Dihydrosanguinarine Enhances Glucose Uptake in Mouse 3T3-L1 Cells

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ABSTRACT: Recently, more studies have aimed at identifying selective peroxisome proliferator-activated receptor gamma (PPARγ) modulators that transactivate the expression of PPARγ-dependent genes as partial agonists to improve diabetic symptoms with fewer side effects compared to classic PPARγ agonists such as thiazolidinediones. We found that dihydrosanguinarine (DHS) treatment induced preadipocyte differentiation and lipid droplet accumulation in 3T3-L1 cells, but this effect is weaker than that elicited by the full PPARγ agonist troglitazone. Furthermore, this effect was reduced by the addition of a PPARγ antagonist, indicating the involvement of PPARγ signaling. Our results suggest that the stimulatory effects of DHS on adipocyte differentiation and insulin sensitivity are mediated by suppressing adenosine monophosphate-activated protein kinase (AMPK) alpha, upregulating the expression of PPARγ and its target genes (particularly Glut-4 and adiponectin) and reducing PPARγ phosphorylation. DHS significantly enhanced the glucose uptake in 3T3-L1 adipocytes without observable cytotoxicity at the effective concentration (5 μM) applied.

1. INTRODUCTION

The adipocyte differentiation process is tightly controlled by molecular and cellular mechanisms, including transcriptional factors and extracellular proteins. Many of the genes associated with the differentiation and maintenance of the adipocyte phenotype could be involved in metabolic disorders, such as type-2 diabetes and obesity.1 Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear receptor superfamily of ligand-inducible transcription factors and is a master regulator of adipocyte differentiation and metabolism, controlling the gene networks involved in lipid metabolism and glucose homeostasis.2,3 PPARγ is the ultimate effector of adipogenesis in a transcriptional cascade that also involves members of the C/EBP (CCAAT enhancer binding protein) transcription factor family.4,5 Together, these proteins regulate downstream target genes involved in adipogenesis.6 Inhibition of PPARγ activity was reported to exert antidiopgenic effects, and PPARγ antagonists have been suggested as candidate drugs for antiobesity.7,8 By contrast, hyperactivation of PPARγ induced adipose tissue expansion, weight gain, and fatty livers in human subjects and animals studies as well as adipogenesis in vitro.2,9 However, activation of PPARγ also yields beneficial effects—it has been shown to improve glucose homeostasis and insulin sensitivity.10,11 Full PPARγ agonists such as thiazolidinediones are an effective class of drugs for treating type-2 diabetes. However, they have serious side effects such as weight gain, bone loss, fluid retention, and cardiac risks, which has led to their restricted use in patients.12

Recently, more studies have focused on finding novel ligands—selective PPARγ modulators—from synthetic and natural compounds that exhibit partial agonism based on selective receptor–cofactor interactions and can target gene regulation. In vivo studies reported that some PPARγ activators from natural compounds, such as honokiol, amorfrutin 1, and amorphastilbol, improved metabolic parameters in diabetic animal models with fewer side effects than full PPARγ agonists such as thiazolidinediones.13 Many plant alkaloids were reported to possess potent pharmacological activities and have been implemented in traditional medicine as well as considered a model for modern drug synthesis. However, most of these natural products, especially the benzylisoquinoline alkaloids (BIAs), remain largely unexplored regarding their pharmacokinetic and pharmacodynamic properties. The pharamacokinetic results of sanguinarine in animal studies demonstrated that its in vivo availability is apparently low and that the main sanguinarine metabolic pathway is iminium bond reduction, which results in dihydrosanguinarine (DHS) formation.14 The pharmacokinetic results of sanguinarine in animal studies demonstrated that its in vivo availability is apparently low and that the main sanguinarine metabolic pathway is iminium bond reduction, which results in dihydrosanguinarine (DHS) formation.15 Previously, we found that BIAs exhibited lipid metabolism-modulating activity in Caenorhabditis elegans. Treatment with...
berberine and sanguinarine reduced lipid droplet accumulation in the worms. This lipid reduction effect was linked to adenosine monophosphate-activated protein kinase (AMPK) activation. AMPK signaling lies upstream of the PPARγ pathway, and AMPK activation can inhibit adipocyte differentiation and modulate energy metabolism-related gene transcription.

