

# LY3009120, a pan-Raf kinase inhibitor, inhibits adipogenesis of 3T3-L1 cells by controlling the expression and phosphorylation of C/EBP- $\alpha$ , PPAR- $\gamma$ , STAT-3, FAS, ACC, perilipin A, and AMPK

SU-MIN YANG<sup>1</sup>, YU-KYOUNG PARK<sup>1</sup>, JEE IN KIM<sup>1</sup>, YUN-HAN LEE<sup>1</sup>,  
TAE-YUN LEE<sup>2</sup> and BYEONG-CHURL JANG<sup>1</sup>

<sup>1</sup>Department of Molecular Medicine, College of Medicine, Keimyung University, Daegu 42601;

<sup>2</sup>Department of Microbiology, College of Medicine, Yeungnam University, Daegu 42415, Republic of Korea

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**Abstract.** Excessive preadipocyte differentiation/adipogenesis is closely linked to the development of obesity. LY3009120 is a pan-Raf kinase inhibitor and is known for its anticancer activities. In the present study, the effect of LY3009120 on 3T3-L1 cell adipogenesis was investigated. The differentiation of 3T3-L1 preadipocytes into adipocytes was measured by Oil Red O staining and AdipoRed assay. Changes of cellular protein expression and phosphorylation levels in differentiating 3T3-L1 preadipocytes in the absence or presence of

LY3009120 were determined by western blotting analysis. Cell count assay was used to assess the cytotoxicity of LY3009120 on 3T3-L1 cells. At 0.3  $\mu$ M, LY3009120 markedly inhibited lipid accumulation and decreased triglyceride content in differentiating 3T3-L1 cells. However, it had minimal effect on the elevated expression and phosphorylation of three Raf kinase isoforms (C-Raf, A-Raf, and B-Raf) observed in the cells. LY3009120 reduced not only the expression of CCAAT/enhancer-binding protein- $\alpha$  (C/EBP- $\alpha$ ), peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ), fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and perilipin A, but also reduced the phosphorylation of signal transducer and activator of transcription-3 (STAT-3) in differentiating 3T3-L1 cells. LY3009120 also increased the phosphorylation of adenosine 3',5'-cyclic monophosphate (cAMP)-activated protein kinase (AMPK), but did not affect the phosphorylation or expression of liver kinase B1 in these cells. In summary, this is the first report, to the best of our knowledge, demonstrating that LY3009120 has an anti-adipogenic effect on 3T3-L1 cells, which may be mediated through control of the expression and phosphorylation of C/EBP- $\alpha$ , PPAR- $\gamma$ , STAT-3, FAS, ACC, perilipin A, and AMPK.

*Correspondence to:* Dr Byeong-Churl Jang, Department of Molecular Medicine, College of Medicine, Keimyung University, 1,095 Dalgubeoldae-ro, Dalseo-gu, Daegu 42601, Republic of Korea  
E-mail: jangbc123@gw.kmu.ac.kr

Dr Tae-Yun Lee, Department of Microbiology, College of Medicine, Yeungnam University, 170 Hyeonchung-ro, Nam-gu, Daegu 42415, Republic of Korea  
E-mail: doxr7p@ynu.ac.kr

**Abbreviations:** ACC, acetyl CoA carboxylase; AMP, adenosine monophosphate; AMPK, cAMP-activated protein kinase; ATP, adenosine triphosphate; CAMKK2, calcium-/calmodulin-dependent kinase kinase 2; cAMP, adenosine 3',5'-cyclic monophosphate; C/EBP- $\alpha$ , CCAAT/enhancer-binding protein- $\alpha$ ; DMEM, Dulbecco's modified Eagle's medium; DR5, death receptor 5; FAS, fatty acid synthase; FBS, fetal bovine serum; LD, lipid droplet; LKB1, liver kinase B1; MDI, IBMX, dexamethasone, and insulin; MEK-1/2, mitogen-activated protein kinase kinase-1/2; PKA, protein kinase A; PPAR- $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; RT, room temperature; SDS, sodium dodecyl sulfate; SE, standard error; STAT-3/5, signal transducer and activator of transcription-3/5; TG, triglyceride

