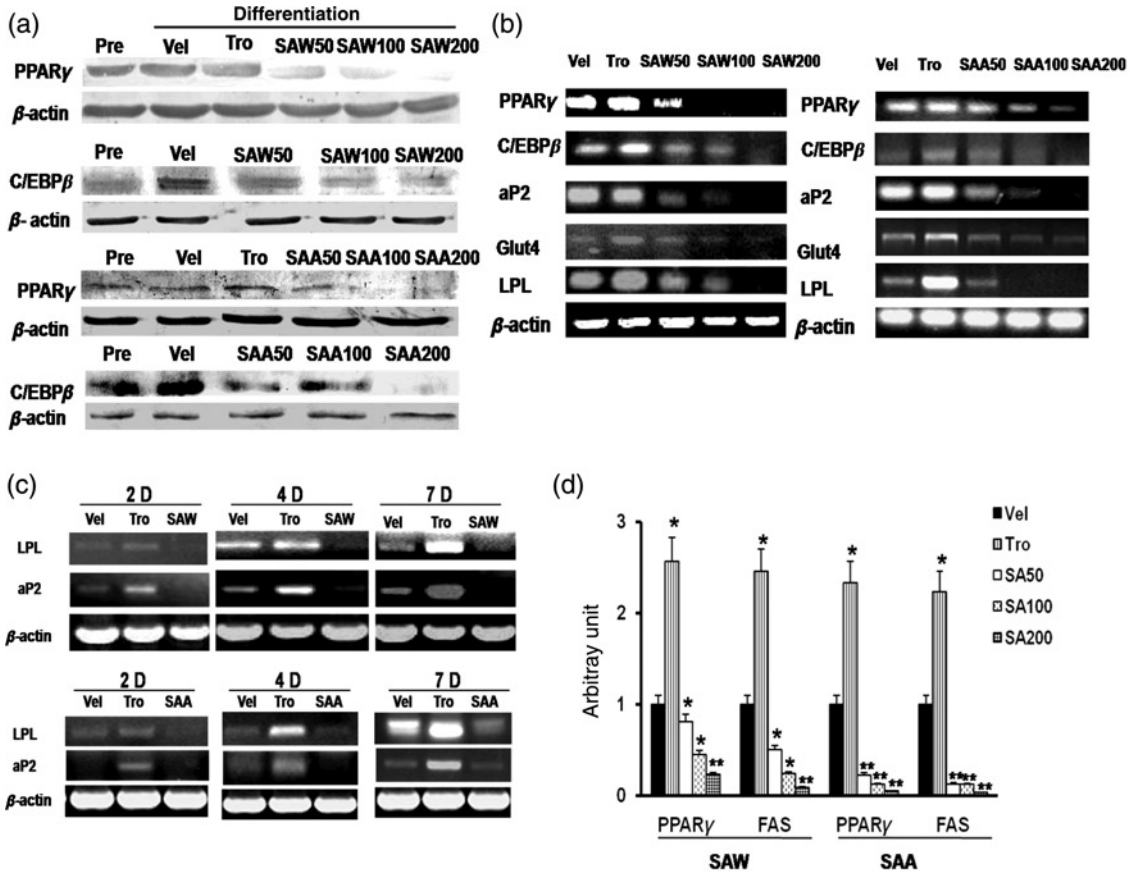

Figure 2 Inhibition of the expansion of key transcriptional regulators of adipocyte differentiation by *Sibiraea angustata* water extract (SAW) and *Sibiraea angustata* acetic ether extract (SAA). (a) Whole-cell extracts were prepared and 50 μg of protein was resolved and probed with CCAAT enhancer binding protein β (C/EBPβ) and peroxisome proliferator activated receptor γ (PPARγ) antibodies. Protein of undifferentiated preadipocytes was loaded as negative control. Pre: undifferentiated preadipocytes. (b) Cells were treated throughout the differentiation period (from day 0 to day 7) with either vehicle alone, 50 mg/mL SAW and SAA, 100 mg/mL SAW and SAA, 200 mg/mL SAW and SAA, or 10 μmol/L Tro. Medium was changed at days 0, 2, 4 and 6. After day 7 of differentiation, total RNA was harvested and reverse transcription polymerase chain reaction (RT-PCR) was performed. (c) Effects of SAW and SAA on lipoprotein lipase (LPL) and adipocyte protein-2 (aP2) mRNA expression