Owing to its potential to differentiate from fibroblasts to adipocytes, the 3T3-L1 cell line has been extensively used to study adipogenesis and the biochemistry of adipocytes. Here, we investigated the adipogenic effect of several BIAs (DHS, sanguinarine, and berberine) in mouse 3T3-L1 adipocytes. Quantitative real-time polymerase chain reaction (RT-PCR) results showed increase of the PPARγ downstream target gene expressions and adipogenesis markers after DHS treatment. However, its induction of PPARγ was not as strong as that of the full agonist, troglitazone. Therefore, we characterized the activity of DHS associated with the PPARγ, AMPK, and insulin signaling pathways in comparison with troglitazone. Interestingly, we found that DHS induced adipocyte differentiation, whereas its oxidized form, sanguinarine, did not. We also investigated the metabolic fate and the cytotoxicity of DHS in 3T3-L1 adipocytes.

2. RESULTS AND DISCUSSION

In this study, we investigated the effects of berberine and sanguinarine on lipid metabolism in mammalian cells. We also examined the activity of DHS (Figure 1a) because it is the main metabolite of sanguinarine. Preadipocytes (day 0) were incubated in a differentiation medium in the presence or absence of 5 μM alkaloid for 2 days and then replaced with a fresh medium containing insulin in the presence or absence of alkaloid every 2 days until day 8. The adipocytes were then stained with Oil Red O. Among the treated cells, berberine-treated cells showed reduced lipid droplet accumulation (Figure 1b), and DHS-treated cells showed a higher density of lipid droplets, but the sanguinarine-treated cells showed no significant change compared to untreated cells. Quantitative measurements of the cellular triglyceride levels also indicated similar results to those of the lipid droplet staining. Next, we treated preadipocytes with troglitazone (a known PPARγ agonist that induces adipogenesis). Both the results of Oil Red O staining and the triglyceride measurement showed that DHS and troglitazone treatments increased lipid droplet accumulation in 3T3-L1 cells. However, DHS exhibited a weaker effect than troglitazone at the same concentration (Figure 1c). DHS showed a dose-dependent adipogenic effect from 2 to 10 μM.
whereas high cytotoxicity was observed at concentrations higher than 10 μM (see below). The conversion of preadipocytes into adipocytes involves the activation of key transcription factors such as PPARγ and C/EBP. During the differentiation process, increased C/EBPβ and C/EBPδ activity induces the transcription of C/EBPα and PPARγ.22 We conducted quantitative RT-PCR to examine the effect of DHS on the expression profiles of genes involved in adipogenesis in 3T3-L1 cells compared with the effects of the same concentration (5 μM) of berberine and troglitazone as well as with the nontreated control.

As shown in Figure 2, the expression of many adipogenesis-related genes was significantly affected. The two main adipogenic transcription factors—PPARγ and C/EBPα—were significantly upregulated by troglitazone and DHS. Some PPARγ target genes, including adipocyte-specific genes, such as insulin-dependent glucose transporter (Glut4), adipose fatty acid-binding protein 2 (aP2), cluster of differentiation 36

![Figure 2](image)

Figure 2. Quantitative RT-PCR of adipogenesis-related pathway genes. 3T3-L1 preadipocytes were treated with 5 μM troglitazone (T), berberine (B), or DHS (D) for 5 days. n = 3 from three independent experiments; error bar = SD. *p < 0.05, **p < 0.005, ***p < 0.001 vs control; two-tailed Student’s t-test.

![Figure 3](image)

Figure 3. (a) Oil Red O staining of 3T3-L1 adipocytes and triglyceride content in 3T3-L1 adipocytes at day 8. n = 3; error bar = SD. *p < 0.05, ***p < 0.001; a—compared to control, b—compared to G10 [analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test]. (b) Quantitative RT-PCR of adipogenic genes in 3T3-L1 preadipocytes treated with 5 μM DHS (D5) or 10 μM GW9662 and 5 μM DHS (G10 + D5) for 5 days. n = 3; error bar = SD. *p < 0.05, **p < 0.005 compared to respective control; two-tailed Student’s t-test. (c) Immunoblotting analyses of 3T3-L1 preadipocytes treated with 5 μM DHS (D5), 10 μM GW9662, and 5 μM DHS (GD), or 5 μM troglitazone (T5) for 5 days. n = 3; error bar = SD. *p < 0.05, ***p < 0.001; a—compared to control, b—compared to D5 (ANOVA followed by Dunnett’s multiple comparisons test).
Our qRT-PCR results showed that the troglitazone treatment (rosiglitazone) treatment of obese mice boosts PGC-1α expression and decrease glucose production. Thiazolidinedione (TZD) acting on the adipose, muscle, and liver to increase glucose utilization and decrease glucose production. In response to dietary availability by coordinating the expression of a wide array of genes involved in glucose and fatty acid metabolism. 25 Antidiabetic drugs such as thiazolidinediones (including troglitazone and rosiglitazone) increase insulin sensitivity by upregulating PPARGC1A expression in 3T3-L1 cells, whereas the effects of berberine inhibit adipocyte differentiation through the PPARγ pathway. 23,24