**Key words:** LY3009120, adipogenesis, adenosine 3',5'-cyclic monophosphate-activated protein kinase, CCAAT/enhancer-binding protein- $\alpha$ , peroxisome proliferator-activated receptor- $\gamma$ , 3T3-L1

## Introduction

Obesity is a serious public health epidemic, with ~2,000,000,000 adults being overweight or obese worldwide, and is considered a major contributor to hyperlipidemia, cardiovascular disease, type 2 diabetes, and cancer (1). As a result, obesity is now among the leading contributors to global morbidity and mortality rates (2). Although the development of obesity is influenced by a number of factors, including excessive food intake, lack of physical activity, genetic susceptibility, medications, and endocrine abnormalities (2,3), increasing evidence indicates that it also arises from excessive preadipocyte differentiation in adipose tissue (4,5). Therefore, any compound that inhibits the disproportionate differentiation of these cells constitutes a potential therapeutic option for obesity.

Preadipocyte differentiation, also known as adipogenesis, is the process during which fibroblast-like preadipocytes develop into mature adipocytes (5). This process is influenced by the adipogenic program, which is controlled by adipogenic transcription factors, including CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptors (PPARs), and signal transducers and activators of transcription (STATs) (6-10), lipogenic enzymes, including fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) (11,12), and lipid droplet (LD)-associated proteins, including perilipin A (13,14). In addition, there is evidence that adenosine 3',5'-cyclic monophosphate (cAMP), cAMP-activated protein kinase (AMPK), protein kinase A (PKA), protein kinase C family members, mitogen-activated protein kinase kinase-1/2, and extracellular signal-regulated protein kinase-1/2, are known to mediate preadipocyte differentiation (15-18). Furthermore, several studies have shown that members of the Raf kinases, including c-Raf, A-Raf, and B-Raf, are expressed and phosphorylated during preadipocyte differentiation, and their expression and activities are crucial to this process (19-21).

In our previous study, 85 known protein kinase inhibitors with anticancer activities were screened using the AdipoRed assay to assess the ability of each to lower lipid (triglyceride, TG) content during the differentiation of 3T3-L1 preadipocytes into adipocytes. Several, including the pan-Raf kinase inhibitor LY3009120 (22), were found to have an anti-adipogenic effect. LY3009120 is known to have anticancer activities (23,24); however, its anti-obesity effect and its mode of action in adipocytes remain to be fully elucidated. The present study investigated the effect of LY3009120 on adipogenesis in 3T3-L1 preadipocytes. The results revealed for the first time, to be the best of our knowledge, that LY3009120 had an anti-adipogenic effect on differentiating 3T3-L1 cells, which appeared to be mediated through modulation of the expression and/or phosphorylation of C/EBP- $\alpha$ , PPAR- $\gamma$ , STAT-3, FAS, ACC, perilipin A, and AMPK.

