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Lanostane triterpenes from *Ganoderma lucidum* suppress the adipogenesis in 3T3-L1 cells through down-regulation of SREBP-1c

IkSoo Lee [a], JinPyo Kim [a], InJa Ryoo [b], YoungHee Kim [b], SooJin Choo [b], IckDong Yoo [b], ByungSun Min [c], MinKyun Na [d], Masao Hattori [e], KiHwan Bae [a,*]

[a] College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea
[b] Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea
[c] College of Pharmacy, Catholic University of Daegu, Gyeongbuk 712-702, Republic of Korea
[d] College of Pharmacy, Yeungnam University, Gyeongbuk 712-749, Republic of Korea
[e] Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

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ABSTRACT

Several lanostane triterpenes [butyl ganoderate A (**1**), butyl ganoderate B (**2**), butyl lucidenate N (**3**), and butyl lucidenate A (**4**)] bearing a butyl ester side chain from the fruiting bodies of *Ganoderma lucidum* exhibited considerable inhibitory effects on adipogenesis in 3T3-L1 cells. The inhibitory mechanism of **1** and **3** on adipogenesis in 3T3-L1 cells was investigated; we found that the mRNA and protein expression levels of SREBP-1c were reduced by treatment with **1** and **3** versus the untreated control. Furthermore, compounds **1** and **3** suppressed the mRNA expression levels of FAS and ACC. These results demonstrate that inhibition of adipogenesis in 3T3-L1 cells by treatment with **1** and **3** may be mediated in part through down-regulation of the adipogenic transcription factor SREBP-1c and its target genes, such as FAS and ACC.

In recent years, obesity, the excess accumulation of adipose tissue, has become a major public health concern worldwide because it is associated with the development of various diseases, including coronary heart disease, hypertension, type 2 diabetes, cancer, respiratory complications, and osteoarthritis.[1–3] Adipose tissue, composed of adipocytes, plays a critical role in lipid homeostasis and energy balance. The primary role of adipose tissue is to store energy in the form of triglycerides when energy intake exceeds energy expenditure and to release it in the form of free fatty acids in starvation.[4] Adipocyte differentiation, known as adipogenesis, is the process of fat cell development, accompanied by coordinated changes in cell morphology, hormone sensitivity, and gene expression.[5] The regulation of adipogenesis has been studied extensively using various cell and animal models. The 3T3-L1 cell line is one of the most well characterized and is a reliable model for studying the adipogenesis process.[6–8] Thus, inhibitors of adipogenesis of 3T3-L1 cells may be effective in treating or preventing obesity.

Ganoderma lucidum (Polyporaceae) has been used in folk medicine throughout the world since ancient times, especially for the treatment of neurasthenia, insomnia, anorexia, dizziness, chronic hepatitis, hypercholesterolemia, coronary heart disease, hypertension, and carcinomas.[9–11] Because of the potential medicinal value and wide acceptability, much attention has been paid to the search for bioactive compounds from this mushroom. The fruiting body, mycelia, and spores of *G. lucidum* contain approximately 400 different bioactive compounds, including primarily triterpenes, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins, peptides, and trace elements. Among these, triterpenes have attracted considerable attention because they exhibit diverse and potentially significant pharmacological effects, such as anti-HIV,[12] anti-hypertensive,[13] hepatoprotective,[14] cytotoxic,[15] anti-allergic,[16] cholesterol-reducing,[17] platelet aggregation-inhibiting,[18] and histamine release-inhibiting[19] properties.

In the course of searching for anti-obesity agents from natural sources, we found several lanostane triterpenes from the fruiting bodies of *G. lucidum* that exhibit considerable inhibition of lipid droplet formation, indicative of 3T3-L1 cell differentiation, during differentiation of 3T3-L1 preadipocytes, as compared to untreated controls.[20] However, the mechanism underlying this effect of lanostane triterpenes remains unknown. The present report describes the effects of lanostane triterpenes from the fruiting bodies of *G. lucidum* on adipogenesis in 3T3-L1 cells, together with some interesting structural requirements for the activity and the inhibitory mechanism of butyl ganoderate A (**1**) and butyl lucidenate N (**3**).