PPARγ coactivator 1-alpha (PPARGC1A or PGC-1α) is a transcriptional coactivator for steroid receptors and nuclear receptors. It increases the transcriptional activity of PPARγ and plays an essential role in metabolic reprogramming in response to dietary availability by coordinating the expression of a wide array of genes involved in glucose and fatty acid metabolism. 25 Antidiabetic drugs such as thiazolidinediones (including troglitazone and rosiglitazone) increase insulin sensitivity by acting on the adipose, muscle, and liver to increase glucose utilization and decrease glucose production. Thiazolidinedione (rosiglitazone) treatment of obese mice boosts PGC-1α transcription in the white adipose tissue, which is accompanied by increased mitochondrial function and insulin sensitivity. 26 Our qRT-PCR results showed that the troglitazone treatment upregulated PPARGC1A expression in 3T3-L1 cells, whereas the effects of berberine and DHS treatment were marginal.

To investigate whether the effect of DHS is mediated by PPARγ, we cotreated preadipocytes with DHS (5 μM) and GW9662 (10 μM), a PPARγ antagonist. Lipid droplet accumulation and triglyceride levels were significantly reduced in the presence of GW9662 compared to DHS alone (Figure 3a). However, simultaneous treatment with DHS and GW9662 did not completely eliminate the adipogenic effect of DHS on 3T3-L1 cells. This suggests the possibility of an additional mechanism involved in the adipogenic effect of DHS.

Next, we examined the impact of GW9662-mediated PPARγ inhibition on the upregulation of adipogenesis-related genes observed in DHS-treated cells. The qRT-PCR results showed that cotreatment of DHS with GW9662 suppressed the mRNA levels of the adipogenic factor C/EBPα and the target genes of PPARγ, that is, aP2 and CD36. The transcript levels of PPARγ were reduced in cells treated with addition of GW9662 compared to when treated with DHS alone (Figure 3b). Furthermore, the immunoblotting results showed that the PPARγ protein levels were significantly reduced in cells cotreated with DHS and GW9662, whereas the C/EBPα levels showed a trend of reduced expression (Figure 3c). These results indicate that the adipogenic effect of DHS is partially dependent on PPARγ signaling.

Because DHS induced adipogenesis and upregulated PPARγ and its downstream adipogenic marker gene expressions, we determine its potential as a ligand for PPARγ using a nuclear receptor cofactor assay system in comparison to GW1929, a PPARγ agonist which was supplied with the assay kit as the reference positive control. The half-maximal effective concentration (EC50) values for DHS and GW1929 are approximately 30 μM and 9 nM, respectively (Figure 4a), whereas the reported EC50 value for troglitazone is 780 nM. 27 This indicates that DHS has poor affinity to PPARγ. We also carried out a PPARγ transactivation assay to evaluate the specificity and transactivicity of DHS by expressing a GAL4 DNA-binding domain/PPARγ ligand-binding domain (LBD) chimera protein using a pGAL4-PPARγ LBD plasmid and a luciferase reporter plasmid pUAS-tk-Luc containing the target sequence of GAL4 in HepG2 cells. In our luciferase reporter assay, DHS did not activate PPARγ in the range of 2–10 μM (Figure 4b). The transactivation assay was repeated using 3T3-L1 cells. Rosiglitazone and troglitazone showed PPARγ transactivation with EC50 values approximately 80 and 800 nM, respectively. The reported EC50 values for these full agonists using the mouse PPARγ receptor were 60–90 and 780 nM, respectively, 27 close to our results (Figure 4c). However, the DHS treatment showed no transactivation activity relative to the control (Figures 4c and S1). These results indicate that the adipogenic effect of DHS is distinct from that of full PPARγ agonists.