## Materials and methods

**Materials.** LY3009120 was purchased from Apexbio Technology (Houston, TX, USA). Polyclonal C/EBP- $\alpha$  (cat. no. sc-61), monoclonal PPAR- $\gamma$  (cat. no. sc-7273), monoclonal STAT-3 (cat. no. sc-8019), and monoclonal phosphorylated (p)-STAT-3 (cat. no. sc-8059) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal FAS (cat. no. 610962), monoclonal  $\beta$ -actin (cat. no. A5441), and polyclonal perilipin A (cat. no. 3948-200) antibodies were purchased from BD Biosciences (San Jose, CA, USA), Sigma, EMD Millipore (Billerica, MA, USA), and BioVision, Inc. (Milpitas, CA, USA), respectively. Polyclonal p-AMPK (T172, cat. no. 2535), monoclonal AMPK (cat. no. 2793), polyclonal p-ACC (S79, cat. no. 3661), polyclonal ACC (cat. no. 3662), polyclonal liver kinase B1 (LKB1; cat. no. 3047), polyclonal p-LKB1 (S428, cat. no. 3482), polyclonal p-A-Raf (S299, cat. no. 4431), polyclonal A-Raf (cat. no. 4432), polyclonal p-B-Raf (S445, cat. no. 2696), polyclonal B-Raf (cat. no. 9433), polyclonal p-C-Raf (S259, cat. no. 9421), and monoclonal C-Raf (cat. no. 12552) antibodies were acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA). Monoclonal procaspase-9 (cat. no. ADI-AAM-139), polyclonal poly (ADP-ribose)

polymerase (PARP; cat. no. 11 835 238 001), and polyclonal death receptor 5 (DR5; cat. no. NBPI-45951) antibodies were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), Roche Diagnostics (Basel, Switzerland), and Novus Biologicals, LLC (Littleton, CO, USA), respectively.

**Culture and differentiation of 3T3-L1 cells.** Murine white preadipocytes of the 3T3-L1 line (ATCC, Manassas, VA, USA) were cultured to the contact-inhibition stage and maintained at 37°C and 5% CO<sub>2</sub> in the post-confluent stage for 2 days in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and penicillin-streptomycin (WelGENE, Inc., Daegu, Korea). Differentiation was then induced in the presence or absence of LY3009120 at the indicated concentrations (0.1, 0.2, 0.3, and 0.4  $\mu$ M) by replacing the medium with DMEM supplemented with 10% FBS (WelGENE, Inc.) and a hormone cocktail (MDI; Sigma, EMD Millipore) comprising 0.5 mM IBMX (M), 0.5  $\mu$ M dexamethasone (D), and 5  $\mu$ g/ml insulin (I). Following 48 h of exposure to MDI, the differentiation medium was replaced with DMEM supplemented with 10% FBS and 5  $\mu$ g/ml insulin, with or without LY3009120 at the indicated concentrations. The cells were then provided every other day with DMEM containing 10% FBS with or without LY3009120 at the indicated concentrations until day 8, by which point, the preadipocytes had become mature adipocytes with a rounded morphology and filled with LDs.

**Oil Red O staining.** On day 8 of differentiation, the control and LY3009120-treated 3T3-L1 cells were washed twice with PBS, fixed with 10% formaldehyde for 2 h at room temperature (RT), washed with 60% isopropanol, and dried completely. The fixed cells were then stained with Oil Red O working solution for 1 h at RT, and then washed twice with distilled water. The LDs were observed by light microscopy (Nikon Corporation, Tokyo, Japan).

**Cell count analysis.** The preadipocytes (3T3-L1 cells) were seeded in 24-well plates and cultured under the above-mentioned differentiation conditions. On day 8 of differentiation, the control and LY3009120-treated 3T3-L1 cells, which cannot be stained with trypan blue dye, were counted under an Olympus phase contrast microscope equipped with a digital camera (Nikon Corporation). The assay was performed in triplicate, and the data are presented as the mean  $\pm$  standard error (SE) of three independent experiments.

**Quantification of intracellular TG content by AdipoRed assay.** On day 8 of differentiation, the lipid content of control and LY3009120-treated 3T3-L1 was measured using the commercially available AdipoRed Assay Reagent kit according to the manufacturer's protocol (Lonza Group AG, Basel, Switzerland). Following incubation for 10 min, fluorescence was measured on a Victor<sup>3</sup> plate reader (PerkinElmer, Inc., Waltham, MA, USA) with excitation and emission wavelengths of 485 and 572 nm, respectively.

