Bioorganic & Medicinal Chemistry Letters 20 (2010) 6076–6081

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

The selected flavonol glycoside derived from Sophorae Flos improves glucose uptake and inhibits adipocyte differentiation via activation AMPK in 3T3-L1 cells

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ARTICLE INFO

Article history:
Received 2 June 2010
Revised 7 August 2010
Accepted 11 August 2010
Available online 16 August 2010

Keywords:
Sophorae Flos
Glucose uptake
AMPK
Adipocyte differentiation
Anti-obesity
3T3-L1

ABSTRACT

Among nine flavonols (**1**–**9**) obtained from Sophorae Flos, we first isolated compounds **4**, **5**, **8**, and **9**. These isolates (**1**–**9**) were evaluated for the phosphorylation of AMPK and ACC. Administered at 10 μM, **9** possessed high potent activity. Compound **9** displayed a dose-dependent stimulation of glucose uptake in 3T3-L1 cells, and this increase was obviously attenuated by compound C, an AMPK inhibitor. In addition, **9** also phosphorylated AMPK and its downstream substrate ACC in 3T3-L1 cells in a time- and dose-dependent manner. Moreover, we discovered that compound C inhibits **9**-stimulated ACC phosphorylation and motivated the **9**-inhibited C/EBPα and PPARγ, and FAS gene expression, significantly. These results revealed the role of the AMPK downstream signaling pathway in **9**-improved glucose metabolism in 3T3-L1 cells and **9**-inhibited adipocyte differentiation. Differentiation was investigated by Oil Red O staining activity after **9** administration (0–20 μM) in 6 days. Compound **9** decreased mean droplet size in a dose-dependent manner. The results revealed that **9** blocked adipogenic conversion in 3T3-L1 cells together with several significant downregulating adipocyte-specific transcription factors, including PPARγ, C/EBPα, and SREBP1. It also reduced FAS gene expression in a dose-dependent manner, which is crucial for adipogenesis in vitro.

An insidious increase in metabolic syndrome, with symptoms including obesity, insulin resistance, and dyslipidemia, has produced a worldwide epidemic of type 2 diabetes.[1] Obesity is a significant risk factor for major metabolic disorder diseases, including type 2 diabetes, coronary heart disease, hypertension, and certain forms of cancer.[2] The mouse 3T3-L1 preadipocyte system provides a well-characterized cell culture model for the study of adipocyte-specific differentiation.[3] The differentiation process is accompanied by sequential expression and activation of a set of transcription factors governing expression of adipocyte-specific markers, such as CCAT/enhancer-binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor-gamma (PPARγ), and the basic helix-loop-helix family (ADD1/SREBP1c).[4] Overexpression of C/EBPα or C/EBPβ promotes adipogenesis through cooperation with PPARγ, a member of the nuclear hormone receptor family, and is predominantly expressed in brown and white adipose tissues.[5] SREBP1c, which also appears to be involved in adipocyte differentiation, is highly expressed in adipose tissue as well as the liver and is also expressed early in adipocyte differentiation.[6] SREBP1c stimulates the expression of several lipogenic gene products, including FAS, LPL, ACC, SCD-1, and SCD-1.[7] Thus, it is likely that SREBP1c plays major roles in both fatty acid and glucose metabolism to orchestrate energy homeostasis.

Data from AMPK deficiency models suggest that AMPK activity might influence the pathophysiology and therapy of diabetes and obesity.[8] Indirect evidence implies that AMPK activation can inhibit preadipocyte differentiation. Moreover, previous studies revealed that AMPK activators, AICAR and several natural compounds, inhibit adipocyte differentiation significantly, block the expression of late adipogenic markers (such as fatty acid synthase and several transcription factors including PPARγ, SREBP1c, and C/EBPα), promote apoptosis, and improve glucose uptake.[9] Thus, identification of compounds that activate the AMPK pathway would significantly contribute to our ability to treat type 2 diabetes and obesity.

