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Inhibition of adipogenesis and leptin production in 3T3-L1 adipocytes by a derivative of meridianin C

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ABSTRACT

Meridianin C, a marine alkaloid, is a potent protein kinase inhibitor and has anti-cancer activity. We have recently developed a series of meridianin C derivatives (compound 7a–7j) and reported their proviral integration Moloney Murine Leukemia Virus (pim) kinases' inhibitory and anti-proliferative effects on human leukemia cells. Here we investigated the effect of these meridianin C derivatives on adipogenesis. Strikingly, among the derivatives tested, compound 7b most strongly inhibited lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes. However, meridianin C treatment was largely cytotoxic to 3T3-L1 adipocytes. On mechanistic levels, compound 7b reduced not only the expressions of CCAAT/enhancer-binding protein-α (C/EBP-α), peroxisome proliferator-activated receptor-γ (PPAR-γ), and fatty acid synthase (FAS) but also the phosphorylation levels of signal transducer and activator of transcription-3 (STAT-3) and STAT-5 during adipocyte differentiation. Moreover, compound 7b repressed leptin, but not adiponectin, expression during adipocyte differentiation. Collectively, these findings demonstrate that a meridianin C derivative inhibits adipogenesis by down-regulating expressions and/or phosphorylations of C/EBP-α, PPAR-γ, FAS, STAT-3 and STAT-5.

1. Introduction

Obesity is a high risk factor for developing many human pathologies, including insulin resistance, type 2 diabetes, hyperlipidemia, and cancer [1]. It has been shown that obesity can be induced by many factors, such as genetic and endocrine abnormalities, some medicines, a low metabolic rate, nutritional and environmental factors, as well as imbalanced energy homeostasis [2,3]. Recent evidence strongly suggests that adipose tissue serves as an energy store and also plays a critical role in the control of energy metabolism by secreting adipokines [3,4]. However, abnormal expansion/accumulation of adipose tissue, which is largely attributable to excessive adipocyte differentiation and increase in the fat cell number and size, is closely linked to the development of obesity [5,6]. Thus, any compound that inhibits excessive adipocyte differentiation and/or adipocyte hyperplasia/hypertrophy may have preventive and/or therapeutic potential against obesity and related diseases.

The differentiation of preadipocyte into adipocyte, also called adipogenesis, is controlled by a variety of cellular proteins, including transcription factors, adipocyte-specific genes, lipogenic enzymes, and signaling proteins. For instance, expressions and/or activities of the family of C/EBPs (C/EBP-α, -β and -δ) and PPARs (PPAR-γ, -α and -β) are critical for adipocyte differentiation [7,8]. Moreover, there is strong evidence that JAK-2/STAT-3 and STAT-5 signaling complexes are involved in adipocyte differentiation

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; C/EBP-α, CCAAT/enhancer-binding protein-α; ERK-1/2, extracellular signal-regulated protein kinase-1/2; FAS, fatty acid synthase; JAK-2, janus-activated protein kinase-2; IBMX, 3-isobutyl-1-methylxanthine; Pim, proviral integration Moloney Murine Leukemia Virus; PKA, protein kinase A; PKC, protein kinase C; PPAR-γ, peroxisome proliferator-activated receptor-γ; STAT-3/5, signal transducer and activator of transcription-3/5.

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[9,10]. In addition, a growing number of signaling proteins and factors, including PKA, cAMP, PKCs, and ERK-1/2, have been found to be important for controlling adipocyte differentiation [11–13].

Meridianins (A–G), the marine-derived indole alkaloids, are a new family of protein kinase inhibitors isolated from *Aplidium meridianum* [14,15], and some of meridianins and/or their synthetic derivatives are also shown to have anti-cancer activities [16,17]. Interestingly, there is evidence further demonstrating that a meridianin C derivative, 3,5-disubstituted indole, is a potent and selective inhibitor of the family of pan-pim kinases comprising of pim-1, pim-2 and pim-3 [18]. We have recently synthesized meridianin C and a series of meridianin C derivatives (compound 7a–7j) and reported their pim kinases' inhibitory and anti-proliferative activities with different potency on human leukemia cells [19]. In this study, we investigated the anti-adipogenic effect of these meridianin C derivatives on 3T3-L1 adipocytes. We report here that among the meridianin C derivatives screened, compound 7b strongly inhibits adipogenesis and the inhibition is largely associated with the reduced expressions and/or phosphorylations of C/EBP-α, PPAR-γ, FAS, STAT-3, and STAT-5.