* Corresponding author. Tel.: +82 42 821 5925; fax: +82 42 823 6566.
E-mail address: baekh@cnu.ac.kr (KiHwan Bae).

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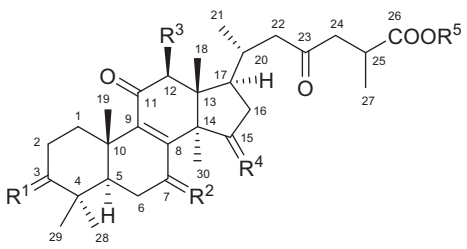

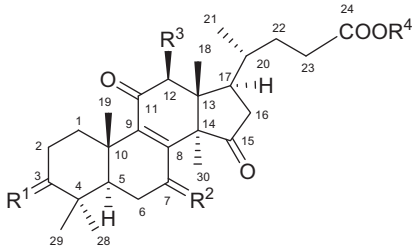

	R^1	R^2	R^3	R^4	R^5
1	O	βOH	H	αOH	Bu
2	βOH	βOH	H	O	Bu
5	O	βOH	H	αOH	H
6	O	βOH	H	αOH	Me
7	βOH	βOH	H	O	H
8	βOH	βOH	H	O	Me
9	O	βOH	H	O	Me
10	O	O	H	O	H
11	O	O	H	O	H
12	O	H_2	H	αOH	H
13	βOH	O	OAc	O	Me

	R^1	R^2	R^3	R^4
3	βOH	βOH	H	Bu
4	O	βOH	H	Bu
14	βOH	βOH	H	H
15	O	βOH	H	Me
16	βOH	βOH	OAc	Me
17	βOH	O	OAc	Me
18	O	O	H	Me

Figure 1. Structures of compounds **1**–**18** isolated from *Ganoderma lucidum*.