Sophorae Flos (SF), the dried flower buds of *Sophora japonica* L. (Leguminosae), is a well-known traditional Chinese medicine. It

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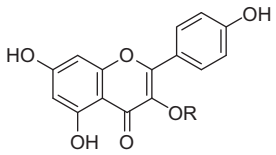
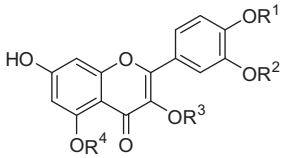
doi:10.1016/j.bmcl.2010.08.054



		R
1	Kaempferol	H
2	Kaempferol 3-*O*-β-D-glc	Glc



X: β-D-xyl-(1→3)-*O*-α-L-rha-(1→6)-*O*-β-D-glc
Y: β-D-glc-(1→3)-*O*-α-L-rha-(1→6)-*O*-β-D-glc

	R^1	R^2	R^3	R^4
3 Quercetin	H	H	H	H
4 Quercetin 3-*O*-β-D-glc	H	H	glc	H
5 Quercetin 5-*O*-β-D-glc	H	H	H	glc
6 Rutin	H	H	rutinose	H
7 Narcissin	H	CH_3	rutinose	H
8 Quercetin 3-*O*-β-D-xyl-(1→3)-*O*-α-L-rha-(1→6)-*O*-β-D-glc	H	H	X	H
9 Quercetin 3-*O*-β-D-glc-(1→3)-*O*-α-L-rha-(1→6)-*O*-β-D-glc	H	H	Y	H

Figure 1. Chemical structures of the isolated compounds (**1**–**9**).

has been used to be treat bleeding-related disorders, such as hematochezia, hemorrhoidal bleeding, dysfunctional uterine bleeding, and diarrhea.[10a] Several phytochemical investigations have been reported examining the chemical constituents of the root, seed, fruit, and pericarp of *S. japonica*, which include flavonoids and isoflavonoids,[10] coumaronochromone,[11] and terpenoids.[12] Pharmacological studies and clinical practice have revealed that *S. japonica* has anti-tumor,[13] anti-inflammatory,[14] anti-platelet,[15] anti-obesity,[16] and anti-hemorrhagic activities.[10a] The mature fruits of *S. japonica* can also considerably decrease blood glucose levels as well as thiobarbituric acid-reactive compounds in streptozocin-induced diabetic rats.[17] However, to our knowledge, anti-obesity effect of compounds isolated from Sophorae Flos have not yet been studied in detail.

Phytochemical investigation of the EtOAc and BuOH fractions led to the isolation of nine flavonoids (Fig. 1).[18] Of these, **4**, **5**, **8**, and **9** have been isolated for the first time from Sophorae Flos. The chemical structures of the isolated compounds were identified by analyzing UV–vis, IR, ^{1}H and ^{13}C NMR, and mass spectroscopic data as well as by comparison of these spectroscopic data with values reported in the literatures for kaempferol (**1**),[19] kaempferol 3-*O*-β-D-glucopyranoside (**2**),[20] quercetin (**3**),[21] quercetin 3-*O*-β-D-glucopyranoside (**4**),[22] quercetin 5-*O*-β-D-glucopyranoside (**5**),[23] quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside (rutin, **6**),[20,23] 3′-*O*-methylquercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside (narcissin, **7**),[24] quercein 3-*O*-[β-D-xylopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside (camellianoside, **8**),[25] and quercein 3-*O*-[β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside (**9**).[26]

The focus of this study was to isolate and identify potentially active compounds from SF and to evaluate their ability to improve glucose uptake as well as inhibit adipocyte differentiation associated with the AMPK and PPARγ signaling pathway in 3T3-L1 cells utilizing the cell culture and adipocyte differentiation,[27] western blot analysis,[28] reverse transcription-polymerase chain reaction (RT-PCR),[28] measurement of 2-deoxy-D-[^{3}H]-glucose uptake,[29] Oil red O staining assays.[30] One-way analysis of variance (ANOVA) was used to determine the significance of the differences between the treatment groups. The Newman–Keuls test was used for multi-group comparisons. The results were conducted in triplicate. Data are expressed as the means ± S.D. Differences were considered significant at p <0.05.

