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# Boiogito Increases the Synthesis and Secretion of Adiponectin by Promoting Differentiation in Cultured Human Adipocytes

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## Abstract

Boiogito (BOT) ameliorates insulin resistance and diabetes in several animal models; however, the underlying mechanisms for these *in vivo* effects remain unclear. Thiazolidine derivatives, which are peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) agonists for the treatment of type II diabetes, promote adiponectin production by inducing adipocyte differentiation, thereby reducing insulin resistance. This study aimed to evaluate the effect of BOT on adipocyte differentiation using cultured human visceral preadipocytes (HVPAds) compared with the thiazolidine derivative troglitazone (TRG). We investigated the effects of BOT (0.125 - 1 mg/mL) and TRG (10 μM) on the differentiation of adipocytes treated with or without tumor necrosis factor-$\alpha$ (TNF-$\alpha$: 5 ng/mL). On day 14 of culture, the following adipocyte differentiation marker levels were measured: intracellular lipids, extracellular (*i.e.*, medium) adiponectin, and intracellular differentiation-related genes (PPAR$\gamma$, CCAAT/enhancer binding protein, adiponectin, differentiation cluster 36, glucose transporter type 4). BOT and TRG increased factors associated with differentiation including lipid, adiponectin, and differentiation-related gene expression levels compared with the controls. The increases in these differentiation markers were inhibited by the PPAR$\gamma$ antagonist GW9662 (20 μM). Furthermore, TNF-$\alpha$ decreased all differentiation marker levels. The decreases in differentiation markers were inhibited by BOT and TRG; however, these inhibitory effects were blocked by GW9662. The results suggest that BOT increases the synthesis and secretion of adiponectin by promoting differentiation similar to TRG. This study is the first to demonstrate that adipocyte differentiation-promoting activity is a mechanism for the beneficial effects of BOT on diabetes and insulin resistance.

**Keywords**

Adipocyte, Adiponectin, Boiogito, Differentiation, Troglitazone

## 1. Introduction

Obesity is a condition where excess fat accumulates in adipose tissue and is considered to be a chronic low-grade inflammatory state [1]. In particular, visceral fat accumulation contributes to the development of obesity-related diseases, especially metabolic dysfunction [2]. The accumulation of excess fat in patients with obesity causes abnormal secretion of physiologically active substances (adipocytokines) derived from adipose tissue, leading to insulin resistance and diabetes [3] [4]. Histopathologically, most adipocytes in the adipose tissue are hypertrophic, and infiltration of numerous macrophages that produce and secrete tumor necrosis factor-$\alpha$ (TNF-$\alpha$) is observed [4] [5]. TNF-$\alpha$ acts on hypertrophic adipocytes and promotes the production and secretion of the proinflammatory cytokine interleukin-6 (IL-6) and the chemokine monocyte chemoattractant protein-1 (MCP-1) by activating the intracellular nuclear factor $\kappa$B (NF-$\kappa$B) pathway [6] [7]. Macrophage migration and invasion into the adipose tissue through MCP-1 promotes TNF-$\alpha$ secretion from the adipose tissue and aggravates adipose tissue inflammation [6]. TNF-$\alpha$ also suppresses the production of the adipocyte-specific adipocytokine adiponectin, which has an antagonistic effect on TNF-$\alpha$ by inhibiting adipocyte differentiation [8]. Adipocytokine imbalance caused by excess TNF-$\alpha$ and insufficient adiponectin in the adipose tissue inhibits cellular glucose uptake by inhibiting insulin signaling in insulin-sensitive tissues, such as the adipose tissue and skeletal muscle, causing insulin resistance [9] [10].

Adiponectin is a specific protein produced in small mature adipocytes that differentiate from preadipocytes in normal adipose tissue [3] [4]. However, in obese adipose tissue filled with hypertrophic adipocytes, adiponectin production and secretion are reduced because of the inhibition of adipocyte differentiation by TNF-$\alpha$ [7] [8]. The disruption of adiponectin production contributes to the development of chronic inflammation, resulting in insulin resistance, thereby causing diabetes [11]. Conversely, promoting adiponectin production improves insulin resistance and diabetes [3] [11]. Adiponectin production is positively regulated by peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$). The transcription of PPAR$\gamma$ is promoted through the PPAR$\gamma$ response element (PPRE) present on the adiponectin promoter, and its upregulation occurs through the binding of PPAR$\gamma$ agonists [12]. Therefore, PPAR$\gamma$ agonists may improve diabetes by activating PPRE and enhancing the production of adiponectin. Thiazolidine derivatives, PPAR$\gamma$ agonists for the treatment of type II diabetes, induce apoptosis of hypertrophic adipocytes and differentiation of preadipocytes into small mature adipocytes through PPAR$\gamma$ activity, thereby decreasing hyper-

trophic adipocytes and increasing small mature adipocytes in the adipose tissue. Consequently, adiponectin production is increased, thereby improving insulin resistance [13] [14] [15] [16]. In Japan, some traditional Kampo medicines are commonly used alongside Western medicines such as thiazolidine derivatives to treat diabetes and obesity [17].

Boiogito (BOT), which is composed of six crude drugs (**Table 1**), is a representative Kampo medicine approved by the Ministry of Health, Labor, and Welfare as a treatment for obesity and insulin resistance [17]. BOT was shown to exert hypoglycemic effects in streptozotocin-induced diabetic mice [18], antiobesity effects in ovariectomized rats [19], and ameliorate the effects of metabolic syndromes, such as obesity, hyperinsulinemia, fasting-induced hyperglycemia, and dyslipidemia in spontaneous obesity type II diabetes mice [20]. However, the molecular mechanisms and roles of adipocytes underlying the antiobesity and anti-insulin resistance effects of BOT remain unelucidated. As mentioned above, BOT, similar to thiazolidine derivatives of PPAR$\gamma$ agonists, effectively alleviates diabetes and insulin resistance [13] [18] [20]. Therefore, we hypothesize that the effects of BOT may involve a molecular mechanism related to adiponectin production by inducing adipocyte differentiation, similar to that of thiazolidine derivatives.

This study aimed to clarify whether adiponectin production induced by adipocyte differentiation is involved in the anti-insulin resistance and antidiabetic effects of BOT. Therefore, we investigated the effects of BOT on the differentiation and adiponectin production of cultured human visceral preadipocytes (HVPAds) treated in the presence or absence of TNF-$\alpha$, with the thiazolidine derivative troglitazone (TRG) as a positive control.

## 2. Materials and Methods

### 2.1. Test Substances and Agents

BOT extract (lot number 2100020010) was obtained from Tsumura & Co. (Tokyo, Japan) and consisted of a mixture of the following six crude drugs (**Table 1**): Astragalus Root (Ogi: *Astragalus membranaceus* Bunge), Sinomenium Stem (Boi: *Sinomenium acutum* Rehder et Wilson), Atractylodes Lancea Rhizome (Sojutsu: *Atractylodes lancea* De Candolle), Jujube (Taiso: *Ziziphus jujube* Miller var. *inermis* Rehder), Glycyrrhiza (Kanzo: *Glycyrrhiza uralensis* Fisher), and Ginger (Shokyo: *Zingiber officinale* Roscoe), with a weight ratio (g) of 5:5:3:3:1.5:1.