AMPK functions like biological fuel gauge, which is activated under conditions that deplete cellular adenosine triphosphate (ATP), and is phosphorylated on the catalytic α-subunit at Thr172 by the upstream kinase LKB1. It can also be stimulated by other stimuli that do not cause a detectable change in the AMP/ATP ratio, including hyperosmotic stress and drugs such as thiazolidinediones, metformin, and 5-aminoimidazole-4-carboxamide ribonucleotide. 28 AMPK is activated during the lipolysis process in adipocytes and has been reported to modulate the transcription of many genes involved in energy metabolism, including lipogenesis, triglyceride synthesis, and fatty acid oxidation. 29 Studies have reported that the lipid-reducing effect of natural products acts via AMPK activation. These phytochemicals include polyphenols such as resveratrol.
ginsenoside, and epigallocatechin gallate as well as plant alkaloids such as berberine and sanguinarine.\textsuperscript{18,30}

AMPK\(\alpha\) regulates preadipocyte differentiation by providing an upstream signal for PPAR\(\gamma\) that inhibits adipocyte differentiation\textsuperscript{19,20} We investigated the involvement of the AMPK pathway, which lies upstream of PPAR\(\gamma\) and C/EBP\(\alpha\), in the adipogenic effect of DHS. The immunoblotting results showed that DHS significantly reduced the AMPK\(\alpha\) levels at 5 \(\mu\)M but not at 10 \(\mu\)M (Figure 5a). The effect of 5 \(\mu\)M troglitazone on AMPK\(\alpha\) levels was also insignificant (Figure 5b). Although the mechanism of how certain plant natural products activate AMPK signaling while others suppress this signaling pathway is still unknown, our result implies that the DHS treatment stimulates adipocyte differentiation via upregulation of PPAR\(\gamma\) by inhibiting AMPK\(\alpha\)-mediated signaling.

Studies have found that PPAR\(\gamma\) is also regulated by posttranslational modification, resulting in insulin sensitization independent of transactivation. Phosphorylation of PPAR\(\gamma\) at Ser273 by cyclin-dependent kinase 5 (CDKS) is linked to obesity. Agonists and antagonists of PPAR\(\gamma\) have been shown to exert their insulin-sensitizing effects by blocking CDKS/ERK phosphorylation. PPAR\(\gamma\) agonists with antidiabetic effects such as thiazolidinediones were found to inhibit CDKS-mediated PPAR\(\gamma\) phosphorylation. As such, the ability of a ligand to suppress PPAR\(\gamma\) Ser273 phosphorylation was suggested for developing new classes of antidiabetic drugs.

Here, we investigated the effect of DHS on PPAR\(\gamma\) Ser273 phosphorylation in comparison to troglitazone and berberine at day 5. The immunoblotting results showed that DHS increased PPAR\(\gamma\) protein expression of the treated cells, but the overall PPAR\(\gamma\) phosphorylation was significantly decreased (Figure 6a). The reduction of the PPAR\(\gamma\) phosphorylation level by DHS was more apparent after 24 h treatment than at day 5, most probably because of the in vivo metabolism of DHS over time. At 5 \(\mu\)M, DHS significantly reduced AMPK\(\alpha\) and PPAR\(\gamma\) phosphorylation levels. Treatment with 10 \(\mu\)M DHS had no effect on AMPK\(\alpha\) and showed weaker reduction of PPAR\(\gamma\) phosphorylation than with 5 \(\mu\)M DHS (Figures 5b and 5a). These results suggest that DHS acts via PPAR\(\gamma\)- and AMPK\(\alpha\)-mediated signaling, and the effects are dose-dependent. The decrease of PPAR\(\gamma\) phosphorylation suggested the potential of DHS as an antidiabetic agent; therefore, we next performed a glucose uptake assay to evaluate this effect.

In the adipose tissue, PPAR\(\gamma\) induces the expression of genes involved in glucose uptake and controls adipocyte-secreted factors such as adiponectin to affect whole-body insulin sensitivity. Adiponectin has been shown to play an important role in mediating glucose uptake in adipocytes and muscle cells. Recent genome-wide scans have mapped a susceptibility locus for type-2 diabetes and metabolic syndrome to chromosome 3q27, where the gene-encoding adiponectin is located.\textsuperscript{33} Decreased adiponectin expression was correlated with insulin sensitivity.
resistance in mouse models of altered insulin sensitivity and in type-2 diabetes subjects.³⁴