The cytotoxicity of **1**–**9** to 3T3-L1 cells was examined under the conditions described by Mosmann.[31] 3T3-L1 cells were treated with various concentrations of these compounds (5–100 μM) for 24 h (data not shown). These compounds did not influence the viability of 3T3-L1 cells at concentrations up to 20 μM. Therefore, we employed the test compounds at less than 20 μM in subsequent experiments. We evaluated all the isolated compounds for their

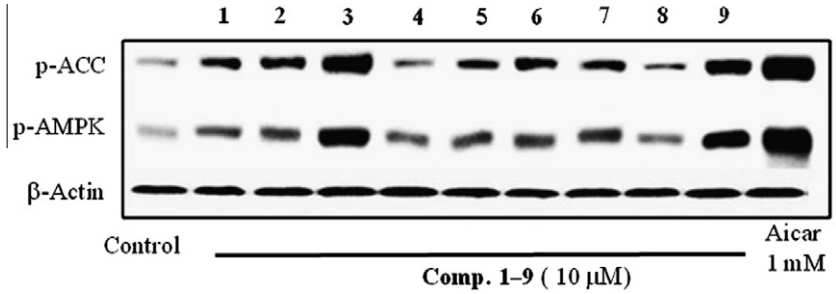


Figure 2. Effects of the isolated compounds (**1**–**9**) (at 10 μM) on the phosphorylation of AMPK and ACC in 3T3-L1 cells.