**Figure 1** shows a three-dimensional high-performance liquid chromatography (3D-HPLC) chromatogram with ultraviolet (UV) detection of the BOT extract provided by Tsumura Co. At least 22 ingredients were identified.

TRG and GW9662 were purchased from Cyman Chemical (Ann Arbor, MI, USA). TNF-$\alpha$ was purchased from Fujifilm Wako Pure Chemical Corporation.

The BOT, TRG, GW9662, and TNF-$\alpha$ concentrations used in each experiment were prepared by dissolving them in differentiation medium.

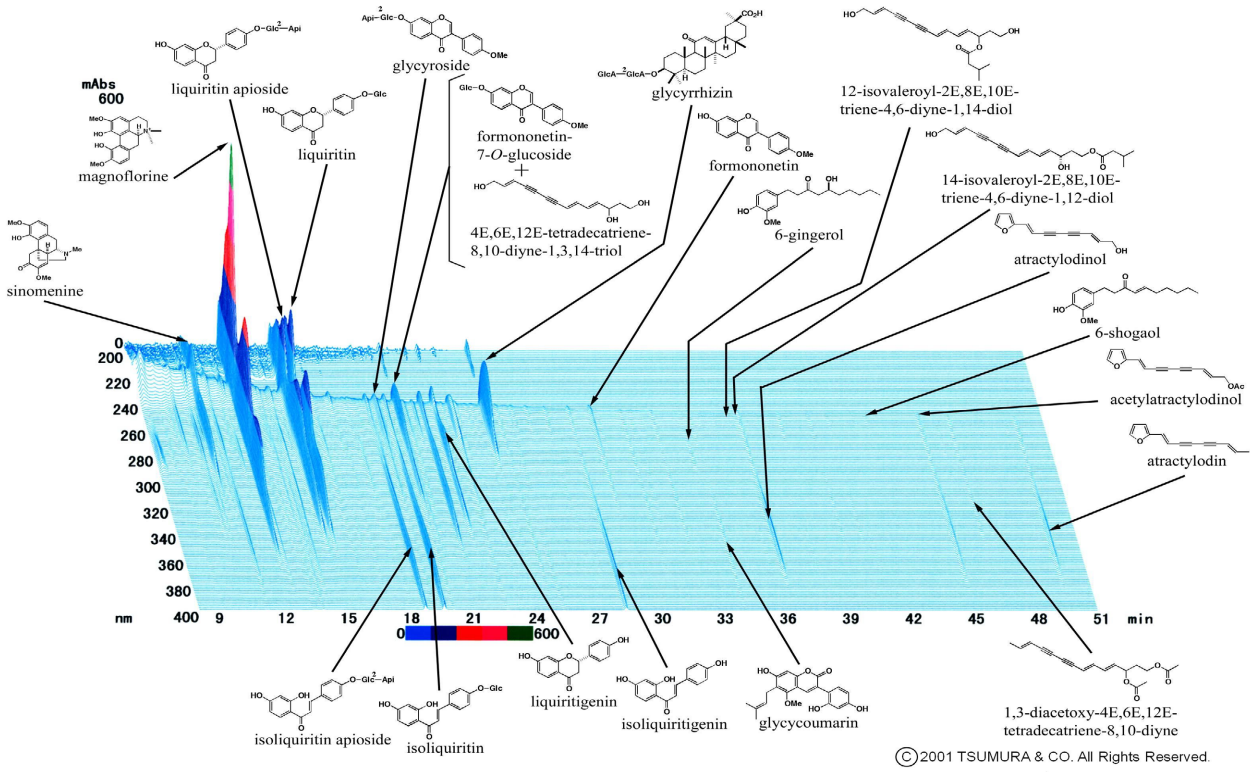


**Figure 1.** Three-dimensional high-performance liquid chromatogram with ultraviolet detection of the BOT extract.

**Table 1.** Composition of BOT.

| Japanese name | English name (Scientific name) | Composition ratio |
|---|---|---|
| Ogi | Astragalus Root (*Astragalus membranaceus* Bunge) | 5.0 g (27.0%) |
| Boi | Sinomenium Stem (*Sinomenium acutum* Rehder et Wilson) | 5.0 g (27.0%) |
| Sojutsu | Atractylodes Lancea Rhizome (*Atractylodes lancea* De Candolle) | 3.0 g (16.2%) |
| Taiso | Jujube (*Ziziphus jujuba* Miller var. *inermis* Rehder) | 3.0 g (16.2%) |
| Kanzo | Glycyrrhiza (*Glycyrrhiza uralensis* Fisher) | 1.5 g (8.1%) |
| Shokyo | Ginger (*Zingiber officinale* Roscoe) | 1.0 g (5.4%) |

Oil Red O and 2-propanol were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA). The 0.36% Oil Red O solution used in the intracellular lipid assay was prepared by dissolving it in 60% 2-propanol adjusted with distilled water.

## 2.2. Preadipocytes and Media

Human visceral preadipocytes (HVPAds) derived from human pericardiac adipose tissue, preadipocyte growth medium (Cat No. 811K-500), and adipocyte differentiation medium (Cat No. 811D-250) were purchased from Cell Applications, Inc. (San Diego, CA, USA). The growth medium contained 10% fetal bovine serum (FBS), 100 U/mL penicillin, 250 ng/mL amphotericin B, 10 μg/mL

insulin, and 100 μg/mL streptomycin. The differentiation medium contained the components of growth medium plus 0.5 μM dexamethasone and 0.25 mM isobutylmethylxanthine. Culture flasks (75 $cm^2$) and collagen-coated 12/48-well microplates were purchased from Corning (Corning, NY, USA) and AGC Techno Glass Co., Ltd. (Shizuoka, Japan).

## 2.3. Experimental Design

### 2.3.1. Cell Culture for Preadipocytes Proliferation

HVPAds ($1 \times 10^6$ cells) were seeded into 75 $cm^2$ culture flasks with the growth medium (15 mL) and cultured at 37˚C and 5% $CO_2$. After the cells reached full confluency, they were treated with 0.25% trypsin (Gibco, Grand Island, NY, USA) and passaged. Second and third-passage cells were used for the subsequent experiments.

### 2.3.2. Validation of the Noncytotoxic Concentration of BOT during Differentiation

HVPAds ($4 \times 10^4$ cells/well) were seeded into 48-well microplates containing the growth medium (500 μL/well), and cultured at 37˚C and 5% $CO_2$ for 2 - 3 days until 100% confluent. Differentiation was initiated by replacing the growth medium with differentiation medium without (control) and with BOT (final concentrations: 0.125 - 1 mg/mL). Cultures were performed at 37˚C and 5% $CO_2$ for 14 days. The medium was replaced every 3 days. The effect of BOT on differentiated cells was determined by measuring cell viability using the Cell Counting Kit-8 (CCK-8) assay on Day 14.