In mammalian cells, facilitative diffusion of glucose across the plasma membrane is mediated by a family of glucose transporters.³⁵ GLUT4 is a glucose transporter isofrom, which is only expressed in peripheral tissues that are targets for insulin action, that is, the adipose tissue, cardiac muscle, and skeletal muscle. When stimulated by insulin, GLUT4 translocates from its intracellular compartment to the plasma membrane and, therefore, is responsible for insulin-stimulated glucose uptake.³⁶

To evaluate whether DHS enhances glucose uptake, we used an enzymatic method to measure 2-deoxyglucose (2DG) uptake in differentiated 3T3-L1 adipocytes. Our results (Figure 6c) showed a significant stimulatory effect of insulin-dependent glucose uptake by DHS in a dose-dependent manner (5 and 10 μM). The berberine treatment also significantly increased glucose uptake in the adipocytes, whereas the troglitazone treatment only showed a trend of increased glucose uptake. Berberine has been reported to increase glucose uptake possibly through a mechanism other than PPARγ activation, as this compound showed antagonistic activity. The expression and cellular localization of GLUT4 were not altered by berberine; instead, it was suggested to stimulate glucose uptake in 3T3-L1 adipocytes and preadipocytes by increasing GLUT1 activity.³⁷ These results indicate that the effects of DHS and berberine on glucose uptake act through distinct mechanisms. Future studies should elucidate the involvement of GLUT1 and GLUT4 plant alkaloid-mediated glucose uptake. It is worth noting that while both DHS and troglitazone treatment strongly upregulated Glut-4 gene expression, induction of the proadipogenic gene adiponectin by DHS was less pronounced (Figure 2). This suggests that DHS promotes glucose uptake but exhibits weaker adipogenicity compared to troglitazone, a thiazolidinedione for treating type-2 diabetes.

In drug development, findings from many studies proposed that the ligand occupancy time on the receptor would improve the potency of PPARγ modulators by creating an extended period of protection against kinase action, that is, longer residence times of a drug occupying a target result in improved duration of action times for that drug.³⁸

Here, we investigated the metabolic fate of DHS and sanguinarine in 3T3-L1 cells by measuring their accumulation in vivo and in the culture medium after 48 h using both alkaloids as standards. A total of 5.7 μmol/g protein of DHS and 0.1 μmol/g protein of sanguinarine were detected in the DHS-treated cell extract, whereas 2.7 μmol/g protein of DHS and 0.3 μmol/g protein of sanguinarine were detected in the sanguinarine-treated cell extract. In the cell culture medium, 0.1 μM each of DHS and sanguinarine were detected in the DHS-treated sample, whereas 0.1 μM DHS and 0.3 μM sanguinarine were detected in the sanguinarine-treated sample. This indicates high accumulation of DHS in both the cell extract samples, whereas sanguinarine, the oxidized form, was prevalent in the culture medium (Figure 7a). This result correlates with those reported in vivo pharmacokinetic studies that the main sanguinarine metabolism pathway is the reduction of its iminium bond to form DHS.³⁹ It also indicates that DHS has higher accumulation in vivo than sanguinarine and may yield high bioavailability. The cells treated with 10 μM DHS showed an insignificant reduction of the AMPKα1 level and weaker effects on the reduction of the PPARγ phosphorylation level and on adipogenesis compared to 5 μM DHS. Toxicity was also observed with the 10 μM treatment on the 3T3-L1 cells. These findings led us to infer that with 10 μM DHS, the cells accumulated a higher amount of sanguinarine than the cells with the 5 μM treatment. As described in our previous work,¹⁸ sanguinarine exerts an antiadipogenic effect, activates AMPK, and thus elicits an opposing effect to DHS.

The observed different effects of sanguinarine and DHS on the adipogenesis of 3T3-L1 cells suggest that the metabolic pathway of a bioactive compound dictates the ultimate biological effects in vivo. In our previous study, the sanguinarine treatment activated the AMPK pathway, which led to the inhibition of lipid synthesis in C. elegans.⁵⁷ Although the mechanism of how sanguinarine activated AMPK signaling is still not fully elucidated, other studies suggested that this activity was due to the inhibition of mitochondrial respiratory complex I, which resulted in an increase of the AMP/ATP ratio. Sanguinarine has also been reported to activate the mitochondrial apoptosis pathway.³⁹ We found that the sanguinarine treatment exhibited toxicity in C. elegans.¹⁸ Evaluation of 3T3-L1 cell viability showed that the cytotoxicity of DHS was insignificant at 5 μM (Figure 7b). This suggests that the cytotoxicity of a compound could contribute to lipid metabolism because of its effect on AMPK pathway activation. Thus, it would be interesting to investigate the effect of bioactive compounds and their metabolites on the multiple signaling pathways involved in adipogenesis and