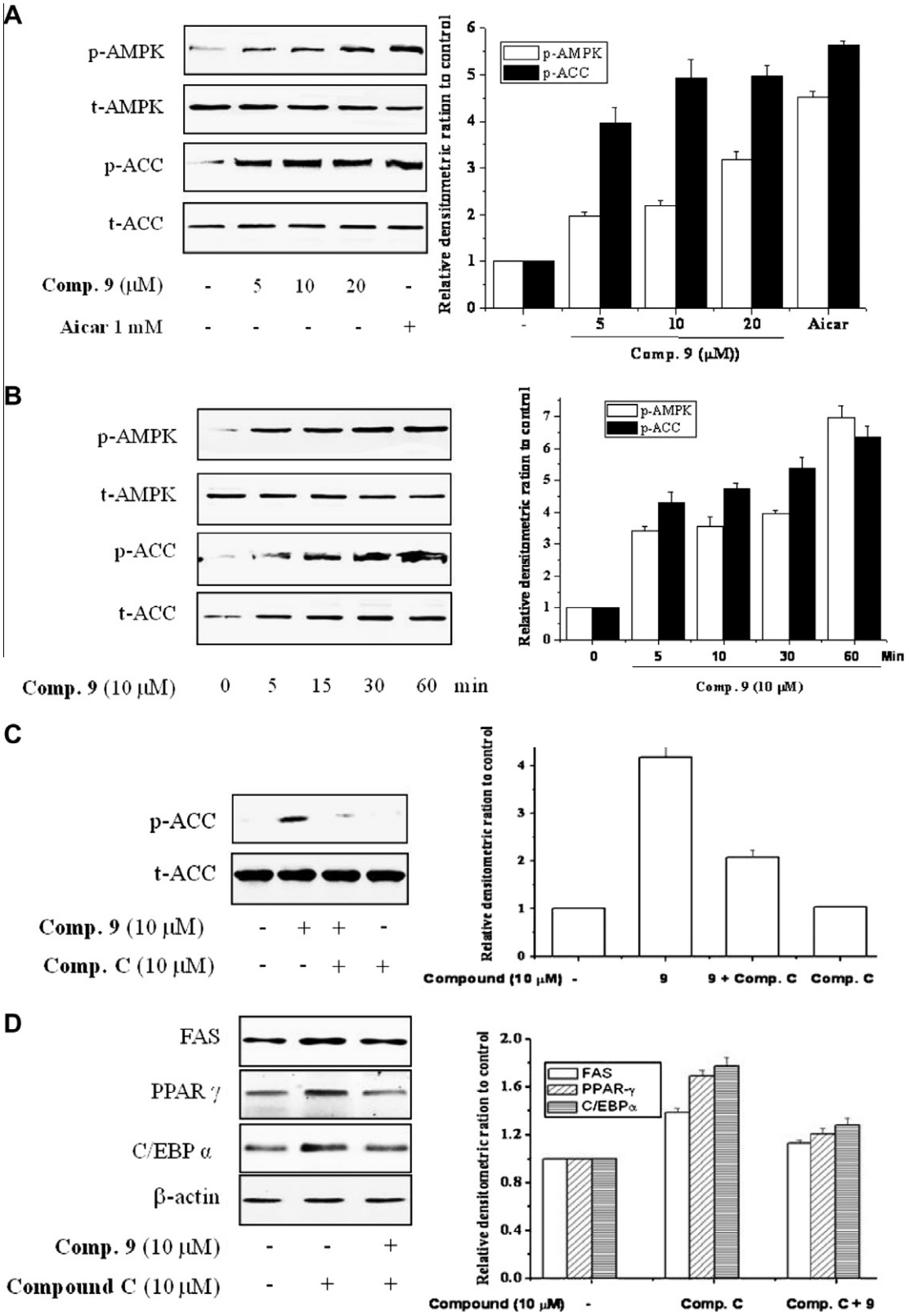


Figure 3. Compound **9** stimulated the phosphorylation of AMPK and ACC in 3T3-L1 cells. (B) Compound **9** increased the phosphorylation of AMPK and ACC in 3T3-L1 cells after the indicated time. (C) The cells were treated for 30 min with 10 μM of compound C (AMPK inhibitor) and then treated with 10 μM of **9** for 60 min. Phosphorylated ACC was detected by western blot quantitative analysis. (D) Effect of comp. C on the expression of key adipogenic markers during adipocyte differentiation. 3T3-L1 cells were treated with comp. C (10 μM) during differentiation and mRNA levels of key adipogenic markers including C/EBPα, PPARγ, and FAS were estimated by Western blot quantitative analysis.

effects on the phosphorylation of AMPK and its downstream target, ACC. As shown in Figure 2, **3** and **9** showed strong induction of phosphorylation of both AMPK and ACC at 10 μm administration. At the same concentration, **1**, **2**, and **5**–**7** increased AMPK and