**Preparation of whole cell lysates.** At the designated time point, the 3T3-L1 cells were washed twice with PBS and exposed to a

modified RIPA buffer [50  $\mu$ M Tris-Cl (pH 7.4), 150  $\mu$ M NaCl, 0.1% SDS, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and 1X proteinase inhibitor cocktail]. The cell lysates were then collected and centrifuged at 12,074 x g for 20 min at 4°C. The supernatants were retained and their protein concentrations were determined with Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Western blot analysis.** The proteins (50  $\mu$ g) were separated by SDS-PAGE on 10% gels and transferred onto nitrocellulose membranes (EMD Millipore). The membranes were washed with TBST [10 mM Tris, and 150 mM NaCl supplemented with 0.05% (vol/vol) Tween 20] and subsequently blocked with TBST containing 5% (wt/vol) non-fat dried milk. The membranes were then incubated overnight with antibodies specific to C/EBP- $\alpha$  (1:1,000), PPAR- $\gamma$  (1:1,000), STAT-3 (1:1,000), p-STAT-3 (1:1,000), FAS (1:1,000), perilipin A (1:2,000), p-AMPK (1:1,000), AMPK (1:1,000), p-ACC (1:2,000), ACC (1:2,000), p-LKB1 (1:1,000), LKB1 (1:1,000), p-A-Raf (1:1,000), A-Raf (1:1,000), p-B-Raf (1:1,000), B-Raf (1:1,000), p-C-Raf (1:1,000), C-Raf (1:1,000), procaspase-9 (1:1,000), PARP (1:5,000), DR5 (1:1,000) or  $\beta$ -actin (1:10,000) at 4°C. The membranes were then exposed to horseradish peroxidase-conjugated secondary antibodies (an anti-rabbit IgG (H+L) (1:2,000), catalog no. 111-035-045 or anti-mouse IgG (H+L) (1:2,000), catalog no. 115-035-062, Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at RT, and were then washed three times with TBST at RT. The immunoreactivity was detected using enhanced chemiluminescence reagents, and variations in protein loading were assessed by observing actin protein levels using ImageJ software (ImageJ version 1.8.0; National Institutes of Health, Bethesda, Maryland, USA).

**Statistical analysis.** The cell count analysis was performed in triplicate and repeated three times. Data are expressed as the mean  $\pm$  SE. Differences were analyzed by one-way analysis of variance (SPSS software, version 11.5; SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**LY3009120 inhibits 3T3-L1 cell adipogenesis.** The present study first investigated the effect of LY3009120 at different concentrations (0.1, 0.2, 0.3, and 0.4  $\mu$ M) on lipid accumulation in differentiating 3T3-L1 cells using the Oil Red O staining. The protocol followed to induce 3T3-L1 preadipocyte differentiation is shown in Fig. 1A. Treatment of these preadipocytes with LY3009120 for 8 days decreased the number of LDs in the corresponding differentiated adipocytes in a concentration-dependent manner (Fig. 1B, upper panels). This LY3009120-mediated inhibition of LD accumulation was also observed in differentiating 3T3-L1 cells by light microscopy on days 2, 5 and 8 (Fig. 1B, lower panels). Subsequently, whether exposure of the differentiating 3T3-L1 cells to LY3009120 for 8 days affects their TG content was determined using the AdipoRed assay. Although cellular TG content was marginally enhanced by treatment with 0.1  $\mu$ M LY3009120, it was

effectively reduced by administration of this drug at 0.2, 0.3, or 0.4  $\mu$ M in a concentration-dependent manner (Fig. 1C). A cell counting assay was used to determine whether treatment with LY3009120 for 8 days at the concentrations assessed was cytotoxic to the differentiating 3T3-L1 cells. As shown in Fig. 1D, 3T3-L1 cell survival was marginally reduced by 0.1, 0.2, or 0.3  $\mu$ M, and markedly decreased following exposure to this drug at 0.4  $\mu$ M (Fig. 1D), indicating that the latter concentration was cytotoxic to differentiating 3T3-L1 cells. In addition, whether 8 days of LY3009120 administration at the concentrations assessed induces the apoptosis of differentiating 3T3-L1 cells was analyzed by measuring the levels of procaspase-9, PARP, and DR5, which are known apoptotic proteins. The results of the western blotting analysis revealed that, at the concentrations used, LY3009120 did not markedly affect the expression of procaspase-9, PARP, or DR5. As 0.3  $\mu$ M of LY3009120 markedly reduced lipid accumulation and TG content with minimal cytotoxicity, this concentration was selected for further experiments.