during the course of differentiation. Cells were harvested at days 2, 4 and 7 after initiating differentiation and RT-PCR analysis was performed. (d) Cells were treated throughout the differentiation period (from day 0 to day 7) with either vehicle alone, 50 mg/mL SAW and SAA, 100 mg/mL SAW and SAA, 200 mg/mL SAW and SAA or 10 μmol/L Tro. Medium was changed at days 0, 2, 4 and 6. After day 7 of differentiation, total RNA was harvested and realtime PCR was performed. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$. FAS, fatty acid synthetase

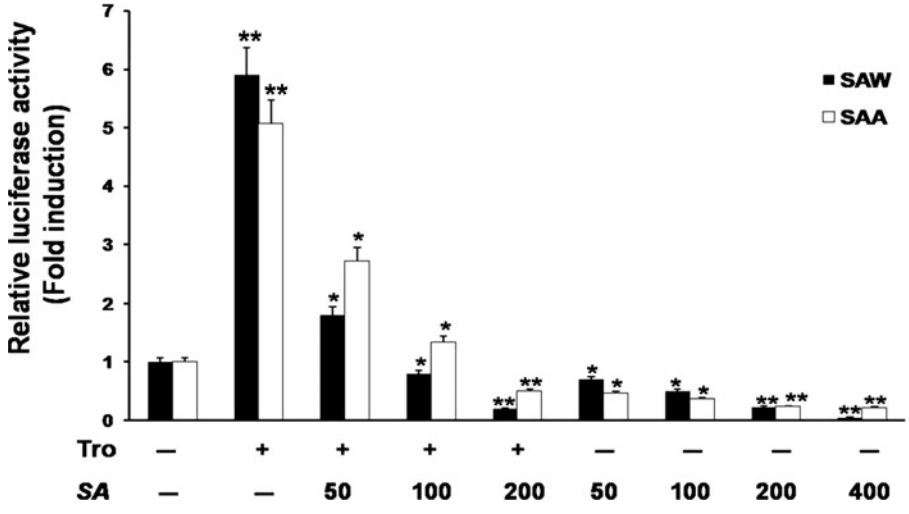

Figure 3 Inhibition of transcriptional activity of peroxisome proliferator activated receptor γ (PPARγ) by *Sibiraea angustata* water extract (SAW) and *Sibiraea angustata* acetic ether extract (SAA). 3T3-L1 cells were transfected as described under Materials and methods. Cells were treated with troglitazone (Tro), SAW or SAA for 24 h. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$. SA, *Sibiraea angustata*