### 2.3.3. Validation of the Adipocyte Differentiation Process

Confluent HVPAds ($4 \times 10^4$ cells/well) prepared following the procedure described in the previous subsection 2.3.2 were cultured in differentiation medium (500 μL/well) for 21 days in 48-well plates. On days 0, 7, 14, and 21, the state of the differentiation was examined by observing the morphology of adipocytes stained with Oil Red O. Intracellular lipid concentrations were measured using the Oil Red O assay. Extracellular (*i.e.*, medium) adiponectin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA).

### 2.3.4. Effects of BOT on Adipocyte Differentiation Markers

**1) Adipocyte lipid and medium adiponectin concentrations**

Confluent HVPAds ($4 \times 10^4$ cells/well) prepared following the procedure described in previous subsection 2.3.2 were cultured for 14 days in differentiation medium (500 μL/well) containing BOT (final concentration: 0 as control, 0.125, 0.25, 0.5, and 1.0 mg/mL) in 48-well plates. On day 14 of culture, the intracellular lipid and extracellular adiponectin concentrations were measured.

**2) Comparison of BOT and TRG on adipocyte differentiation**

Confluent HVPAds ($4 \times 10^4$ cells/well) prepared following the procedure described in previous subsection 2.3.2 were cultured for 14 days in differentiation media (500 μL/well) containing BOT, TRG, BOT + GW9662, and TRG +

GW9662 in 48 well plates. The final concentrations of test substances in the media were 0.25 and 0.5 mg/mL for BOT, 10 nM for TRG, and 20 nM for GW9662. The 10 nM TRG and 20 nM GW9662 concentrations were previously effective for promoting and antagonizing differentiation, respectively, in cultured adipocyte experiments [21]. Intracellular lipid and extracellular adiponectin concentrations were determined on day 14.

**3) Comparison of BOT and TRG on the expression of differentiation-related genes**

Confluent HVPAds ($1.8 \times 10^5$ cells/well) prepared following the procedure described in previous subsection 2.3.2 were cultured for 14 days in differentiation media (2 mL/well) without (control) and with BOT (final concentration: 0.25 and 0.5 mg/mL) or TRG (final concentration: 10 nM) in a 12-well plate. The mRNA expression levels of PPAR$\gamma$, CCAAT/enhancer-binding protein $\alpha$ (C/EBP$\alpha$), adiponectin, cluster of differentiation 36 (CD36), and glucose transporter type 4 (GLUT4) were measured on day 14 of culture. Gene expression levels were determined using real-time polymerase chain reaction (PCR).

#### 2.3.5. Effects of BOT and TRG on TNF-$\alpha$-Induced Suppression of Adipocyte Differentiation

Confluent HVPAds ($4 \times 10^4$ cells/well) prepared following the procedure described in previous subsection 2.3.2 were cultured for 14 days in differentiation media (500 µL/well) without (control) and with TNF-$\alpha$ (final concentration: 5 ng/mL) or TNF-$\alpha$ (final concentration: 5 ng/mL) + BOT (final concentrations: 0.125 - 1 mg/mL) in 48-well plates. The 5 ng/mL TNF-$\alpha$ concentration used in the experiment was consistent with a previously study that examined inflammation in cultured adipocytes [22]. On day 14 of culture, intracellular lipid and extracellular adiponectin concentrations were measured.

To further compare the effects of BOT and TRG on cell differentiation, HVPAds were cultured in differentiation media containing TNF-$\alpha$, TNF-$\alpha$ + BOT, TNF-$\alpha$ + TRG, TNF-$\alpha$ + BOT + GW9662, and TNF-$\alpha$ + TRG + GW9662, following the same procedure described above. Lipid and adiponectin concentrations were measured on day 14. The final concentrations of test substances in the media were 5 ng/mL for TNF-$\alpha$, 0.25 and 0.5 µg/mL for BOT, 10 nM for TRG, and 20 nM for GW9662. The expression of differentiation-related genes was measured on day 14 in a separate set of experiments using 12-well plates.

### 2.4. Assay Methods

#### 2.4.1. Cell Viability Assay

Cell viability was determined according to the manufacturer's instruction for the CCK-8 assay kit (Dojindo Molecular Technologies, Gaithersburg, MD, USA). CCK-8 solution (15 µL) was added to wells containing cells cultured for 14 days in a medium without (control) or with BOT. The cells were incubated for 2 h at 37˚C and 5% $CO_2$. The absorbance (450 nm) was measured using a plate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell viability of BOT-treated cells was calculated as a percentage of control.

#### 2.4.2. Intracellular Lipid Assay

After removing the medium in the wells, the adipocytes were rinsed twice with phosphate-buffered saline (500 μL/well). A 0.36% Oil Red O solution (200 μL) was added to the wells and incubated for 15 min at room temperature to stain intracellular lipids. After washing the cells three times with distilled water, the dye was extracted with 250 μL of 2-propanol. The absorbance of the extract was measured at 540 nm.

#### 2.4.3. Extracellular Adiponectin Assay

The AssayMaxTM human adiponectin ELISA kit (Assaypro, LLC, St. Louis, MO, USA) was used to determine adiponectin concentration in the medium as previously reported [22].

#### 2.4.4. Adipocyte Differentiation-Related Gene Assay

Real-time PCR analysis was performed with some modifications of a previously reported method [22]. FastGeneTM RNA Basic Kit (Nippon Genetics Co., Ltd., Tokyo, Japan) and ReverTra AceR qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan) were used for isolation of total adipocyte RNA and reverse transcription to cDNA, respectively. The gene expression was quantified using an Applied BiosystemsR 7500 fast real-time PCR system (Thermo Fisher Scientific K.K., MA, USA). PCR reaction conditions were set as described in our previous report [22]. The base sequences of PPAR$\gamma$, adiponectin, CD36 and GLUT4 were described in a previous report [22]. $\beta$-Actin was used as an internal control gene. The base sequences of the forward (F) and reverse (R) primers for $\beta$-actin were as follows: (F) 5'-CACCAGGGTGTGATGGTGGGAAT-3' and (R) 5'-GGTCTTTACGGATGTCAACGT CACA-3'. The mRNA expression levels of target genes were expressed as fold change of the control levels.

### 2.5. Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). The statistical significance of differences among groups was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey's multiple test. The significance level was accepted at $P < 0.05$.

## 3. Results and Discussion

Prior to evaluating the effects of BOT on adipocyte differentiation, BOT (0.125 - 1 mg/mL) was administered to the cells over the 14-day differentiation period to establish noncytotoxic concentrations (**Figure 2**). Cell viability in this concentration range was >93% and not significantly different compared with the controls. This suggests that BOT concentrations in this range are largely non-toxic to differentiating adipocytes. Therefore, we used noncytotoxic BOT concentrations (0.125 - 1 mg/mL) in subsequent experiments.