**LY3009120 does not substantially alter the expression and phosphorylation of Raf kinases in differentiating 3T3-L1 cells.** As LY3009120 targets Raf kinases, western blotting analysis was performed to assess whether C-Raf, A-Raf, and B-Raf, members of this protein kinase family, are expressed and phosphorylated in differentiating 3T3-L1 cells and whether this is affected by LY3009120. Substantial expression and phosphorylation of C-Raf, A-Raf, and B-Raf was observed in these cells (Fig. 2A); however, LY3009120 treatment had no notable effect on the expression and phosphorylation of these Raf kinases. Densitometry data obtained from Fig. 2A are shown in Fig. 2B.

**LY3009120 reduces the expression and/or phosphorylation of C/EBP- $\alpha$ , PPAR- $\gamma$ , and STAT-3 in differentiating 3T3-L1 cells.** To obtain a better understanding of the mechanisms associated with the anti-adipogenic effect of LY3009120, the present study aimed to determine whether this drug modulates the expression and/or phosphorylation (activation) of C/EBP- $\alpha$ , PPAR- $\gamma$ , and STAT-3 in differentiating 3T3-L1 cells. As shown in Fig. 3A, LY3009120 treatment considerably lowered the levels of C/EBP- $\alpha$  and PPAR- $\gamma$  in the cells. Furthermore, it reduced the phosphorylation of STAT-3 without affecting the expression of total STAT-3 (Fig. 3B). Densitometry data from the experiments performed in triplicate confirmed that LY3009120 reduced the expression of C/EBP- $\alpha$  and PPAR- $\gamma$  (Fig. 3C) and phosphorylation of STAT-3 (Fig. 3D) during 3T3-L1 preadipocyte differentiation.

**LY3009120 alters the expression and/or phosphorylation of FAS, perilipin A, AMPK, ACC, and LKB1 in differentiating 3T3-L1 cells.** The effects of LY3009120 on the expression of FAS and perilipin A in differentiating 3T3-L1 cells were also examined. As shown in Fig. 4A, LY3009120 treatment led to a decrease in the levels of these proteins in the cells. Subsequently, the effect of LY3009120 on the expression and/or phosphorylation of AMPK and ACC proteins in 3T3-L1 cells during differentiation was investigated. LY3009120 markedly increased the phosphorylation of AMPK but decreased that of ACC. LY3009120 did not affect the levels