2. Materials and methods

2.1. Materials

Polyclonal C/EBP-α, monoclonal PPAR-γ, monoclonal STAT-3, monoclonal phospho-STAT-3 (p-STAT-3), polyclonal STAT-5, and polyclonal p-STAT-5 antibodies were purchased from Santa Cruz Biotechnology (Delaware, CA). Monoclonal FAS antibody was purchased from BD Bioscience (San Jose, CA). Monoclonal β-actin antibody was purchased from Sigma (St. Louis, MO).

2.2. Cell culture and differentiation

3T3-L1 preadipocytes (ATCC, Manassas, VA) were grown up to the contact inhibition stage and remained in the post-confluent stage for 2 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf bovine serum (Gibco) and penicillin–streptomycin (Welgene, Daegu). Differentiation was then induced by changing the medium to DMEM supplemented with 10% FBS (Welgene, Daegu) plus a cocktail of hormones (MDI) that include 0.5 mM IBMX (M) (Sigma), 0.5 μM dexamethasone (D) (Sigma), and 5 μg/ml insulin (I) (Sigma) in the presence or absence of meridianin C or compound 7a–7j at the indicated concentrations. After 48 h MDI-induction, the differentiation medium was replaced with DMEM supplemented with 10% FBS and 5 μg/ml insulin in the presence or absence of meridianin C or compound 7a–7j at the indicated concentrations. The cells were then fed every other day with DMEM containing 10% FBS in the presence or absence of meridianin C or compound 7a–7j at the indicated concentrations until day 8. On day 8, the preadipocytes became mature adipocytes that rounded-up and filled with many oil droplets.

2.3. Oil red O staining

On day 8 of differentiation, control or drug (meridianin C or compound 7b)-treated 3T3-L1 cells were washed twice with PBS, fixed with 10% formaldehyde for 2 h at room temperature, washed with 60% isopropanol and dried completely. The fixed cells were then stained with Oil red O working solution for 1 h at room temperature (RT) and then washed twice with distilled water. Lipid droplets were observed by light microscopy (Nikon).

2.4. Cell count analysis

3T3-L1 preadipocytes that were seeded in 24-well plates were similarly grown under the above-mentioned differentiation conditions. On day 8 of differentiation, meridianin C or compound 7b-treated 3T3-L1 cells, which cannot be stained with trypan blue dye, was counted under microscope. The cell count assay was done in triplicates. Data are mean ± standard error (SE) of three independent experiments.

2.5. Quantification of intracellular triglyceride (TG) content by AdipoRed assay

On day 8 of differentiation, lipid content in control or compound 7b-treated 3T3-L1 cells was measured using a commercially available AdipoRed Assay Reagent kit according to the manufacturer's instructions (Lonza). After a 10 min incubation, fluorescence was measured on Victor^3 (Perkin Elmer) with an excitation at 485 nm and an emission at 572 nm.

2.6. Preparation of whole cell lysates

At the designated time point, 3T3-L1 cells were washed twice with PBS and exposed to a modified RIPA buffer [50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1×)]. The cell lysates were collected and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was saved and protein concentrations were determined with Bradford reagent (Bio-Rad).