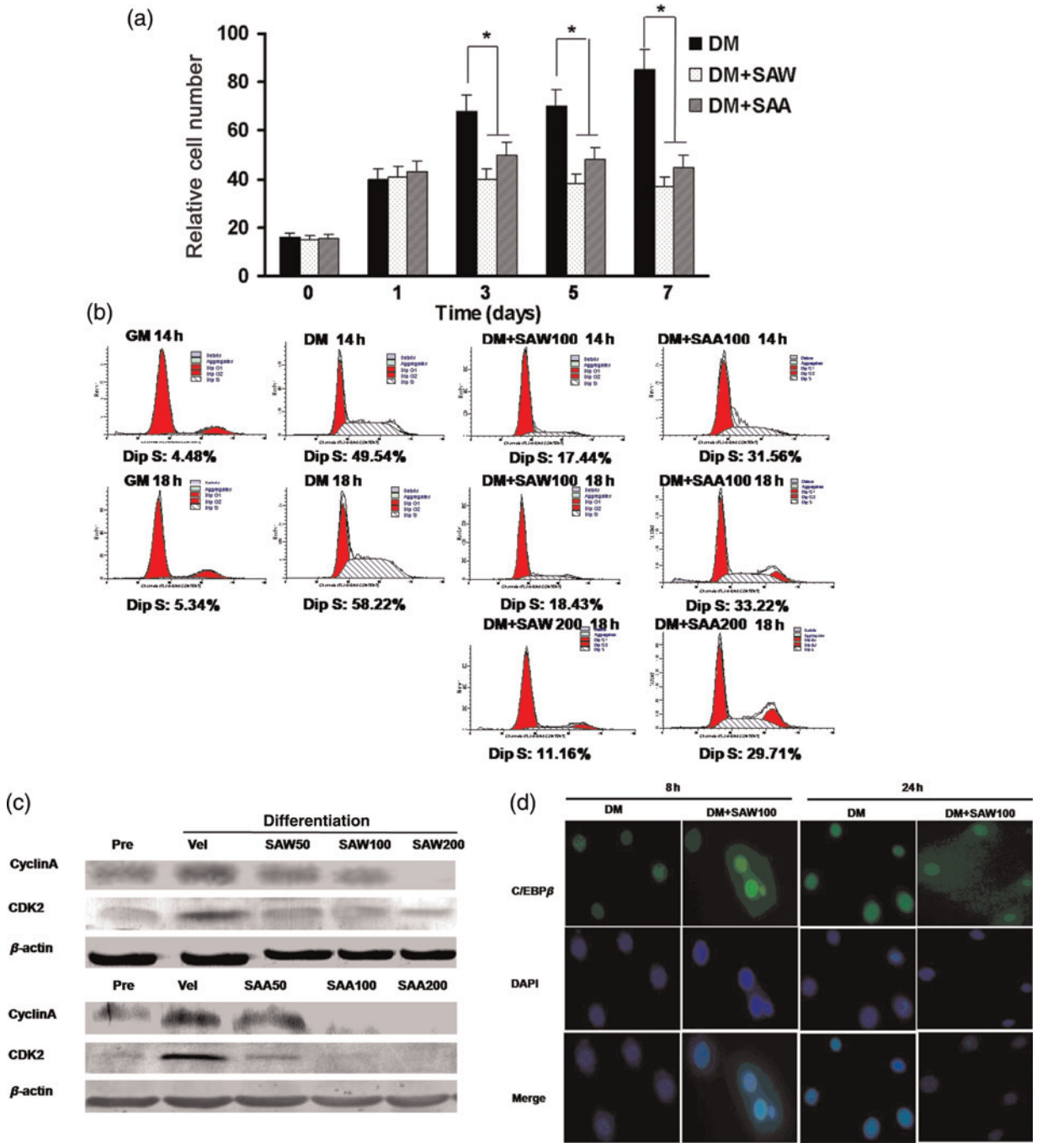

Figure 4 Inhibition of mitotic clonal expansion (MCE) and the expansion of CyclinA and cyclin-dependent kinase 2 (CDK2) by *Sibiraea angustata* water extract (SAW) and *Sibiraea angustata* acetic ether extract (SAA) and the inhibition of the localization of CCAAT enhancer binding protein β (C/EBPβ) to centromeres by SAW. (a) 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of SAW (100 mg/mL) and SAA (100 mg/mL). Cell numbers were determined 0, 1, 3, 5 and 7 days after induction. (b) 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of SAW (100 mg/mL) and SAA (100 mg/mL). Cells were harvested and stained with propidium iodide to analyze cell cycle distribution at 14 and 18 h after induction. (c) Postconfluent 3T3-L1 cells were differentiated in the presence or in the absence of 50, 100 and 200 mg/mL SAW or SAA. Proteins were harvested after 24 h. (d) 3T3-L1 preadipocytes were plated on coverslips, and treated in the presence or absence of SAW (100 mg/mL). Cells were fixed and stained with C/EBPβ antibody and fluorescein isothiocyanate-conjugated secondary antibody to detect localization of C/EBPβ at the indicated times after induction. Cells were also stained with 40,6-diamidino-2-phenylindole and photographed with a fluorescent microscope. Statistical significance: $^{*}P < 0.05$ (A color version of this figure is available in the online journal)