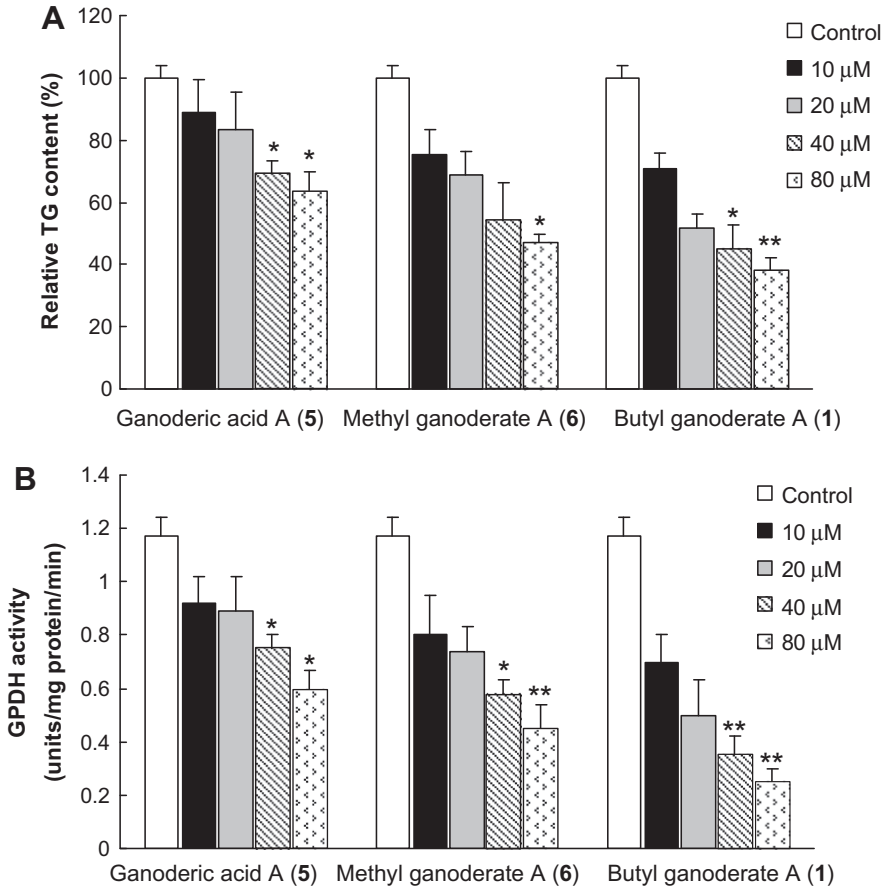

Figure 2. Effect of ganoderic acid A derivatives (**1**, **5**, and **6**) on triglyceride (TG) accumulation and GPDH activity in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were untreated, as a control, or treated with different concentrations of compounds **1**, **5**, and **6** (10–80 μM) during differentiation. On day 8 of differentiation, TG accumulation (A) and GPDH activity (B) in cells treated with **1**, **5**, and **6** were measured. Results are expressed as the mean ± SD of three individual experiments. $^{*}p$ <0.05, $^{**}p$ <0.001, versus control.

In a previous study, we reported the isolation of 18 lanostane-type triterpenes from the fruiting bodies of *G. lucidum* (Fig. 1).[20] They were divided into two groups (I and II) based on their carbon number and oxidation state. Compounds **1**, **2**, and **5**–**13** (group I) have one double bond at C-8, which is in conjugation with an oxo group at C-11 or with two oxo groups at C-7 and C-11. They are lanostane 26-acids, ganoderic acids, having a carboxyl group at the end of the side chain at C-26. Compounds **3**, **4**, and **14**–**18** (group II) have one double bond at C-8, which is in conjugation with an oxo group at C-11. They are trinortriterpenic acids (C_{27} compounds), lucidenic acids, which have a carboxyl group at C-24.

To test whether the isolated compounds had cytotoxic effects in 3T3-L1 cells, the viability of 3T3-L1 cells treated with **1**–**18** was measured; no cytotoxic effect was detectable for any of the compounds at concentrations of up to 40 μg/mL (data not shown). Thus, cells were untreated, as a control, or treated with 40 μg/mL of **1**–**18** during differentiation, and on day 8 of differentiation, the effects of **1**–**18** on adipogenesis in 3T3-L1 cells were evaluated. Among them, butyl ganoderate A (**1**), butyl ganoderate B (**2**), butyl lucidenate N (**3**), and butyl lucidenate A (**4**), having a butyl ester side chain, significantly reduced lipid accumulation in 3T3-L1 adipocytes by 48%, 41%, 56%, and 45% at 40 μg/mL, respectively, versus the untreated control.[20] These findings suggest that esterification of the carboxyl group in the side chain of the isolated lanostane triterpenes with BuOH may enhance the anti-adipogenic effect. However, other structural features did not seem to significantly influence the adipogenic effect of the lanostane triterpenes.

To examine whether the butyl ester side chain affected the process of adipogenesis in 3T3-L1 cells, the intracellular triglyceride (TG) content and glycerol-3-phosphate dehydrogenase (GPDH) activity of the cells treated with different concentrations of ganoderic acid A derivatives [**1**, **5**, and **6** (10–80 μM); no cytotoxic effect was observed with these compounds up to 80 μM] were evaluated.[21] As shown in Figure 2A, butyl ganoderate A (**1**), with a butyl ester side chain, reduced the amount of accumulated TG in the cells most effectively. This compound, at the highest concentration used (80 μM), reduced the amount of stored TG by more than 60%. The GPDH activity of the cells treated with butyl ganoderate A (**1**) was also significantly decreased (Fig. 2B). The least efficient was ganoderic acid A (**5**), which reduced the amount of stored TG by only 36% and suppressed GPDH activity by 48% at the highest concentration used (80 μM). Similar behavior was also observed for ganoderic acid B derivatives (**2**, **7**, and **8**; data not shown) and lucidenic acid N derivatives (**3** and **14**; data not shown), demonstrating that an ester side chain does influence the inhibitory potential of these lanostane triterpenes during adipogenesis in 3T3-L1 cells. Furthermore, this result indicated that the length of the ester side chain in these lanostane triterpenes may be a factor that affects the process of adipogenesis in 3T3-L1 cells.