2.7. Western blot analysis

Proteins (50 μg) were separated by SDS–PAGE (10%) and transferred onto nitrocellulose membranes (Millipore). The membranes were washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (vol/vol) Tween 20 (TBST) followed by blocking with TBST containing 5% (wt/vol) non-fat dried milk. The membranes were incubated overnight with antibodies specific for C/EBP-α (1:1000), PPAR-γ (1:1000), STAT-3 (1:1000), p-STAT-3 (1:1000), STAT-5 (1:1000), p-STAT-5 (1:1000), FAS (1:1000) or β-actin (1:10,000) at 4 °C. The membranes were then exposed to secondary antibodies coupled to horseradish peroxidase for 2 h at RT. The membranes were washed three times with TBST at RT. Immunoreactivities were detected by ECL reagents. Equal protein loading was assessed by the expression level of actin protein.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

At the designated time point, total cellular RNA in control or compound 7b-treated 3T3-L1 cells was isolated with the RNAzol-B (Tel-Test, Friendswood, TX). Three micrograms of total RNA were reverse transcribed using a random hexadeoxynucleotide primer and reverse transcriptase. Single stranded cDNA was amplified by PCR with the following primers. Primer sequences used for amplifications were as follows: C/EBP-α sense 5′-TTACAACAGGC CAGGTTTCC-3′; C/EBP-α antisense 5′-CTCTGGGATGGATCGATTGT-3′; PPAR-γ sense 5′-GGTGAAACTCTGGGAGATTC-3′; PPAR-γ antisense 5′-CAACCATTGGGTCAGCTCTC-3′; FAS sense 5′-TTGCTGGCA CTACAGAATGC-3′; FAS antisense 5′-AACAGCCTCAGAGCGACAAT-3′; Leptin sense 5′-CCAAAACCCTCATCAAGACC-3′; Leptin antisense 5′-CTCAAAGCCACCACCTCTGT-3′; Adiponectin sense 5′-GGAGATGC AGGTCTTCTTGGT-3′; Adiponectin antisense 5′-TCCTGATACTG GTCGTAGGTGAA-3′; Actin antisense 5′-GGTAGGAACACGGAAGG CCA-3′. The PCR conditions applied were: C/EBP-α, 30 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and

extension at 72 °C for 30 s; PPAR-γ, 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s; FAS, 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 40 s, and extension at 68 °C for 45 s; leptin, 30 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min; adiponectin, 30 cycles of denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min; Actin, 25 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min. Expression levels of actin mRNA were used as an internal control to evaluate the relative mRNA expression of adipocyte-specific genes and adipokines.

2.9. Statistical analyses

Cell count analysis was done in triplicates and repeated three times. Data were expressed as mean ± SE. The significance of difference was determined by One-Way ANOVA. All significance testing was based upon a P value of <0.05.

3. Results

3.1. Compound 7b, a derivative of meridianin C, has strong anti-adipogenic effects

In order to identify compounds that can inhibit adipogenesis in 3T3-L1 adipocytes, we investigated the effect of a series of meridianin C derivatives (compound 7a–7j) on lipid accumulation at a final concentration of 10 μM during adipocyte differentiation by an AdipoRed assay. Timescale of 3T3-L1 preadipocyte differentiation is shown in Fig. 1A. Among the derivatives tested, compound 7b most strongly reduced levels of triglyceride (TG) contents during adipocyte differentiation (data not shown). Due to a strong inhibitory effect of compound 7b on TG synthesis, we further tested its lipid-lowering effect on 3T3-L1 adipocytes by an Oil Red O staining. As shown in Fig. 1B (upper panels), many lipid droplets were formed in 3T3-L1 preadipocytes in induction medium only on day 8 of cell differentiation, compared with no lipid droplet in undifferentiated cells. However, treatment of the preadipocytes with compound 7b for 8 days strikingly reduced the amounts of lipid droplets in a dose-dependent manner, compared with the mock-treated cells. Apparently, compound 7b maximally reduced lipid droplets at 10 μM. The compound 7b-mediated lipid-reducing effect was also observed by light microscopic measurement (Fig. 1B, lower panels). Meridianin C also dose-dependently reduced the amounts of lipid droplets. However, light microscopic measurement demonstrated that meridianin C at either 5 or 10 μM induced cell toxicity, as evidenced by morphological change and formation of many cytoplasmic vacuoles. Meanwhile, the meridianin C (5 or 10 μM)-mediated cell toxicity was easily visible even on day 2 of differentiation (data not shown). Results of AdipoRed assay and cell count assay, respectively, showed that compound 7b dose-dependently reduced levels of TG contents (Fig. 2A) without changing the number of adipocytes (Fig. 2C), whereas meridianin C reduced both TG contents (Fig. 2B) and the cell numbers (Fig. 2D) in a concentration-dependent manner.