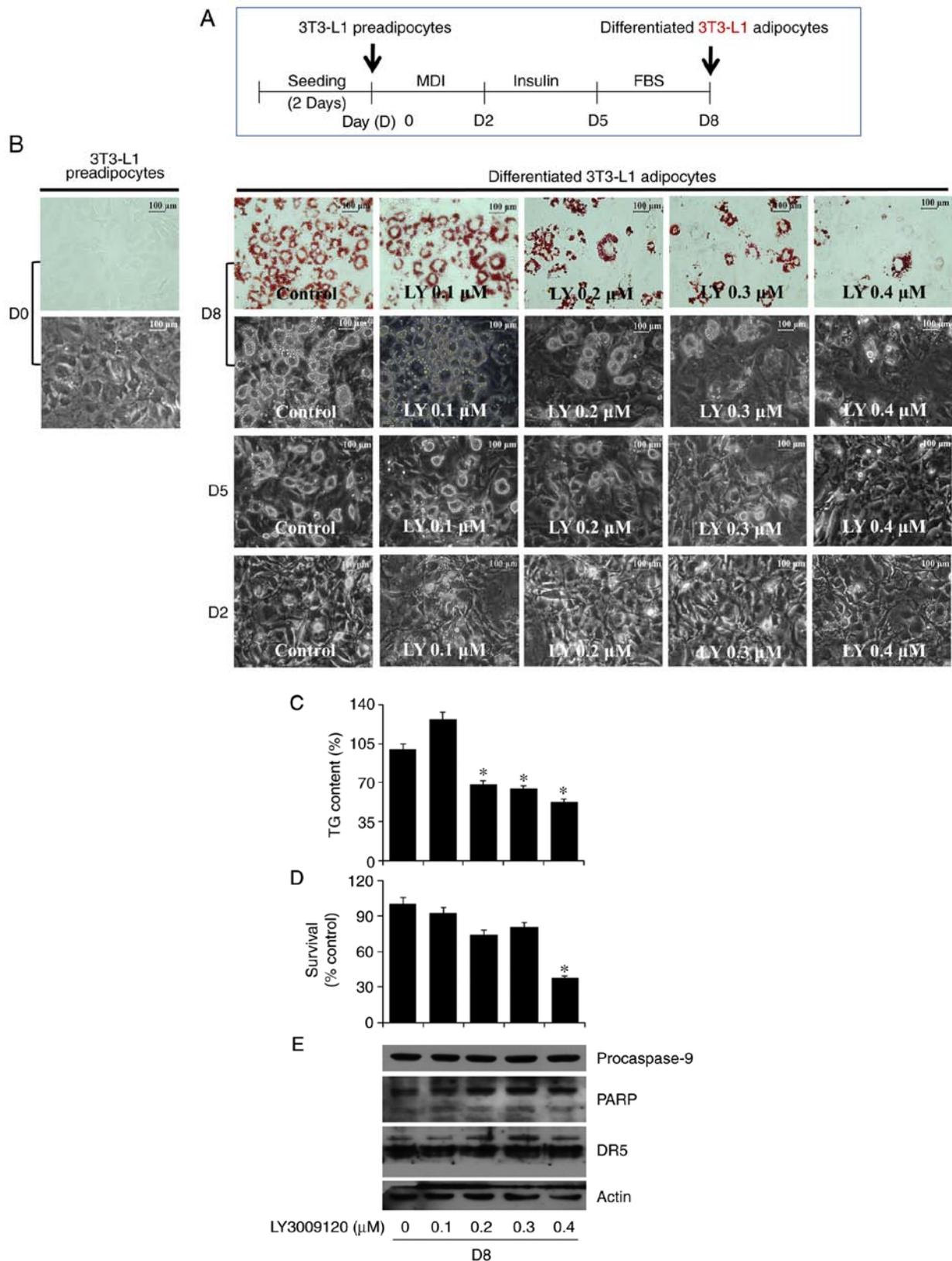


Figure 1. Effects of LY3009120 on the adipogenesis and growth of differentiating 3T3-L1 cells. (A) Protocol used to induce 3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes were induced to differentiate with induction medium in the presence or absence of LY3009120 at the indicated concentrations and for the indicated times. (B) On D8, cellular lipid content was assessed by Oil Red O staining (upper panels). Phase-contrast images of the cells were also recorded following treatment (lower panels). (C) On D8, cellular TG content was quantified using the AdipoRed assay. Values are presented as the mean  $\pm$  standard error of the mean of data from three independent experiments with three replicates. \* $P < 0.05$ , vs. control. (D) On D8, LY3009120-treated 3T3-L1 cells, which are not stained by trypan blue dye, were counted under a microscope. The cell count assay was performed in triplicate. Data are presented as the mean  $\pm$  standard error of the mean of data from three independent experiments. \* $P < 0.05$ , vs. control. (E) 3T3-L1 preadipocytes were induced to differentiate with induction medium in the presence or absence of LY3009120 at the indicated concentrations for 8 days. Cellular proteins were extracted and analyzed by western blot analysis. LY, LY3009120; MDI, IBMX, dexamethasone, and insulin; FBS, fetal bovine serum; D, day; TG, triglyceride; PARP, polyclonal poly (ADP-ribose) polymerase; DR5, death receptor 5.