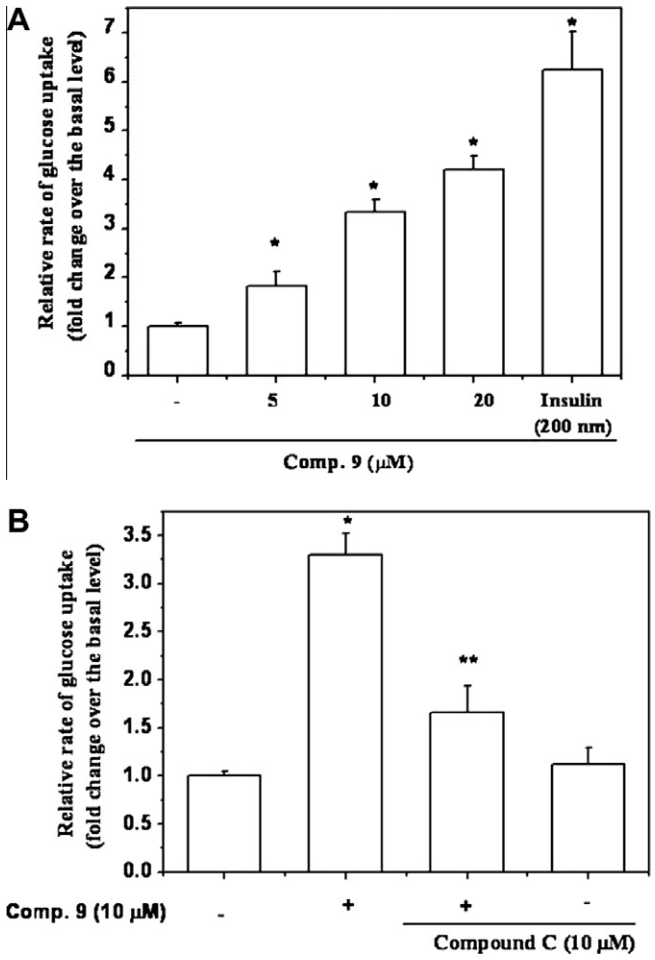


Figure 4. (A) Compound **9** increased glucose uptake in differentiated adipocyte 3T3-L1 cells. Differentiated 3T3-L1 cells were incubated in 60-mm dishes for 1 h with either **9** (5, 10, or 20 μM) or insulin (200 nM). The cells were then assayed for glucose uptake; *p <0.05, as compared with the control values. (B) Under identical conditions, differentiated 3T3-L1 cells were incubated in 60-mm dishes for 1 h with **9** (10 μM) in the presence or absence of compound C (10 μM) and then assayed for glucose uptake. *Significantly different from untreated cells (p <0.05), **significantly different from cells treated with **9** (p <0.05).

ACC phosphorylation significantly. Compounds **4** and **8** exhibited weak activity under the same experimental conditions. Several biological studies of the molecular mechanisms underlying the anti-obesity and anti-type 2 diabetic activities of the well-known compound quercetin (**3**) have been reported.[9c,32a] Therefore, we further tested **9**, the major compound derived from the BuOH fraction of SF, for anti-type 2 diabetic and anti-obese effects at the molecular level.

As illustrated in Figure 3A and B, the levels of phosphorylation of AMPK and ACC increased in a dose- and time-dependent manner. Recently, Gao et al. reported the effect of an AMP-activated protein kinase inhibitor, compound C, on adipogenic differentiation of 3T3-L1 cells. The study revealed the suppression of AMPK by compound C markedly reduced the expression of C/EBPα and PPARγ, and FAS during differentiation.[32b] Thus, we used compound C to confirm the effects of **9** on phosphorylation of ACC and key adipogenic markers (C/EBPα and PPARγ, and FAS) whether or not associate to AMPK. As shown in Figure 3C and D, compound C attenuated the **9**-stimulated phosphorylation of ACC and motivated the **9**-inhibited C/EBPα and PPARγ, and FAS gene expression, significantly. The results clarified the involvement of AMPK on suppression of adipogenic differentiation. To examine whether **9** affected glucose uptake, we treated 3T3-L1 cells with **9** and measured glucose uptake as described in the materials and methods section. As shown in Figure 4A, **9** enhanced the uptake of glucose in differentiated adipocyte 3T3-L1 cells in a dose-dependent manner. To test whether the presence of AMPK is necessary for the uptake of glucose induced by **9**, we pretreated the 3T3-L1 cells with compound C. Figure 4B showed that glucose uptake, which increased in the presence of **9**, was attenuated in 3T3-L1 cells that had been pretreated with compound C. Taken together, these data suggest that AMPK is involved in **9**-induced glucose uptake in 3T3-L1 cells.

To test the effects of **9** on adipocyte differentiation, adipocytes undergoing MDI-induced differentiation were exposed to different concentrations of **9** (0, 5, 10, and 20 μM) on day 0. The cells were differentiated using differentiation medium. At day 6, the differentiation was terminated, and fat drops were detected by Oil red O staining. As shown in Figures 5A and B, **9** markedly blocked adipocyte differentiation in a dose-dependent manner. To further investigate the effects of **9** on the initial phase of adipogenic differentiation, we examined the expression pattern of early adipogenic transcription factors and markers, including SREBP-1c, PPARγ, C/EBPα, and FAS using western blot analysis and RT-PCR. Among these, SREBP-1c and FAS were AMPK downstream substrates. The usual increases in the levels of SREBP-1c, FAS, PPARγ, and C/EBPα protein and gene expression were attenuated markedly by treatment with 20 μM of **9**, and the results were dose-dependent (Fig. 5C and D). Thus, **9** inhibits adipogenic transcription factors involved in the AMPK pathway. At a concentration of 100 μM, its aglycone (quercetin) reduced lipid accumulation in differentiated 3T3-L1 cells and at a concentration of 75 μM, quercetin attenuated markedly the transcription factors, including SREBP-1, C/EBPα, and PPARγ (Ref. 32c: Fig. 2C, 4–6).[32c] Thus, compound **9** inhibited adipogenic differentiation more stronger than that of quercetin.