Adipocyte differentiation is characterized by the production of intracellular lipids and adiponectin [23]. We first measured changes in the lipid concentration in Oil Red O-stained adipocytes and adiponectin concentration in the me-

dium as differentiation markers to verify the differentiation process (Figure 3). The images show that intracellular lipid droplets increased concomitantly with increased days of culture. The lipid concentration increased up to day 14 and the levels almost reached a plateau on day 21. The medium adiponectin concentration (*i.e.*, the secretion level of cell-to-medium) also increased with increasing days of culture.

Significant increases in both parameters were observed on days 7, 14, and 21 ($P < 0.001$) compared with day 0. Our results are in good agreement a previous report [22], indicating that the experimental conditions for adipocyte differentiation used to evaluate the test substances were appropriate.

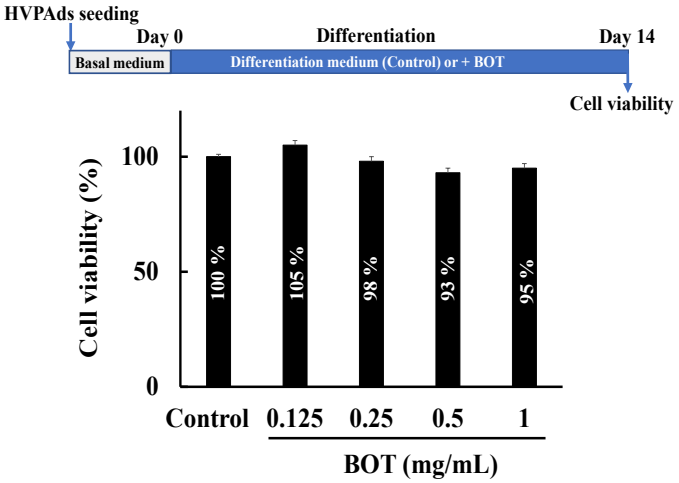


Figure 2. The viability of adipocytes treated with BOT during differentiation. Each data represents the mean ± SEM (n = 14). No significant difference was detected between the control and BOT groups (one-way ANOVA + Dunnett's test).

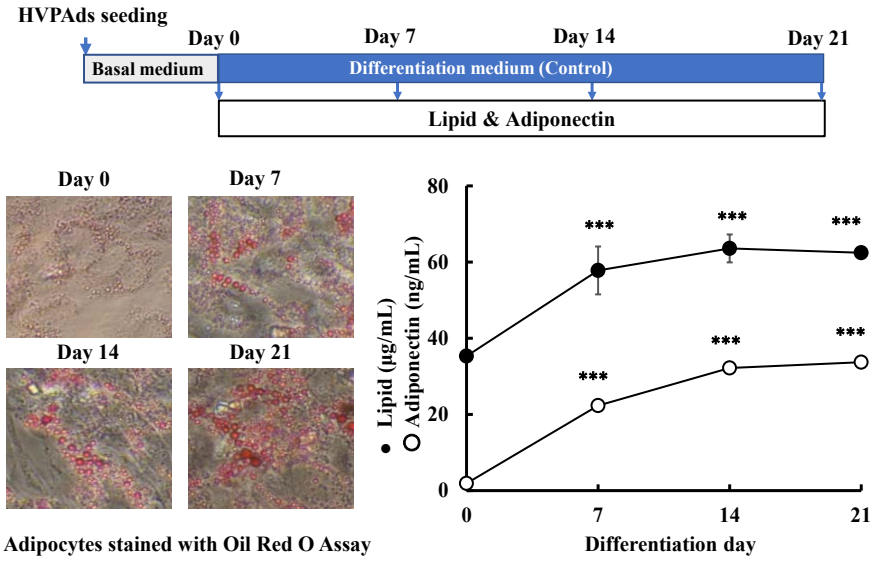


Figure 3. Changes in adipocyte lipid and medium adiponectin concentrations during differentiation. The images show typical adipocytes stained with Oil Red O on days 0, 7, 14, and 21. Each data represents the mean ± SEM (n = 6). ***$P < 0.001$ vs. day 0 (one-way ANOVA + Dunnett's test).

The thiazolidine derivatives, which are used to treat type II diabetes, promote adipocyte differentiation through PPAR-$\gamma$ agonist activity, thereby promoting adiponectin production and improving insulin resistance [13]. We hypothesized that the underlying mechanisms of BOT, which is effective at ameliorating obesity, diabetes, and insulin resistance, may be associated with enhanced adiponectin production by inducing adipocyte differentiation, similar to the thiazolidine derivative TRG.

The effects of BOT on adipocyte differentiation were examined by culturing HVPAds in differentiation medium without (control) and with BOT for 14 days (**Figure 4**). BOT (0.125 - 1 mg/mL, $P < 0.05$ - 0.001) significantly increased lipid concentration in a dose-dependent manner (**Figure 4(A)**). Furthermore, the effects of BOT and TRG on adipocyte lipid concentrations were compared (**Figure 4(B)**). BOT (0.25 and 0.5 mg/mL, $P < 0.001$) and TRG (10 μM, $P < 0.001$) significantly increased lipid concentration and both were significantly suppressed by the PPAR$\gamma$ antagonist GW9662 ($P < 0.001$). **Figure 4(C)** shows the medium adiponectin concentrations. BOT (0.125 - 1 mg/mL) increased adiponectin levels in a dose-dependent manner compared with the controls. A significant increase was observed at concentrations of 0.5 ($P < 0.05$) and 1 mg/mL ($P < 0.05$). When the effects of BOT and TRG on adiponectin concentrations were compared (**Figure 4(D)**), BOT (0.25 and 0.5 mg/mL, $P < 0.05$ - 0.001) and TRG (10 μM, $P < 0.001$) significantly increased adiponectin concentration and these levels were significantly suppressed by GW9662 exposure ($P < 0.001$). Combining the results of lipid and adiponectin concentration, BOT increased both adipose differentiation markers, suggesting that it may have a differentiation-promoting effect in a manner similar to that of TRG, which is known to exhibit adipocyte differentiation effects [23].

There are several types of C/EBP family such as $\alpha$, $\beta$ and $\delta$. Adipocyte differentiation is regulated by transcriptional factors such as C/EBP family members and PPAR$\gamma$ [24]. The differentiation process is initiated by increased expression of C/EBP$\beta$ and $\delta$. Both C/EBPs upregulate the expression of the master regulators, PPAR$\gamma$ and C/EBP$\alpha$. Master regulators complement each other and induce the expression of adipocyte-specific genes, such as fatty acid transport proteins (CD36 and FABP4), adiponectin, and GLUT4. Consequently, they differentiate into mature cells with various functions, such as lipid synthesis, adiponectin synthesis and secretion, and intracellular uptake of fatty acids and glucose [24]. The differentiation-promoting action of thiazolidine derivatives was reported to be caused by promoting the expression of master regulators [23].