Based on these screening results, two compounds (**1** and **3**) were chosen for further investigation to examine the mechanism(s) involved in the anti-obesity effect. Adipogenesis is regulated by several transcription factors, including peroxisome proliferators-activated receptors (PPARs), CCAAT/enhancer-binding proteins (C/EBPs), and sterol regulatory element-binding

Table 1
The primer sequence used for RT-PCR

Gene name	Forward	Reverse
SREBP-1c	GGAGACATCGCAAACAAGCTGA	AAAGCAGACTGCAGGCCA GATCCA
FAS	TGGTGGGTTTGGTGAATTG	GCTTGTCCTGCTCTAACTG
ACC	GAGTGACTGCCGAAACATCTCTG	GCCTCTTCCTGACAAACGAGT
β-Actin	CACCCACACTGTGCCCATCTAC	CGATTTCCCTCTCAGCTGTGGT

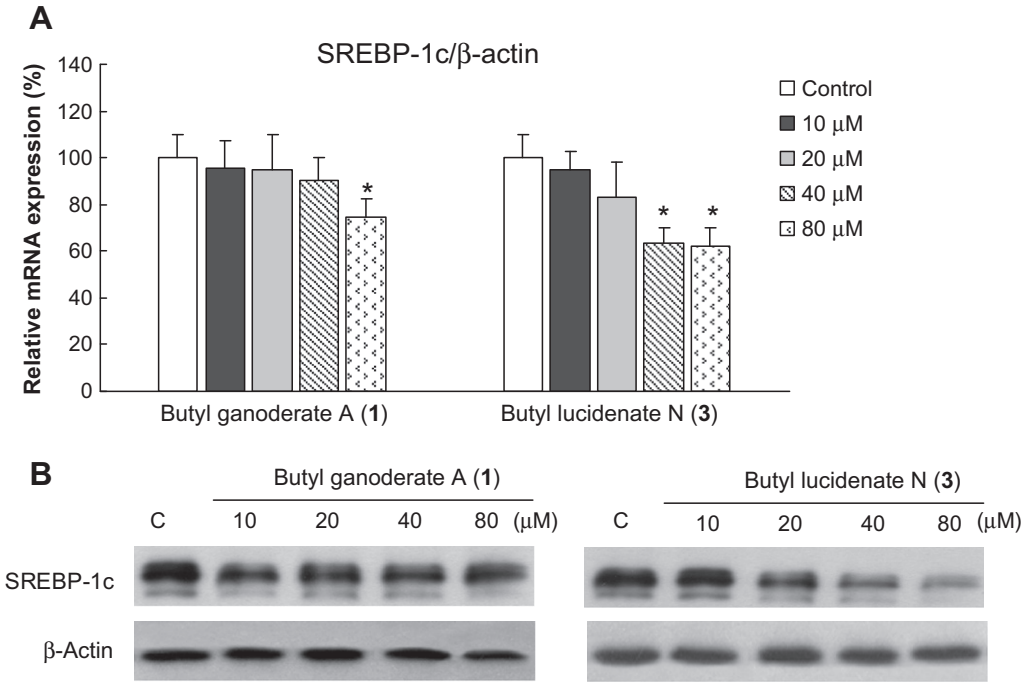

Figure 3. Effect of compounds **1** and **3** on gene and protein expressions of SREBP-1c in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were untreated, as a control, or treated with different concentrations of butyl ganoderate A (**1**) and butyl lucidenate N (**3**) (10–80 μM) during differentiation. On day 8 of differentiation, total RNA and total protein were isolated from differentiated adipocytes. (A) Gene expression was quantified by quantitative RT-PCR in three independent experiments. The relative mRNA expression level of SREBP-1c was normalized to the β-actin mRNA level. The sequences of the primers are shown in Table 1. Results are expressed as the mean ± SD of three individual experiments. $^{*}p$ <0.05, $^{**}p$ <0.001, versus control. (B) Twenty micrograms of protein was subjected to SDS–PAGE, transferred, and immunoblotted with an antibody to SREBP-1c (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000). A representative result from two independent experiments is presented.