3.2. Compound 7b reduces the expressions of C/EBP-α and PPAR-γ and the phosphorylation levels of STAT-3 and STAT-5 during adipocyte differentiation

To understand molecular and cellular mechanisms underlying the compound 7b-mediated anti-adipogenic effect, we next examined the effect of compound 7b (10 μM) on expression and/or activity (phosphorylation) of the family of C/EBPs, PPARs, and STATs involved in adipogenesis. Western blot analysis showed a time-dependent increase in the protein levels of C/EBP-α and

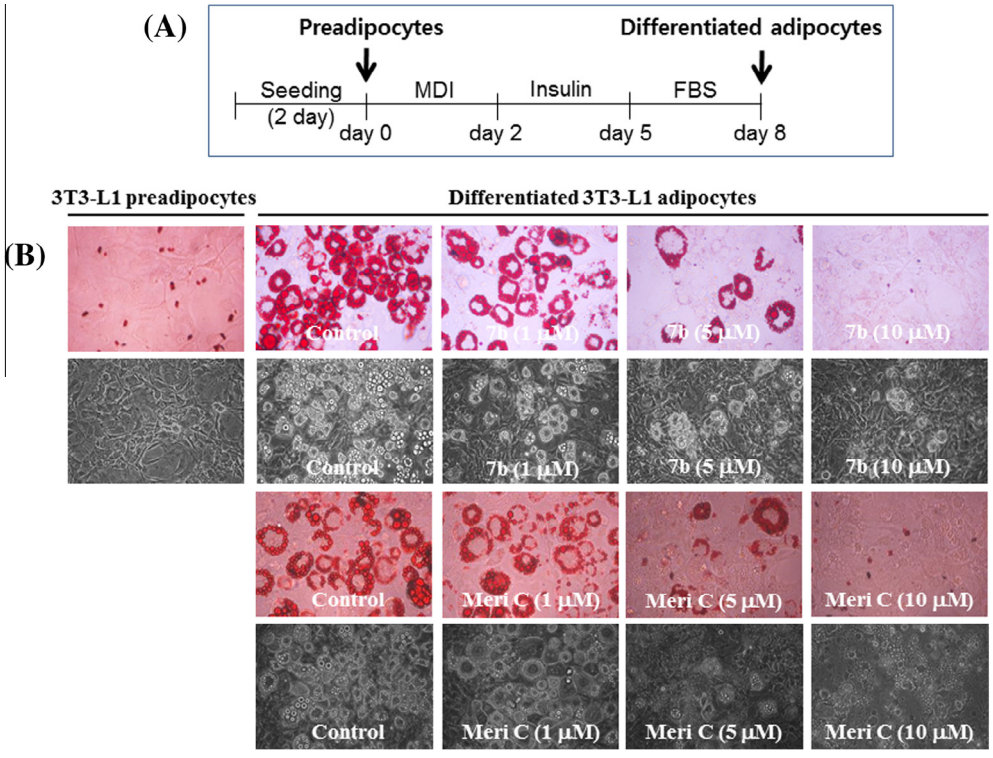

Fig. 1. Effects of compound 7b and meridianin C on lipid accumulation during adipocyte differentiation. (A) Scheme for 3T3-L1 preadipocyte differentiation. (B) Measurement of the cellular lipid droplets in 3T3-L1 preadipocytes (undifferentiated) or differentiated adipocytes on day 8 by Oil Red O staining. Phase-contrast images of the cells were also taken after the treatment (lower panels in B). Each picture in (B) is a representative of three independent experiments.