Therefore, to further validate the adipocyte differentiation-promoting effects of BOT, we analyzed the expression of differentiation-associated genes on day 14 [**Figure 5(A)** (PPAR$\gamma$ mRNA), **Figure 5(B)** (C/EBP$\alpha$ mRNA), **Figure 5(C)** (adiponectin mRNA), **Figure 5(D)** (CD36 mRNA), and **Figure 5(E)** (GLUT4 mRNA)]. BOT (0.5 mg/mL) significantly increased the expression of all genes similar to that of TRG ($P < 0.01$ - 0.001). The increase in all gene expression levels was significantly suppressed by GW9662 ($P < 0.001$). These results suggest

that BOT increases differentiation-associated genes through molecular mechanisms, such as that induced by TRG.

In normal adipose tissue, preadipocytes and various-sized normal mature adipocytes reflecting the state of lipid accumulation are mixed. Normal adipocytes synthesize and secrete PPAR$\gamma$-mediated adiponectin, which has anti-inflammatory and anti-insulin resistance properties [25]; however, adipose tissue in obese patients is primarily composed of hypertrophic adipocytes and macrophages that infiltrate between the hypertrophic adipocytes [5].

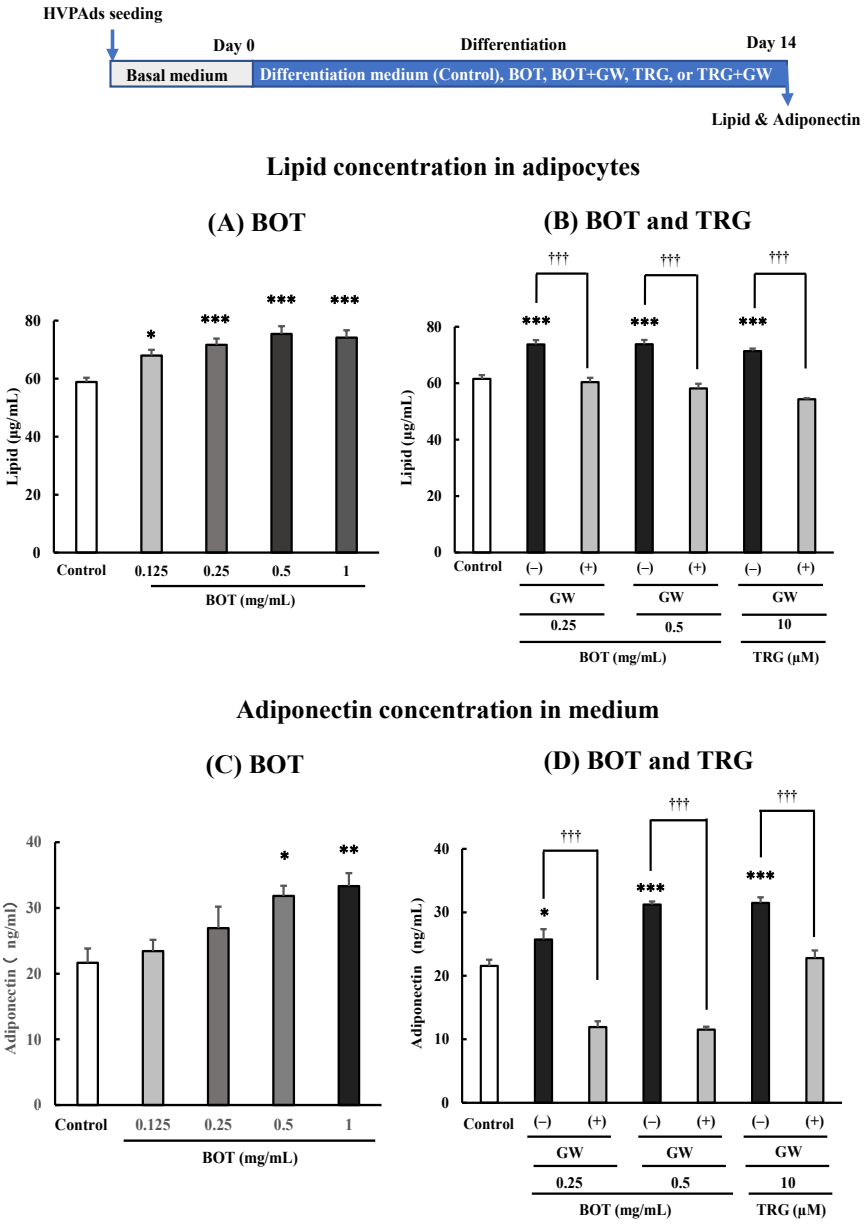


**Figure 4.** Effects of BOT and TRG on adipocyte lipid and medium adiponectin concentrations. Each data represents the mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control. $^{\dagger\dagger\dagger}P < 0.001$: comparison between (–) and (+) groups of 20 μM GW9662 ((A) and (C): one-way ANOVA + Dunnett's test, (B) and (D): one-way ANOVA + Tukey's multiple test).