cells without causing cytotoxicity (Figure 1a–c). Addition of SAW and SAA to the DM also inhibited differentiation as evidenced by the decreased oil red O staining (Figure 1d and e). Together, our results indicate that SAW and SAA can inhibit both the proliferation and differentiation of 3T3-L1 cells.

When the growth inhibitory effect of SAW and SAA was further analyzed, we found that the DM-induced cell cycle progression of preadipocytes was blocked at the G1/S transition in cells treated with SAW or SAA. Because MCE is required for 3T3-L1 preadipocytes to differentiate, our

results suggest that SAW and SAA may inhibit the differentiation of preadipocytes by preventing preadipocytes from traversing the G1–S checkpoint of the cell cycle (Figure 4a and b). Cell cycle protein analysis also supported this, as evidenced by the suppression of CyclinA and CDK2 protein expression by SAW and SAA (Figure 4c).

In order to determine the mechanism of the inhibitory effect of SAW and SAA on 3T3-L1 differentiation, we examined the key regulators involved in adipocyte differentiation, such as C/EBPβ and PPARγ. The expression of C/EBPβ was induced in the early stage of 3T3-L1 differentiation. C/EBPβ activation and localization to centromeres are required for MCE and downstream gene transcription and lipogenesis. PPARγ, a ligand-activated nuclear transcription factor, regulates the expression of genes involved in adipocyte differentiation and adipogenesis.[21] Differentiation of fibroblasts into adipose tissue also requires PPARγ. Inhibition of PPARγ activity can impair adipocyte differentiation and adipogenesis through the inhibition of adipogenic genes, such as LPL, aP2 and Glut4. Consistent with the decrease of lipid content, the expression of PPARγ and C/EBPβ was also reduced by SAW and SAA treatment (Figure 2a). Meanwhile, the expression of LPL, aP2 and Glut4, the PPARγ target genes and signs of mature adipocytes, were also decreased by SAW and SAA treatment (Figure 2b–d). Interestingly, the basal and ligand-dependent activity of PPARγ was also inhibited by SAW and SAA (Figure 3). In addition to a decreased expression of C/EBPβ, the association of C/EBPβ with centromeres was also disrupted by the treatment of SAW, suggesting another mechanism of compromised C/EBPβ activity (Figure 4d).

In addition to SA, several other TCM herbs and natural products have also been suggested to play a role in the inhibition of adipocyte differentiation and adipogenesis. For example, Berberine has been reported to inhibit 3T3-L1 adipocyte differentiation by targeting the PPARγ pathway.[22] In another report, *Rehmannia glutinosa* was shown to inhibit adipocyte differentiation and adipogenesis.[19] EGCG, a green tea cathechin, downregulated preadipocyte proliferation by targeting the ERK pathway.[18] Our results have reinforced the notion that neutraceuticals may represent a practical approach to manage fat cell differentiation and obesity.

In summary, we showed both the water and acetic ether extracts of SA can inhibit the proliferation and differentiation of 3T3-L1 cells. The effects of SA on preadipocyte differentiation were achieved by targeting the C/EBPβ and PPARγ pathways. The active ingredients in SAW and SAA that are responsible for the antiadipogenic effects remain to be determined. Another future direction is to determine whether SA is effective in the prevention and treatment of obesity *in vivo*.

Author contributions: YZ designed the research; LX and JZ performed the research; LH contributed new reagents; LX, JZ and HL analyzed data; LX, JZ, JL and JZ wrote the paper.

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