proteins (SREBPs).[22,23] Of these, SREBPs are known to regulate the transcription of many genes that are important in cholesterol and fatty acid metabolism.[24] Three isoforms of SREBP have been identified and designated SREBP-1a, -1c, and -2. Of them, SREBP-1c is responsible for the transactivation of lipogenic genes that encode enzymes of fatty acid and triglyceride synthesis.[25] Thus, to investigate whether **1** and **3** suppressed TG accumulation and GPDH activity through a SREBP-1c pathway, the mRNA and protein expression levels of SREBP-1c in cells treated with **1** and **3** were investigated by RT-PCR and Western blotting. Consistent with the decrease in TG accumulation and GPDH activity, compounds **1** and **3** suppressed the mRNA expression level of SREBP-1c in the cells, dose-dependently (Fig. 3A). More specifically, the mRNA expression level of SREBP-1c in the cells treated with 80 μM of **1** and **3** decreased to 26% and 38%, respectively, compared with the untreated control. The protein expression level of SREBP-1c in the cells treated with **1** and **3** also decreased in a similar way to mRNA expression (Fig. 3B). This indicated that the decrease in TG accumulation and GPDH activity during differentiation may result in part from a lower level of a SREBP-1c expression, resulting from treatment with **1** and **3**.

The inhibition of SREBP-1c mRNA expression was accompanied by a sharp reduction in the mRNA expression of SREBP-1c target genes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC).[26] Because the mRNA expression level of SREBP-1c was reduced by treatment with **1** and **3**, we next investigated the expression levels of its target genes. As shown in Figure 4, the mRNA expression levels of FAS and ACC were reduced by treatment with **1** and **3**, as compared to the untreated control, indicating that **1** and **3** suppressed the mRNA expression level of SREBP-1c, decreasing expression of the FAS and ACC genes.

In summary, several lanostane triterpenes (**1**–**4**) from the fruiting bodies of *G. lucidum* bearing a butyl ester side chain exhibited considerable inhibition on adipogenesis in 3T3-L1 cells, indicating that the ester side chain influences the inhibitory potential of these lanostane triterpenes during adipogenesis in 3T3-L1 cells. To investigate the inhibitory mechanism of triterpenes (**1** and **3**) on adipogenesis in 3T3-L1 cells, the mRNA expression levels of SREBP-1c and its target genes, such as FAS and ACC, were examined; we found that the mRNA expression level of SREBP-1c, FAS, and ACC was reduced by treatment with **1** and **3** versus the untreated control. These findings demonstrated that the inhibition of adipogenesis in 3T3-L1 cells by treatment with **1** and **3** may be mediated in part through down-regulation of the adipogenic transcription factor SREBP-1c and its target genes, such as FAS and ACC. However, further studies are needed to elucidate more specifically the mechanism(s) involved in the anti-obesity effect.