**Figure 7.** (a) Accumulation of DHS and sanguinarine in cells and in the cell culture medium after 48 h treatment with the respective compound. n = 3 from two independent experiments; error bar = SD. (b) 3T3-L1 adipocyte viability after 24 h treatment with DHS at various concentrations. n = 5 from three independent experiments; error bar = SD. *p < 0.05, **p < 0.005 vs control; two-tailed Student’s t-test.
To a fine in vivo toxicity of sanguinarine was reported to be lower than DHS. For instance, in the human leukemia HL-60 cell line, the half-maximal inhibitory concentration, IC50 values of sanguinarine and DHS were reported as 0.72 and 20 μM, respectively.41 The in vivo toxicity of sanguinarine was reported to be low. We found that sanguinarine was more cytotoxic than DHS in cancer cells, whereas it was found that sanguinarine was more cytotoxic than DHS. For instance, in the human leukemia HL-60 cell line, the half-maximal inhibitory concentration, IC50 values of sanguinarine and DHS were reported as 0.72 and 20 μM, respectively.41 The in vivo toxicity of sanguinarine was reported to be low. We found that sanguinarine was more cytotoxic than DHS in cancer cells, whereas it was found that sanguinarine was more cytotoxic than DHS.

3. CONCLUSIONS This is the first report that evaluated the effect of DHS, a reduced form of sanguinarine, on adipogenesis in 3T3-L1 cells. DHS exhibited a weaker adipogenic effect than the full PPARγ agonist, troglitazone. Our results suggest that DHS induces adipocyte differentiation by upregulating PPARγ and suppressing AMPK signaling pathways. DHS treatment reduced PPARγ Ser273 phosphorylation and upregulated the expressions of Glut-4 and adiponectin genes—activities that are associated with improved insulin sensitivity. We found that 5 μM DHS significantly enhanced glucose uptake in 3T3-L1 adipocytes without exerting cytotoxicity. Further experiments using animal models are needed to evaluate the efficacy and safety of DHS for medical application. Although the interaction of DHS with PPARγ is not fully elucidated, our results show that DHS bound weakly with PPARγ and did not transactivate it. Crystallographic structures of the PPARγ ligand binding revealed two binding modes exist in the same LBD that correspond to potent and weak agonists and affect their transactivation activities. A weak agonist could have low transactivation activity but high phosphorylation inhibition activity on PPARγ Ser273, similar to the characteristics of DHS discovered in this study.44–46 By examining the metabolic fates of DHS and its oxidized form, sanguinarine, in 3T3-L1 cells, we discovered the significance of considering the metabolites of a compound during bioactivity screening. This is because the metabolic pathway of a bioactive compound ultimately dictates the biological effect in vivo. Our findings in this study suggest that BAs could be potential lead compounds for modulators of glucose metabolism, and additional screening of these alkaloids should be carried out in the future.

4. EXPERIMENTAL SECTION

4.1. Chemicals. Berberine sulfate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); troglitazone and GW9662 from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and sanguinarine chloride and rosiglitazone from Sigma-Aldrich (St. Louis, MO, U.S.A.). DHS was prepared by reducing sanguinarine with NaBH₄ reduction.47 The purity was analyzed by LC–MS (see below and Figure S2). Alkaloid samples are diluted in DMSO (Wako) to a final concentration of 0.1% DMSO in cell treatments. All other chemicals were purchased from Wako, unless otherwise stated.

4.2. Purity Check. The purities of dihydrosanguinarine and sanguinarine were analyzed by an LCMS2010 system (Shimadzu, Japan) using a TSK-gel ODS-80Tm 4.6 × 250 mm column. The samples were analyzed under the following conditions: a column temperature of 40 °C; a flow rate of 0.5 mL/min; and a gradient schedule of 0–15 min of AcCN/H₂O = 45:55, 18.5–24.5 min of AcCN/H₂O = 80:20, and 28–33 min of AcCN/H₂O = 45:55 (containing 1% trifluoroacetic acid) in positive SIM-SCAN mode ranging from m/z 100–500. The purities were calculated based on % peak area for each sample.