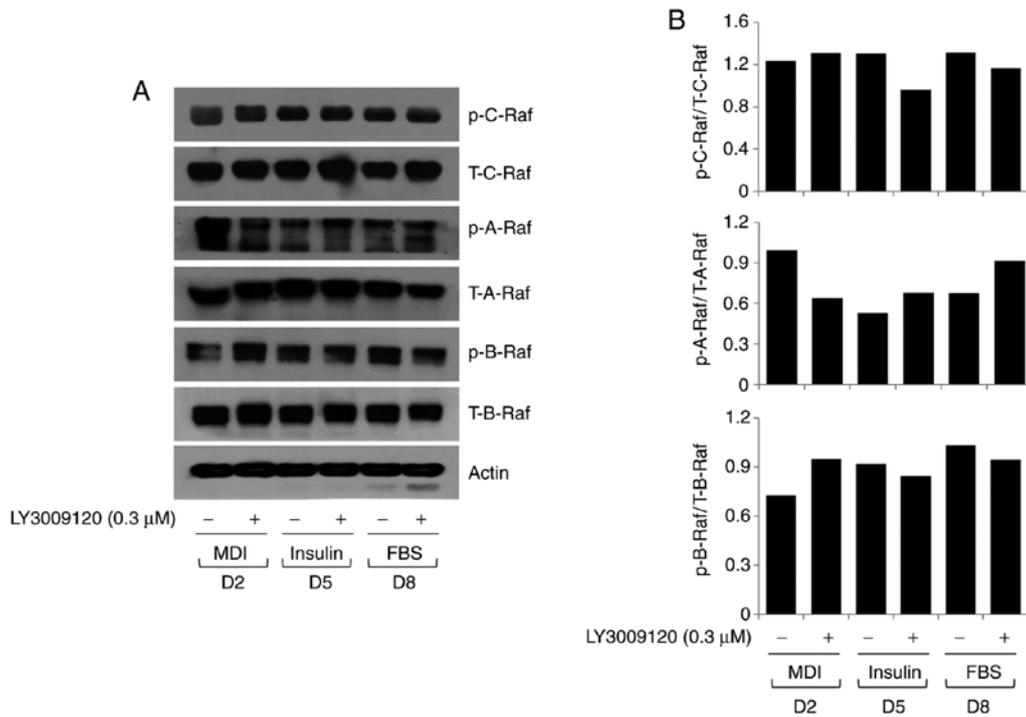


Figure 2. Effect of LY3009120 on the expression and/or phosphorylation of three Raf kinases in differentiating 3T3-L1 cells. (A) 3T3-L1 preadipocytes were induced to differentiate with induction medium in the presence or absence of LY3009120, and harvested on D2, 5, and 8. Proteins were extracted from cells at the indicated time points and analyzed by western blot analysis. (B) Densitometry data from triplicate experiments showing levels of p-C-Raf, p-A-Raf, and p-B-Raf relative to those of T-C-Raf, T-A-Raf, and T-B-Raf, respectively, on D2, 5 and 8. MDI, IBMX, dexamethasone, and insulin; FBS, fetal bovine serum; p-C-Raf, phosphorylated C-Raf; T-C-Raf, total C-Raf; p-A-Raf, phosphorylated A-Raf; T-A-Raf, total A-Raf; p-B-Raf, phosphorylated B-Raf; T-B-Raf, total B-Raf; D, day.