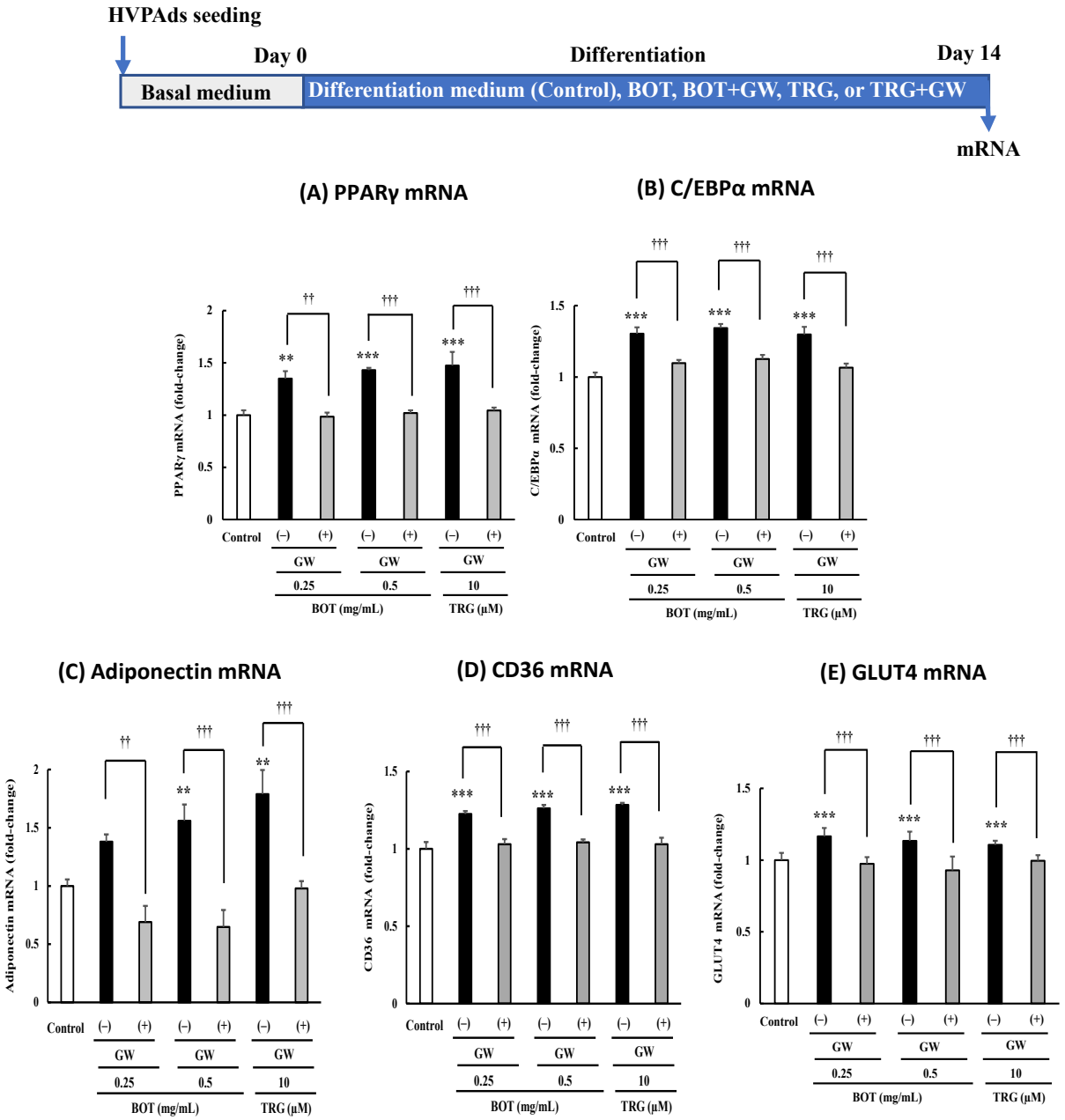


**Figure 5.** Effects of BOT and TRG on the expression of differentiation-related genes ((A): PPAR$\gamma$, (B): C/EBP$\alpha$, (C): adiponectin, (D): CD36, and (E): GLUT4 mRNAs). Each data represents the mean ± SEM (n = 6). $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control. $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$: comparison between (−) and (+) groups of 20 μM GW9662 (one-way ANOVA + Tukey's multiple test).

Infiltrating macrophages secrete TNF-$\alpha$, which suppresses the production of PPAR$\gamma$ and C/EBP$\alpha$ in differentiating cells, thereby suppressing differentiation and subsequent adiponectin production [8]. TNF-$\alpha$ also activates c-Jun N-terminal kinase to suppress adiponectin secretion [26]. Therefore, promoting adipocyte differentiation to increase normal adipocytes with adiponectin-producing ability and reducing hypertrophied adipocytes is necessary to prevent or treat metabolic syndrome, obesity type II diabetes, and insulin resistance. Thiazolidine deriva-

tives promote adipocyte differentiation and increase normal small adipocytes [14]. Thiazolidine derivatives also ameliorate TNF-$\alpha$-induced inhibition of adipocyte differentiation [27]. As mentioned, BOT, like TRG, enhanced the mRNA expression of PPAR$\gamma$ and C/EBP$\alpha$ (**Figure 5**), and increased adiponectin secretion (**Figure 4**). The results suggest that BOT may overcome the suppression of adipocyte differentiation by TNF-$\alpha$ in the same way as TRG.

Next, we examined the effect of BOT on TNF-$\alpha$-induced inhibition of adipocyte differentiation and compared the results with those of TRG. TNF-$\alpha$ significantly decreased lipid concentration ($P < 0.001$) compared with the control on day 14 (**Figure 6(A)**), which was significantly inhibited by BOT (0.125 - 1 mg/mL, $P < 0.001$). When the effects of BOT and TRG on adipocyte lipid concentrations were compared (**Figure 6(B)**), BOT (0.25 and 0.5 mg/mL, $P < 0.001$) and TRG (10 μM, $P < 0.001$) significantly inhibited the TNF-$\alpha$-induced decrease in lipid concentration. Both inhibition effects were significantly blocked by GW9662 ($P < 0.001$). **Figure 6(C)** shows the effects of BOT on the TNF-$\alpha$-induced decrease in adiponectin concentration in the medium. TNF-$\alpha$ significantly decreased adiponectin concentration compared with the control ($P < 0.001$). The decreased adiponectin concentration was inhibited by BOT (0.125 - 1 mg/mL, $P < 0.001$). When the effects of BOT and TRG on the adiponectin concentration in the medium were compared (**Figure 6(D)**), BOT (0.25 and 0.5 mg/mL, $P < 0.001$) and TRG ($P < 0.001$) markedly inhibited the TNF-$\alpha$-mediated decrease in adiponectin concentration. Both inhibition effects were significantly blocked by GW9662 ($P < 0.001$). Combining the results of the lipid and adiponectin concentrations, BOT prevented TNF-$\alpha$-induced inhibition of both adipogenesis markers, suggesting that it prevents TNF-$\alpha$-induced differentiation inhibition in a manner similar to that of TRG.

To validate the preventative effects of BOT, we measured the expression of differentiation-associated genes on day 14 [**Figure 7(A)** (PPAR$\gamma$ mRNA), **Figure 7(B)** (C/EBP$\alpha$ mRNA), **Figure 7(C)** (adiponectin mRNA), **Figure 7(D)** (CD36 mRNA), and **Figure 7(E)** (GLUT4 mRNA)]. BOT (0.25 and 0.5 mg/mL) prevented the TNF-$\alpha$-induced decrease in the expression of these genes, which was similar to the effects of TRG ($P < 0.001$). Both the preventative effects of BOT and TRG were significantly suppressed by GW9662 ($P < 0.05$). The results suggest that BOT prevents TNF-$\alpha$-induced differentiation inhibition in a manner similar to that of TRG.

TNF-$\alpha$ not only suppresses adipocyte differentiation, but also activates NF-$\kappa$B in hypertrophic adipocytes, resulting in increased production and secretion of proinflammatory cytokines and chemokines [6] [7]. Consequently, macrophage activity (TNF-$\alpha$ secretion) and excess free fatty acid generation through lipolysis via the mitogen-activated protein kinase pathway are enhanced [9]. Large quantities of TNF-$\alpha$ and free fatty acids suppress insulin signal-activated insulin receptor substrate-1 activation and downstream phosphoinositide 3-kinase/protein kinase B signaling.