4.3. Cell Culture. 3T3-L1 cells (a generous gift from Dr. M. Nagao, Kyoto University) were cultured at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM; Wako) with 10% fetal bovine serum (FBS; Corning). Cells were passaged twice before used in assays to allow cells to re-establish the normal cell cycle. Cell differentiation was induced at 2 day postconfluence (designated as day 0) by addition of the 3-isobutyl-1-methylxanthine (IBMX) mix which consists of 5 μg/mL insulin (Sigma), 500 μM isobutylmethylxanthine (Sigma), and 0.25 μM dexamethasone for 2 days.48 The culture medium was replaced every 2 days thereafter with DMEM containing 10% FBS and 5 μg/mL insulin.

4.4. Oil Red O Staining. 3T3-L1 adipocytes (day 8 or day 12) in the wells of cell culture plates were rinsed with phosphate-buffered saline (PBS) and then fixed in formalin for 30 min at room temperature. The formalin was removed, and the cells were rinsed twice with PBS. A 0.3 w/v % Oil Red O (Sigma) solution was added at room temperature to stain the cells. After 1 h, cells were rinsed with PBS twice, and lipid droplet accumulation was observed under a microscope.

4.5. Triglyceride and Protein Measurement. 3T3-L1 adipocytes (day 12) in the wells of cell culture plates were rinsed twice with PBS. Cell lysis buffer (1 M Tris-HCl pH7.5, 1 M MgCl₂, 10% Triton X100) was added to each well, and the cells were harvested using a cell scraper into Eppendorf tubes. The cells were disrupted using an ultrasonicator. Triglyceride and protein contents of the cell lysates were measured. The triglyceride concentration was determined using triglyceride E test kit (Wako) and the absorbance at 595 nm was measured using a PowerScan4 plate reader (DS Pharma Biomedical, Japan). Protein concentrations were determined using Bio-Rad DC protein assay (Bio-Rad Laboratories, U.S.A.) reagents and the absorbance at 595 nm was measured using the PowerScan4 plate reader. The triglyceride content of each cell sample was normalized to its corresponding protein content.

4.6. Quantitative RT-PCR. Two day postconfluent 3T3-L1 cells (day 0) were cultured in the differentiation medium IBMX without or with the addition of alkaloids for 2 days, followed by medium change to DMEM/10% FBS/5 μg/mL insulin without or with the addition of alkaloids. The cells were harvested on day 5 following the method for protein as described above. RNAs were extracted using RNeasy Mini Kit (QIAGEN). Reverse transcription was done using 2 μg of total RNA, and RT-PCR was carried out using a CFX96 RT-PCR System (Bio-Rad Laboratories, Inc., Foster City, CA, U.S.A.). The conditions for PCR reactions were 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. Melting curve analysis was performed after each run at 72–95 °C to check the specificity of amplification. Data analysis was done using the ΔΔCT method, and a relative transcript amount was standardized using the TATA box-binding protein...
as the internal control. Fold changes between samples were normalized to control (0.1% DMSO).

4.7. Immunoblot Analysis. 3T3-L1 cells were rinsed twice with PBS. Cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X100, 0.1% sodium dodecyl sulfate (SDS), 10 mM NaF, 1 mM Na3VO4, 50 mM Na2P2O7, and 1% protease inhibitor cocktail) was added to each well, and the cells were harvested using a cell scrapper into Eppendorf tubes. The cells were disrupted using the ultrasonicator. Protein contents of cell lysate samples were measured as mentioned above and adjusted to the same amount using a 2× sample buffer (0.1 M Tris-HCl pH 6.8, 2% SDS, 12% β-mercaptoethanol, 20% glycerol, and 0.2% bromophenol blue). The protein (20 μg) was loaded and separated by SDS-polyacrylamide gel electrophoresis. The proteins were electro-transferred onto a polyvinylidene difluoride membrane (Millipore Immobilon-P). The membranes were blocked for 1 h at room temperature in 5% bovine serum albumin (BSA)/Tris buffered saline with Tween-20 (TBST) and then probed with primary antibody (in 5% BSA/TBST) overnight at 4°C, followed by horseradish peroxidase (HRP)-conjugated secondary donkey antirabbit IgG (in 5% BSA/TBST). Target-specific antibodies were obtained from the following manufacturers: AMPKα, C/EBPα, PPARγ, and β-actin from Cell Signaling Technology (Beverly, MA, U.S.A.); phosphospecific PPARγ (Ser273) from Funakoshi Co., Ltd. (Japan); and HRP-conjugated donkey antirabbit IgG from GE Healthcare (Buckinghamshire, U.K.). Chemiluminescence was detected using a ChemiDoc Touch imaging system (Bio-Rad Laboratories, Inc., U.S.A.). The blots were then stripped and reprobed with other primary antibodies and subjected to chemiluminescence detection. The target protein band intensity was quantified using ImageJ software (NIH). The average values were normalized to control.