Obesity is no longer considered simply a cosmetic problem, and the consequences of obesity are serious.[33] Adipose tissue accumulation is thought to be under the control of a number of adipogenic transcription factors.[2,34] Thus, the etiological link between obesity and type 2 diabetes involves a multitude of factors, including changes in adipose tissue distribution and metabolism, muscle metabolism, and alterations in the levels of carbohydrates, fatty acids, and adipocyte derived factors.[2,33a] Preventing the development of obese disease can reduce many diseases associated with obesity such as type 2 diabetes mellitus, at least in part. A number of treatments have been proposed for obesity, including balanced energy intake and expenditure, decreased lipogenesis, increased lipolysis, and the induction of adipocyte apoptosis.[35] A variety of naturally-occurring flavonoids have drawn attention because of their relative safety and accumulating evidence for their anti-obesity and anti-diabetic effects in animals and humans.[32,36] In the current study, we evaluated nine flavonol derivatives isolated from SF for the activation of AMPK and its downstream substrate ACC in 3T3-L1 cells. Compounds **3** and **9** showed high potent effect on phosphorylation of AMPK and ACC at 10 μM administration. However, quercetin is well-known, and their anti-diabetes and anti-obesity effects have been reported.[9b,32,37] Thus, in this study we chose to evaluate the effects of **9** on glucose uptake and adipocyte differentiation in 3T3-L1 cells. Compound **9** was isolated for the first time from *Camellia sinensis* in 1991 by Finger et al. However, its biological activities have not yet been studied. To investigate the molecular mechanisms underlying the anti-diabetic effects induced by **9** on 3T3-L1 preadipocytes, we measured the levels of phosphorylated AMPK and its substrate, ACC. Compound **9** considerably increased the phosphorylation of AMPK and its substrate, ACC, in a dose-dependent manner. Accumulating evidence indicates that AMPK is likely to mediate the effects of insulin-independent stimuli for glucose uptake.[38] Compound C is a highly-selective AMPK inhibitor that has an effect similar to that of a