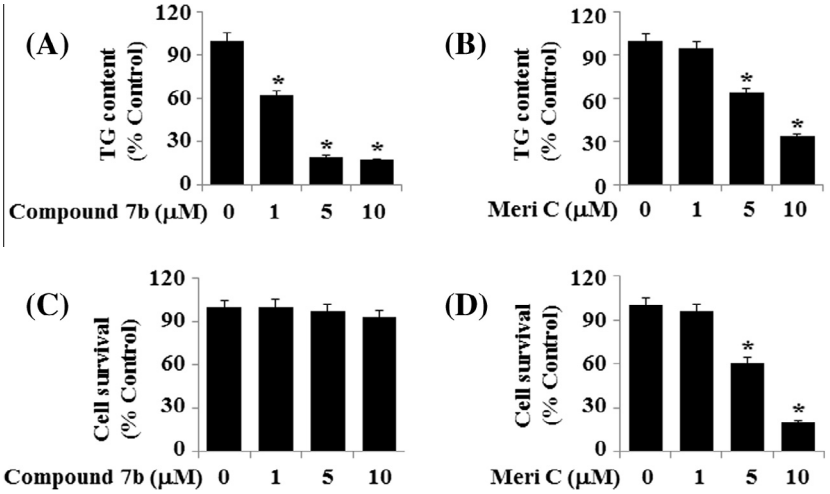

Fig. 2. Effects of compound 7b and meridianin C on triglyceride (TG) synthesis and cell growth during adipocyte differentiation (A and B) Quantification of the cellular TG contents in compound 7b- or meridianin C-treated 3T3-L1 adipocytes on day 8 by AdipoRed assay. Values are mean ± SE of data from three independent experiments with three replicates. $^{*}P < 0.05$ vs. control (no chemical). (C and D) 3T3-L1 preadipocytes were grown under the above-mentioned 3T3-L1 preadipocyte differentiation condition in Fig. 1A. On day 8, meridianin C or compound 7b-treated 3T3-L1 cells, which cannot be stained with trypan blue dye, was counted under microscope. The cell count assay was done in triplicates. Data are mean ± SE of three independent experiments. $^{*}P < 0.05$ vs. control (no chemical).

PPAR-γ in 3T3-L1 preadipocytes produced by treatment with induction medium containing MDI and insulin and FBS on day 2, 5, and 8, respectively (Fig. 3A). Importantly, compound 7b inhibited the adipogenesis-dependent protein expressions of C/EBP-α and PPAR-γ. Results of RT-PCR analysis (Fig. 3B) also revealed a time-dependent increase in the mRNA expressions of C/EBP-α and PPAR-γ during adipocyte differentiation. However, compound 7b repressed the mRNA expressions of C/EBP-α and PPAR-γ. These results suggest that compound 7b down-regulates PPAR-γ and C/EBP-α at the transcriptional levels. Control actin protein and mRNA expressions remained constant under these experimental conditions (Fig. 3A and B). There were also higher phosphorylation levels of STAT-3 and STAT-5 in 3T3-L1 preadipocytes produced by differentiation with induction medium containing MDI and insulin and FBS on day 2, 5, and 8, respectively. However, compound 7b strongly reduced the phosphorylation levels of STAT-3 on day 5 and 8, and of STAT-5 on day 8 during adipocyte differentiation. Compound 7b did not affect the total protein levels of STAT-3 and STAT-5 during adipocyte differentiation at the designated time points.