4.8. PPARγ Binding Assay. The ability of DHS to bind PPARγ was determined using the EnBio receptor cofactor assay system for PPARγ Kit (EnBio Tec. Laboratories Co., Ltd., Tokyo, Japan) following the manufacturer’s protocol. This system employs a cell-free assay using nuclear receptors and cofactors to screen for chemicals. Changes in the absorbance at 450 nm for reactions treated with DHS, and the PPARγ agonist GW1929 (positive control) were measured using the PowerScan 4 plate reader. The EC50 values were determined from the concentration that elicited a half-maximal response concentration on the plotted graphs.

4.9. PPARγ Reporter Assay. The assay was performed as previously described.39 A DNA fragment encoding the LBD (residues 204–505) of mouse PPARγ2 (GenBank U09138) with BamHI and Sall sites at the ends was amplified by PCR using genomic DNA from mouse 3T3-L1 cells as a template and inserted into the pSUG42 plasmid at the BamHI and Sall sites to construct pGal4-PPARγ LBD. pGal4-PPARγ LBD and pUAS-tk-luc were cotransfected into HepG2 or 3T3-L1 cells in separate experiments using HilyMax (Dojindo). The PPARγ agonist activity was determined via luciferase activity using a Luciferase Assay System according to the manufacturer’s protocol (Promega). The β-galactosidase activity was determined using chloronaphenol red-β-D-galactopyranoside (Roche). Relative luciferase activities were normalized to the β-galactosidase activity.

4.10. Glucose Uptake Assay. The assay was performed according to the reported method with modifications. The 3T3-L1 cells were differentiated in six-well culture plates. Test compounds were added to differentiated adipocytes on day 8. Insulin stimulation and glucose uptake were conducted on day 12. Cells were lysed with 10 mM Tris-HCl buffer (pH 8.0) and disrupted using the ultrasonicator. Glucose uptake was determined by 2-deoxyglucose uptake with an enzymatic photometric assay using 2-deoxyglucose uptake measurement kit (Cosmo Bio Co., Ltd., Japan). Measurements were done using the PowerScan4 plate reader at an absorbance of 420 nm in a kinetic program with a read every 1 min for 30 min. Glucose uptake values were then normalized to the protein value of the cell lysates as determined by the Bio-Rad DC Protein Assay method described above. The insulin-induced glucose uptake was calculated by subtracting the values from the negative control (glucose uptake inhibitor added).

4.11. Alkaloid Accumulation Analysis. 3T3-L1 adipocytes (day 4) were treated without or with alkaloids for 48 h and collected as in protein samples. Cell lysis samples and cell culture media were extracted with methanol using Sep Pak C18 cartridges (Millipore). Methanol extracts were concentrated using a rotary evaporator. The samples were analyzed using an LCMS2020 system (Shimadzu) according to the previously reported method.25 Alkaloid concentrations were calculated based on the LC peak area relative to the standard peak area, and those values are normalized to the protein content in each sample.

4.12. Cytotoxicity Assay. The 3T3-L1 cells were cultured at a density of 1.5 × 105 cells/well in a 96-well plate for 16 h. The culture medium was then replaced with a fresh medium with the addition of alkaloids and further cultured for 24 h. Cell viability was determined using Cell Counting Kit-8 (Dojindo). Cells were incubated with the reagent for 2 h and live cells were measured using the PowerScan4 plate reader at an absorbance of 450 nm.

■ ASSOCIATED CONTENT

5 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01134.

Reporter assay showing the transactivation activity of the PPARγ-derived reporter gene in 3T3-L1 cells after treatment with DHS (Figure S1); LC chromatogram showing the UV absorbance at 280 nm; and the mass spectra of dihydrosanguinarine and sanguinarine (Figure S2) (PDF)

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Author Contributions
Y.-L.C. and F.S. designed the study. Y.-L.C. and Y.I. performed the experiments. Y.-L.C., Y.I., and F.S. analyzed the data. Y.-L.C. and F.S. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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