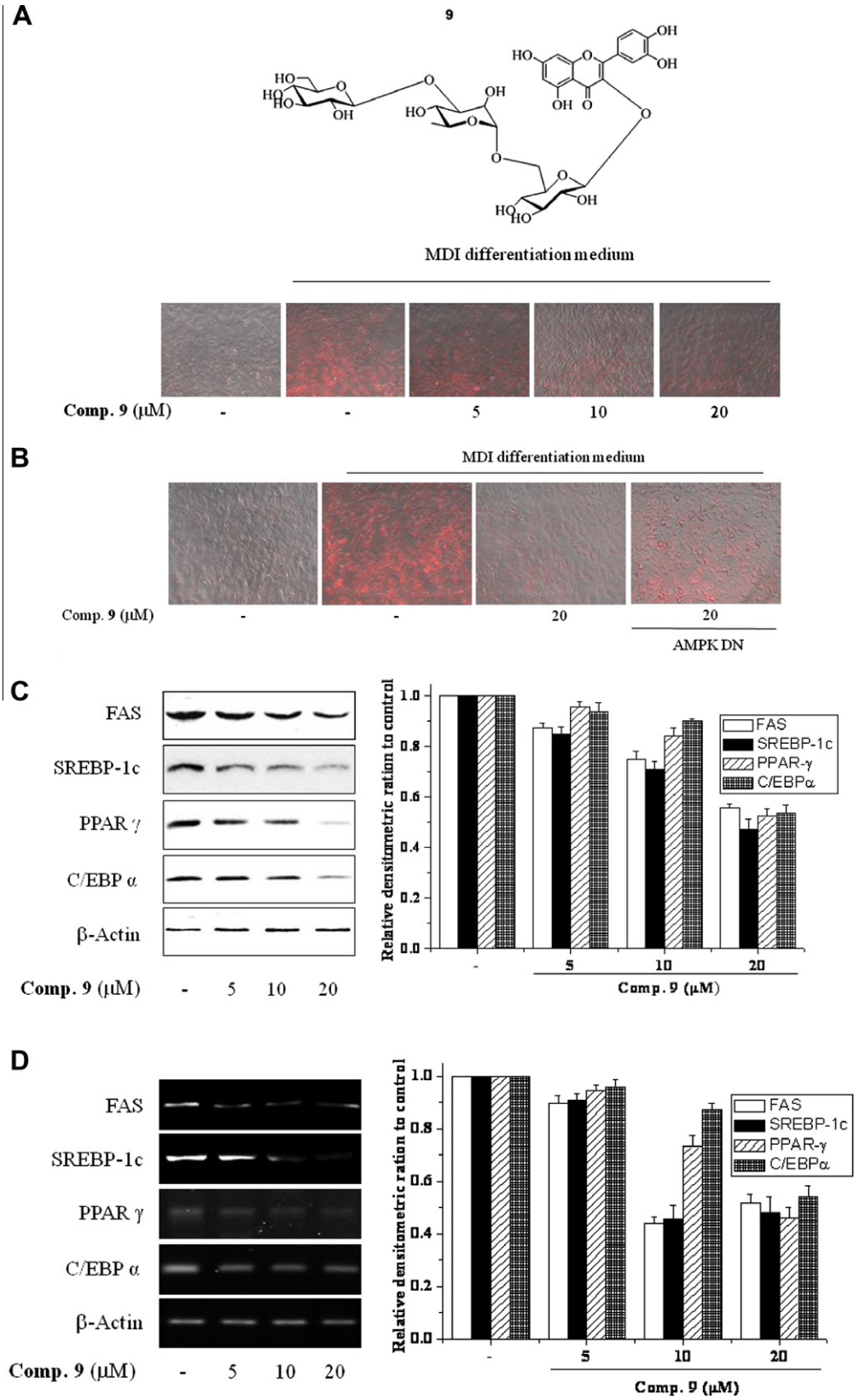


Figure 5. The inhibitory effect of **9** on adipocyte differentiation via the AMPK pathway. (A) Various doses of **9** (0, 5, 10, or 20 μM) were co-applied with MDI differentiation medium for 48 h. After 6 days, the cells were stained with Oil red O. (B) Dominant-negative AMPK blocks adipocyte differentiation by **9** (20 μM) in 3T3-L1 cells. (C) Cell lysates were analyzed using western blots to detect the expression of enzymes and adipogenic transcription factors (FAS, SREBP-1c, C-EBPα, and PPARγ). (D) The cells were treated with **9** for 6 h, RNR was extracted using Trizol reagent, and RT-PCR was performed using specific primers. The expression of these adipogenesis factors was detected by western blot quantitative analysis.

dominant-negative AMPK mutant.[39] This inhibitor has been widely used to evaluate the roles that AMPK plays in various cellular events.[40] In 3T3-L1 adipocyte cells, we have shown that compound C inhibits ACC phosphorylation stimulated by **9** and markedly suppresses **9**-activated glucose uptake. These results indicate that AMPK is a key factor in **9**-induced glucose uptake.

The increase in inactive, phosphorylated ACC, in turn, inhibited adipogenesis in our study. AMPK cascades have been postulated to respond to intracellular levels of AMP or to the AMP/ATP ratio.[41] Previous reports indicate that FAS is a key enzyme in lipogenesis, a fact that renders it an important target of anti-obesity research,[42] and adipocyte differentiation is enhanced by the transcription factor SREBP-1c, which directly or indirectly activates PPARγ.[43] PPARγ is an essential regulator of adipogenesis and a modulator of fat cell function.[44] Anti-adipogenic compounds, such as EGCG, vitisin, and quercetin, were shown to reduce the expression of PPARγ greatly.[32,45] In this study, we found that **9** decreased the expression of the FAS gene in a dose-dependent manner and the downregulated expression of SREBP-1c, PPARγ, and C/EBPα. These results suggest that **9** might possess inhibitory effects on lipid accumulation and adipocyte differentiation.