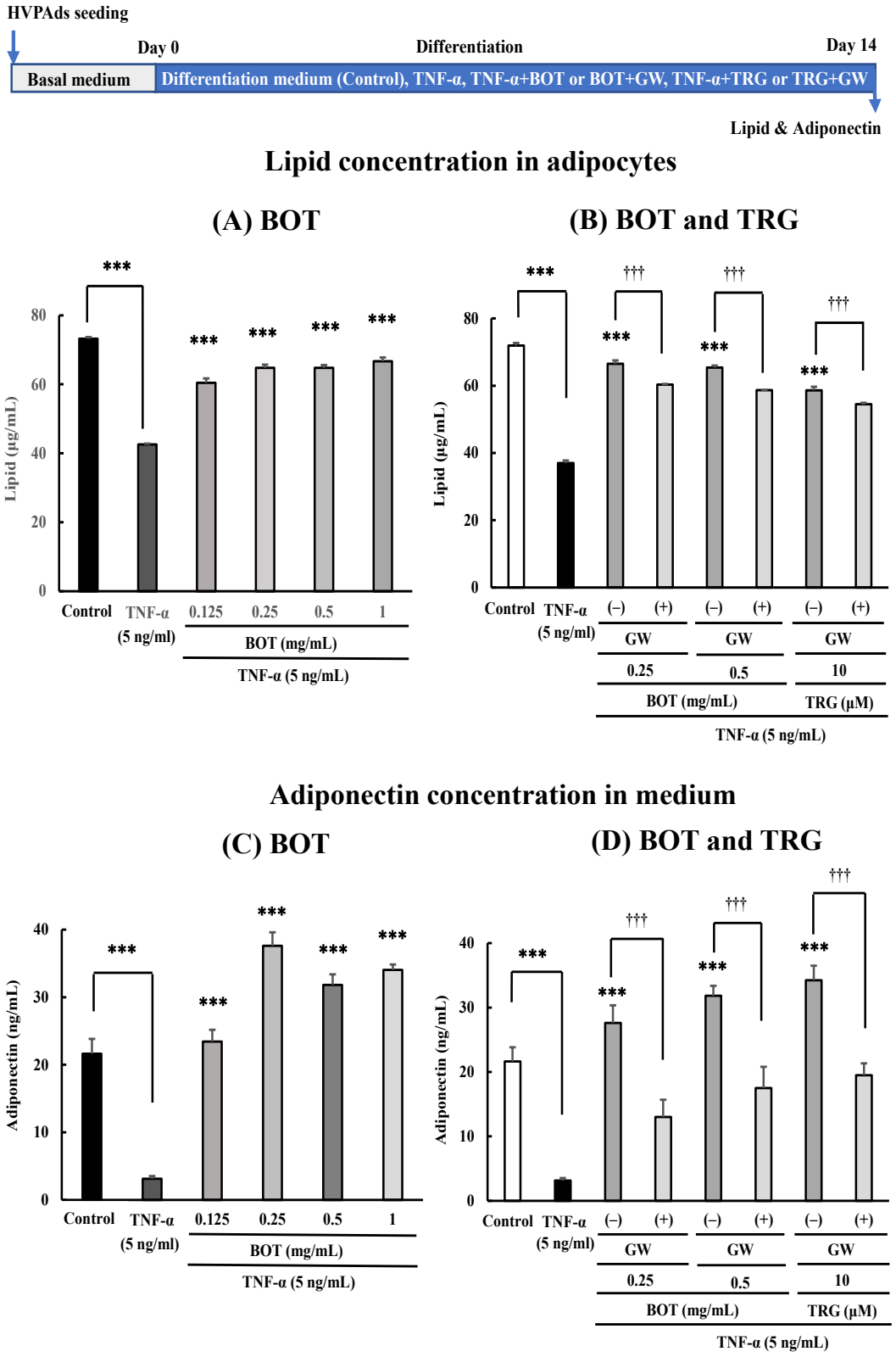


**Figure 6.** Effects of BOT and TRG on the TNF-*α*-induced decrease in adipocyte lipid and medium adiponectin concentrations. Each data represents the mean ± SEM (n = 6). ***$P < 0.001$ vs TNF-*α*. $^{\dagger\dagger\dagger}P < 0.001$: comparison between (−) and (+) groups of 20 μM GW9662 ((A) and (C): one-way ANOVA + Dunnett's test, (B) and (D): one-way ANOVA + Tukey's multiple test).

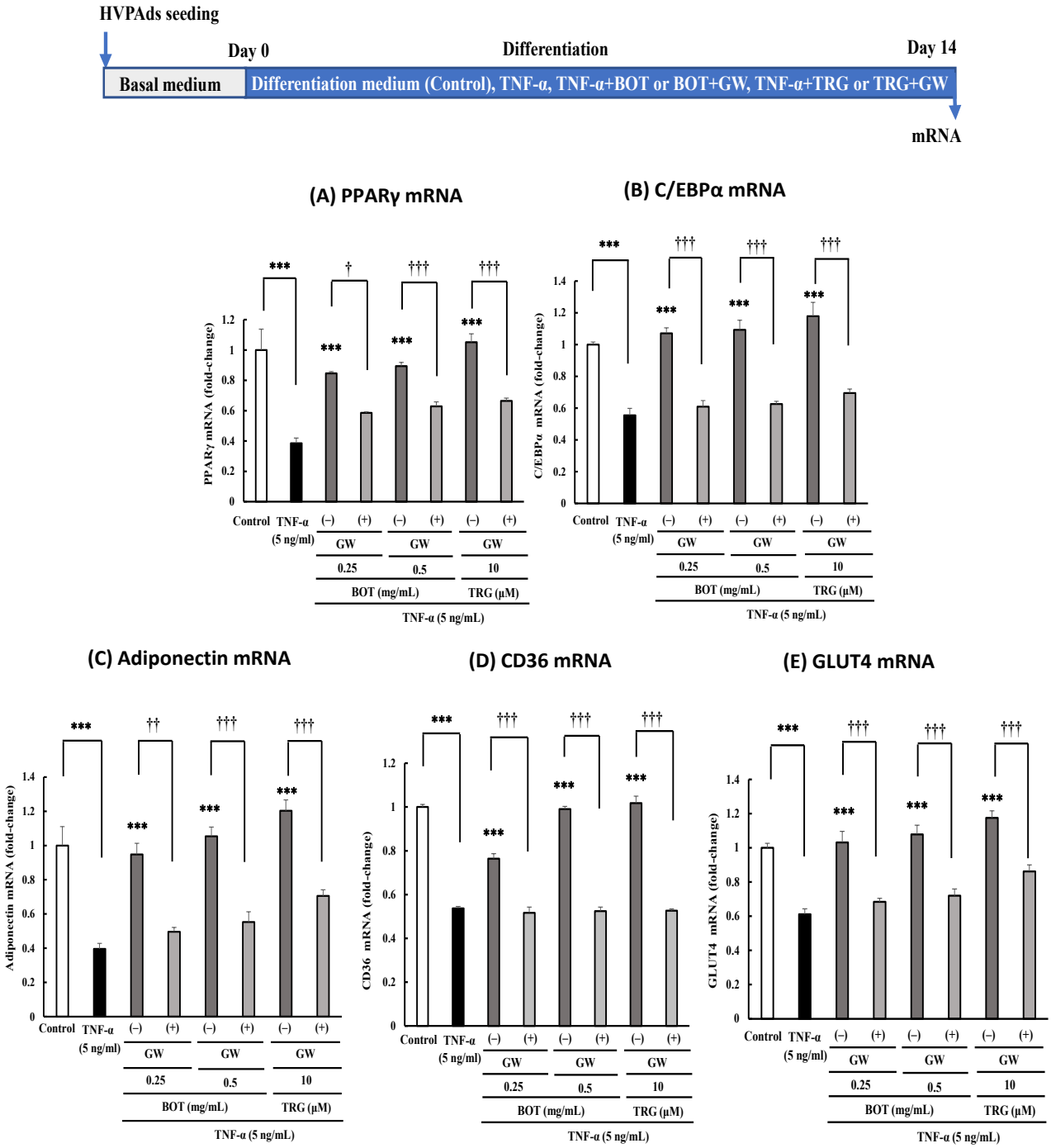


**Figure 7.** Effects of BOT and TRG on the TNF-*α*-induced decrease in the expression of differentiation-related genes ((A): PPAR*γ*, (B): C/EBP*α*, (C): adiponectin, (D): CD36, and (E): GLUT4 mRNAs). Each data represents the mean ± SEM (n = 6). $^{***}P < 0.001$ vs TNF-*α*. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$: comparison between (−) and (+) groups of 20 μM GW9662 (one-way ANOVA + Tukey's multiple test).