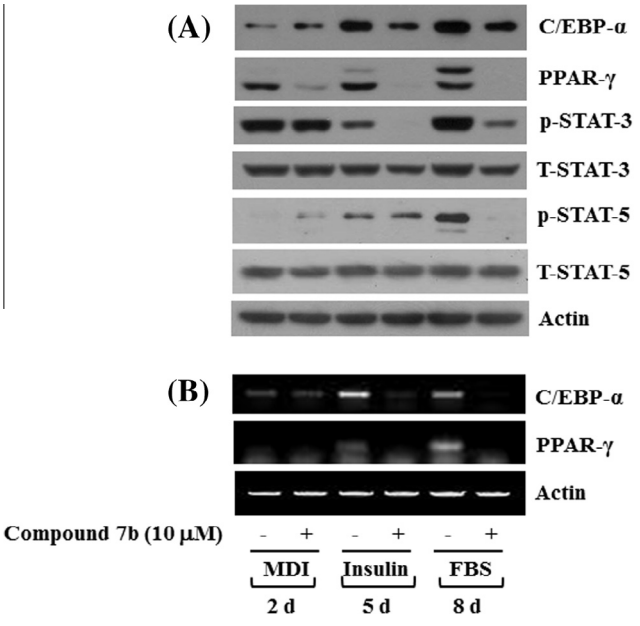

Fig. 3. Effect of compound 7b on expressions and/or activities of C/EBP-α, PPAR-γ, STAT-3, and STAT-5 during 3T3-L1 adipocyte differentiation. (A and B) 3T3-L1 preadipocytes were differentiated with induction medium containing MDI, insulin, and FBS in the presence or absence of compound 7b and harvested at day 2, 5, and 8, respectively. Cellular protein and mRNA at the indicated time point were extracted and analyzed by Western blot (A) and RT-PCR (B) analysis, respectively. Each picture in (A) and (B) is a representative of three independent experiments.

3.3. Compound 7b inhibits the mRNA expressions of FAS and leptin during adipocyte differentiation

We next determined whether compound 7b (10 μM) regulates the expression of adipocyte-specific genes and/or adipokines during adipocyte differentiation. As shown in Fig. 4A and B, there

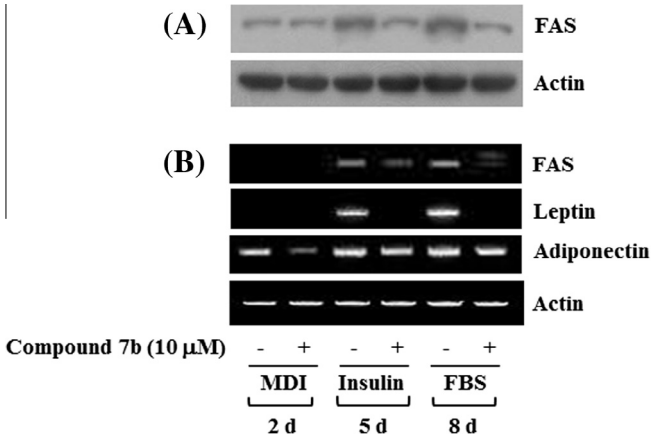

Fig. 4. Effect of compound 7b on protein and/or mRNA expressions of FAS, leptin and adiponectin during 3T3-L1 adipocyte differentiation. (A and B) 3T3-L1 preadipocytes were induced to differentiate with induction medium containing MDI, insulin, and FBS in the presence or absence of compound 7b, and harvested at day 2, 5, and 8, respectively. Cellular protein and mRNA at the indicated time point were extracted and analyzed by Western blot (A) and RT-PCR (B) analysis, respectively. Each picture in (A) and (B) is a representative of three independent experiments.

was a time-dependent increase in the protein and mRNA expressions of FAS during adipocyte differentiation. However, compound 7b largely suppressed the protein and mRNA levels of FAS on day 5 and 8 during adipocyte differentiation. Results of RT-PCR assays also revealed a time-dependent increase in the mRNA expressions of leptin and adiponectin on day 2, 5, and/or 8 during adipocyte differentiation. Notably, compound 7b blocked the insulin- and FBS-induced mRNA expression of leptin on day 5 and 8. Compound 7b also inhibited the MDI-induced mRNA expression of adiponectin on day 2, but had no effect on the insulin- and FBS-induced mRNA expression of adiponectin on day 5 and 8. Control actin mRNA expression remained constant under these experimental conditions.

4. Discussion

Excessive adipocyte differentiation confers abnormal expansion/accumulation of adipose tissue leading to high secretion of pathological factors including adipokines, which are implicated in inflammation, insulin resistance, and metabolic disorders. Thus, potent inhibitors of adipocyte differentiation may have therapeutic potential as anti-obesity drugs. Here we demonstrated that compound 7b, a meridianin C derivative, has a strong anti-adipogenic effect and this effect is mediated through modulation of the expressions and/or phosphorylations of C/EBP-α, PPAR-γ, FAS, STAT-3 and STAT-5.