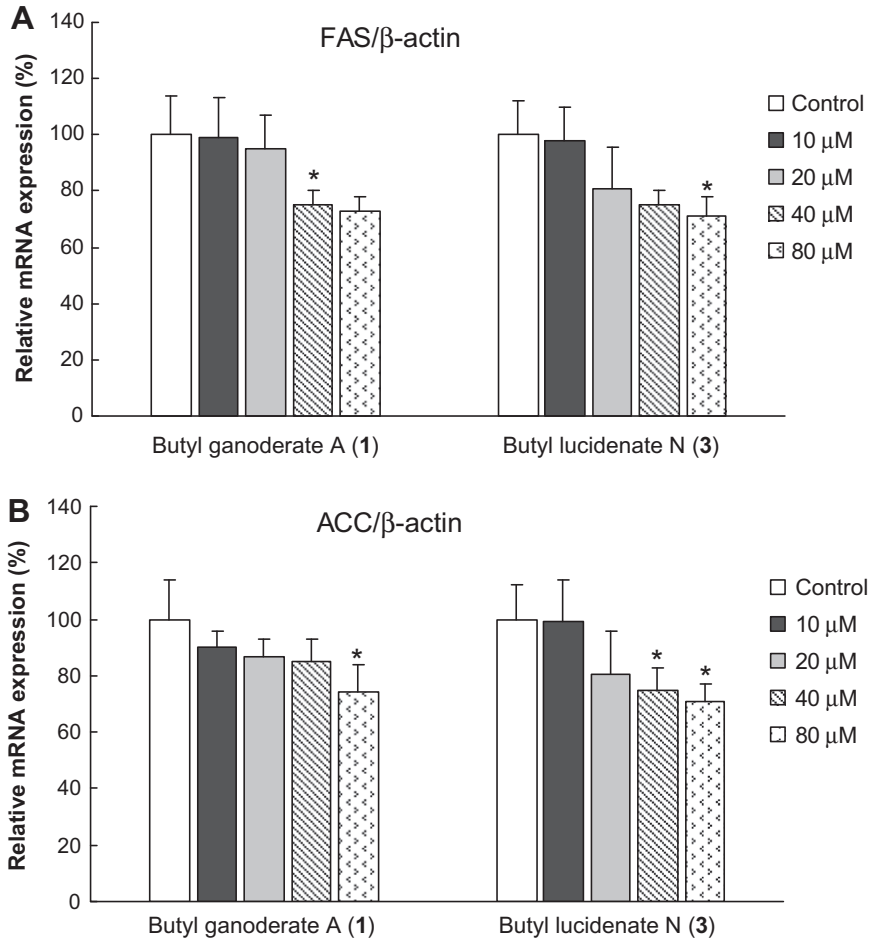

Figure 4. Effect of compounds **1** and **3** on gene expression of FAS and ACC in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were untreated, as a control, or treated with different concentrations of butyl ganoderate A (**1**) and butyl lucidenate N (**3**) (10–80 μM) during differentiation. On day 8 of differentiation, total RNA was isolated from the differentiated adipocytes. Gene expression was quantified by quantitative RT-PCR in three independent experiments. The relative mRNA expression level of FAS and ACC was normalized to the β-actin mRNA level. The sequences of primers are shown in Table 1. Results are expressed as the mean ± SD of three individual experiments. $^{*}p$ <0.05, $^{**}p$ <0.001, versus control.

Acknowledgments

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21. (a) *Measurement of triglyceride (TG) content*: The cellular TG content was determined spectrophotometrically using a TG assay kit (Cleantech TG-S; Asan Pharm Co., Ltd, Whasung, Korea). Briefly, the cells were washed gently twice with PBS and lysed in lysis buffer (1% Triton X-100 in PBS). Cellular lysate (20 μL) were mixed with 3 mL of the enzyme solution supplied, and incubated for 10 min at 37 °C. The absorbance at 550 nm was measured within 60 min. The protein concentration was determined by using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). (b) *Measurement of GPDH activity*: The GPDH activity was measured with a GPDH activity assay kit (Takara, Kyoto, Japan). Briefly, cells were carefully washed twice with ice-cold PBS on 8 days of differentiation period, and enzyme extraction buffer was added, and cells were collected by scraping with a cell scraper. After centrifugation at 12,000 rpm for 5 min at 4 °C, the absorbance reduction of supernatant at 340 nm for 5 min was measured to estimate the rate of NADH oxidation during the GPDH-catalyzed reduction of dihydroxyacetone phosphate. One unit of enzyme activity corresponded to the oxidation of 1 nmol NADH/min and the results were expressed as units per mg protein, and the protein concentration was determined by using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA)
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