The suppression of the intracellular signaling pathway inhibits translocation of intracellularly synthesized GLUT4 to the plasma membrane, which impedes glucose uptake into cells and results in insulin resistance [10]. Adiponectin is well-known to inhibit the activation of the TNF-*α*-induced NF-*κ*B pathway, the-

reby leading to anti-inflammatory effects and anti-insulin resistance [3] [4] [13]. Therefore, the enhancement of adiponectin synthesis and secretion by promoting adipocyte differentiation by BOT is thought to antagonize the effects of TNF-$\alpha$. Furthermore, the de novo expression of CD36 and GLUT4 by promoting differentiation increases the uptake of fatty acids and glucose into cells [24]. These beneficial effects by BOT are expected to improve obesity-induced inflammation, insulin resistance, and diabetes.

Overall, this study's results are summarized in **Figure 8** as a graphic abstract: BOT increases adiponectin production by promoting adipocyte differentiation. Furthermore, it protects against the decrease in adiponectin production caused by TNF-$\alpha$-induced inhibition of differentiation. The results of gene analysis suggest that its molecular mechanism may induce adiponectin production by upregulating the mRNA expression of PPAR$\gamma$ and C/EBP$\alpha$, which are master regulators of the adipocyte differentiation process. These results are similar to those of TGZ. Therefore, the finding that BOT has TNF-$\alpha$ antagonism through adiponectin production suggests that it may inhibit TNF-$\alpha$-induced NF-$\kappa$B pathway activation and improve obesity-induced inflammation, insulin resistance, and diabetes.

Additionally, this result suggests that the production and secretion mechanism of adiponectin by promoting adipocyte differentiation may be involved in the anti-insulin resistance and antidiabetic effects of BOT, which have already been demonstrated [18] [19] [20].

This study had several limitations. First, because this study focused on the effects of BOT, we could not verify the crude drugs and ingredients that contribute to the effects of BOT using a culture model. However, 6-shogaol and 6-gingerol are components of ginger, one of the constituent crude drugs of BOT, have been reported to activate the PPAR$\gamma$ activity of 3T3-L1 adipocytes and promote adipocyte differentiation [21]. These may be candidates for active crude drugs or ingredients responsible for the effects of BOT. Additionally, BOT consists of six crude drugs (**Table 1**). The three-dimensional HPLC chromatogram revealed at least 22 components by UV detection (**Figure 1**). Kampo medicine is a multicomponent system composed of multiple crude drugs, and its clinical effect appears as a combined or synergistic action of the components contained in each crude drug [28]. Therefore, further studies are required to identify the active ingredients. Second, we demonstrated that BOT increased adiponectin production by promoting adipocyte differentiation. Given that increased adiponectin inhibits the activation of the TNF-$\alpha$-induced NF-$\kappa$B pathway, thereby leading to anti-inflammatory effects and anti-insulin resistance [3] [4], they were not examined in this experiment. However, in the future, it will be necessary to ensure under our experimental conditions that the BOT-induced increase in adiponectin production suppresses NF-$\kappa$B activity, thereby leading to anti-inflammatory and anti-insulin resistance effects. Finally, *in vitro* studies are difficult to faithfully reproduce all of the complex physiological states found *in vivo*. Therefore, it is necessary to verify whether the present *in vitro* results are reflected *in vivo*.

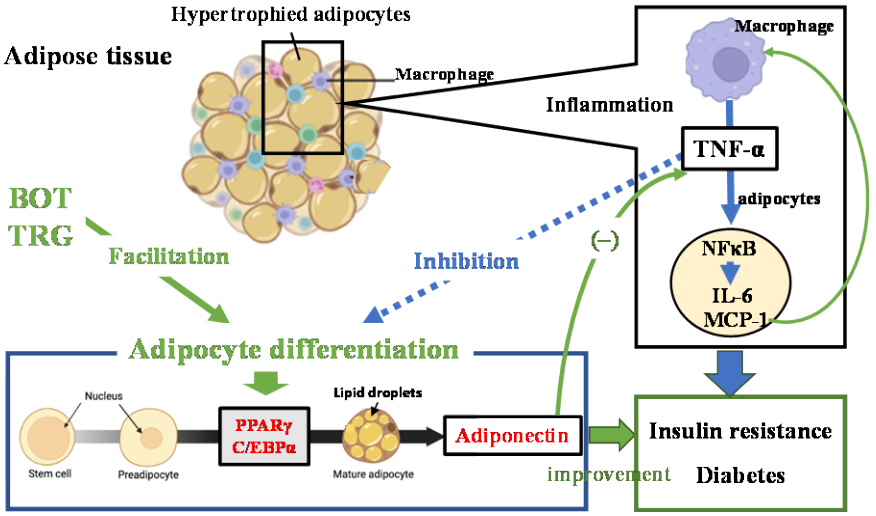


**Figure 8.** Graphic abstract of this study. Boiogito (BOT), similar to the thiazolidine derivative troglitazone (TRG), increases adiponectin synthesis and secretion by promoting adipocyte differentiation by activating PPAR$\gamma$ and C/EBP$\alpha$. This mechanism may be involved in the diabetes and insulin resistance improving effects of BOT.

## 4. Conclusion

BOT promoted differentiation by increasing the expression of differentiation-associated genes and increasing the production and secretion of adiponectin, similar to that of TRG. Likewise, BOT prevented TNF-$\alpha$-induced differentiation inhibition. The present study is the first to demonstrate that the adipocyte differentiation-promoting activity may be one of the underlying mechanisms of the ameliorating effect of BOT on insulin resistance and diabetes.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

## Authors' Contributions

**Yuan Gu:** Conceptualization, Investigation, Visualization, Writing-Original draft preparation. **Ailing Hu:** Conceptualization and Methodology. **Takuji Yamaguchi:** Conceptualization and Resources. **Masahiro Tabuchi:** Conceptualization, Formal analysis. **Yasushi Ikarashi:** Conceptualization, Writing-Original draft preparation. **Hiroyuki Kobayashi:** Supervision.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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