Mature or differentiated adipocytes have a spherical shape and are filled with many lipid droplets, a morphology which is distinguished that of from fibroblast-like preadipocytes [20]. The present study shows that compound 7b (10 μM) largely inhibits lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes of 8 days (Figs. 1B and 2A), suggesting its strong anti-adipogenic effect. Data of cell count analysis further show that compound 7b treatment for 8 days does not affect the number of 3T3-L1 adipocytes (Fig. 2C). On the other hand, although treatment with meridianin C (10 μM) for 8 days also strongly inhibits lipid accumulation (Figs. 1B and 2B), it is likely that the meridianin C's effect is largely due to its cytotoxic properties, as assessed by formation of many cytoplasmic vacuoles (Fig. 1B) and a large loss of the adipocytes (Fig. 2D).

Increasing evidence suggests that adipocyte differentiation is largely influenced by the expressions and/or activities of C/EBP-α and PPAR-γ. It is shown that blockage of their expressions and/or activities by each pharmacological inhibitor or siRNA transfection leads to inhibition of lipid accumulation during adipocyte differentiation and also knocking out C/EBP-α or PPAR-γ decreases or impairs white adipose tissue in mice [7,8,21–23]. It also has been reported that that the family of STATs, including STAT-1, STAT-3, STAT-5, and STAT-6, is expressed in both 3T3-L1 preadipocytes and adipocytes [9], and several members of the STAT family, including STAT-3 and STAT-5, are critical for 3T3-L1 adipocyte differentiation [10,23–25]. Thus, considering the present findings that compound 7b inhibits the protein and mRNA expressions of C/EBP-α and PPAR-γ, and the phosphorylations of STAT-3 and STAT-5 during adipocyte differentiation (Fig. 3A and B), the compound 7b-mediated anti-adipogenic effect is closely linked to the reduced expressions and/or phosphorylations of C/EBP-α, PPAR-γ, STAT-3, and STAT-5.

There is accumulating evidence that increased expressions and/or activities of C/EBPs and PPARs are necessary for the expression of adipocyte-specific genes and adipokines, including FAS, leptin, and adiponectin [26–28]. Among those, FAS is a lipogenic enzyme involved in fatty acid synthesis and its expression is largely increased in cells or tissues with high rates of fatty acid synthesis [29]. It was previously proposed that once 3T3-L1 preadipocytes are differentiated with dexamethasone and isobutylxanthine, the cells acquire the characteristics of bona fide fat cells including responsiveness to insulin and induction of FAS [30]. In this study, we also demonstrate that 3T3-L1 cells treated with insulin and FBS that had primarily been induced to undergo adipocyte differentiation by treatment with MDI largely induce the mRNA expression of FAS (Fig. 3A). However, compound 7b inhibits the insulin- and FBS-induced FAS at the both protein and mRNA levels in 3T3-L1 adipocytes. These results suggest that compound 7b has anti-lipogenic effect by repressing FAS expression, which may further contribute to the compound 7b-mediated anti-adipogenesis. Mature (differentiated) adipocytes are characterized by their ability to synthesize and secrete some of adipokines, which are involved in the endocrine control of energy homeostasis [31]. Furthermore, mounting evidence reveals that abnormal expression/secretion of leptin is linked to obesity and obesity-related disease, whereas adiponectin plays a positive role in control of obesity [3,32,33] and its overexpression enhances insulin sensitivity in part through effects on hepatic glucose production [34]. In this study, compound 7b inhibits the mRNA expression of leptin, but not adiponectin, during adipocyte differentiation. Therefore, specific small molecule inhibitors such as compound 7b identified in this study could serve as the starting point to find a lead compound that ultimately leads to the discovery of new agents for prevention and/or treatment of obesity and related metabolic disorders in which overexpression of leptin plays a pathological role.

Acknowledgments

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