This mechanism is similar to that of the endogenous adipogenesis inhibitor quercetin, which showed potent anti-obesity activity associated with the AMPK and MAPK signaling pathways.[32] Thus, this study examined a flavonol triglycoside (**9**) with promising anti-obesity and anti-type 2 diabetic effects. Its metabolic function was studied, and the results provided (at least partial) insight into the molecular mechanisms by which **9** influences the regulation of fat cell differentiation. The results further suggest that **9** can be used to regulate the adipocyte life cycle. In addition, we demonstrated that the AMPK pathway exerts an intense influence on **9**-mediated glucose metabolism and **9**-inhibited adipocyte differentiation.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (KOSEF2009-007-4808).

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18. *Extraction and isolation*. Sophorae Flos (SF, the flower buds of *Sophora japonica* L., Leguminocae) was purchased from a pharmacy store in Daejeon, Korea, in July 2008. A voucher specimen (CNU 1518-1) has been verified by Prof. Bae and deposited in the Herbarium of the College of Pharmacy, Chungnam National University. SF (5.0 kg) was extracted three times with hot MeOH (12 L each time) using an ultrasonic apparatus, for 3 h each time, and the solvent was removed under reduced pressure. The residue (1.5 kg) was then dissolved in hot water and left in the refrigerator overnight, Next, the resulting sample was filtered, and the precipitate was recrystallized in hot water to yield rutin (**6**, 620 g). The filtrate was partitioned with hexane, EtOAc, and BuOH, successively, and then concentrated exhaustively to give a hexane-soluble fraction (64 g), an EtOAc-soluble fraction (213 g), and a BuOH-soluble fraction (149 g). The EtOAc fraction was subjected to column chromatography (CC) on silica gel using solvents of increasing polarity (EtOAc–MeOH, 1:0, 5:1, 1:1, 1:5, and 0:1 (v/v)) to give fractions SFE1–10. Fraction SFE3 (15 g) was eluted through a Sephadex LH-20 column (MeOH–H_2O, 2:1) to obtain 4 fractions (SFE3.1–3.4). Rechromatography of fraction SFE3.2 (2.2 g) on a YMC column with MeOH–H_2O (3:1) yielded **2** (28 mg) and **3** (1.3 g). Fraction SFE6 (14 g) was resolved using silica gel ($CHCl_3$–MeOH, 5:1) and YMC C-18 (MeOH–H_2O, 1.2:1) chromatography columns to yield **7** (700 mg).
The BuOH-soluble fraction was subjected to chromatography on a Dianion HP-20 column and eluted with a gradient solvent system using a graded series of aqueous methanol solutions (0%, 25%, 50%, 75%, and 100%) to obtain the five fractions, SFB1–5. Fraction 2 (35 g) was resolved using Sephadex LH-20 (MeOH–H_2O, 2:1) and YMC C-18 (MeOH–H_2O, 1:3) columns. This yielded seven fractions (SFB2.1–2.7), and after purification on a silica gel column (EtOAc–MeOH–H_2O, 3:1:0.5), generated **8** (10 mg) and **9** (420 mg). Fraction SFB3 (24 g) was subjected to silica gel CC using ($CHCl_3$–MeOH, 20:1, 10:1, and 5:1) as the eluent. Five fractions were obtained (SFB3.1–3.5). Compounds **1** (25 mg), **4** (8 mg), and **5** (8 mg) were separated from fraction SFB3.2 (8 g) using silica gel CC (EtOAc–MeOH–H_2O, 16:2:1) and an YMC C-18 CC (MeOH–H_2O, 1:1) column.
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30. *Oil red O staining*. The cells were washed with ice-cold PBS twice, fixed with 10% formalin at room temperature for 1 h, and stained with 0.2% Oil red O in isopropanol for 10 min. The cells were then washed with 70% ethanol and water. Images were collected using an Olympus (Tokyo, Japan) microscope.
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