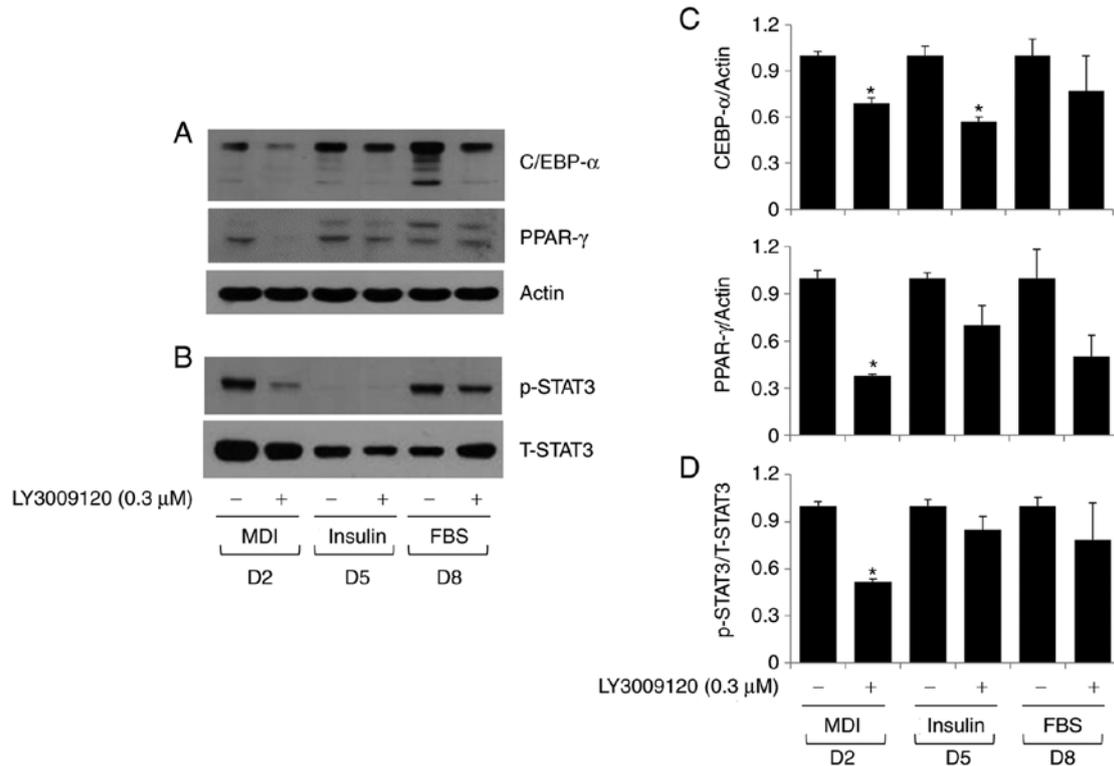


Figure 3. Effect of LY3009120 on the expression and/or phosphorylation of C/EBP- $\alpha$ , PPAR- $\gamma$ , and STAT-3 in differentiating 3T3-L1 cells. 3T3-L1 preadipocytes were induced to differentiate with induction medium in the presence or absence of LY3009120, and harvested on D2, 5, and 8. Proteins were extracted from cells collected at the indicated time points and analyzed by western blot analysis for (A) C/EBP- $\alpha$  and PPAR- $\gamma$  or (B) p-STAT3 and T-STAT3. Densitometry data from the western blot experiments for (C) C/EBP- $\alpha$  and PPAR- $\gamma$  and (D) p-STAT3/T-STAT3, which were performed in triplicate on cells collected on D2, 5, and 8. Data are expressed as the mean  $\pm$  standard error. \* $P$ <0.05 vs. control at the indicated day. MDI, IBMX, dexamethasone, and insulin; FBS, fetal bovine serum; C/EBP- $\alpha$ , CCAAT/enhancer-binding protein- $\alpha$ ; PPAR- $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; p-STAT-3, phosphorylated STAT-3; T-STAT-3, total STAT3; D, day.

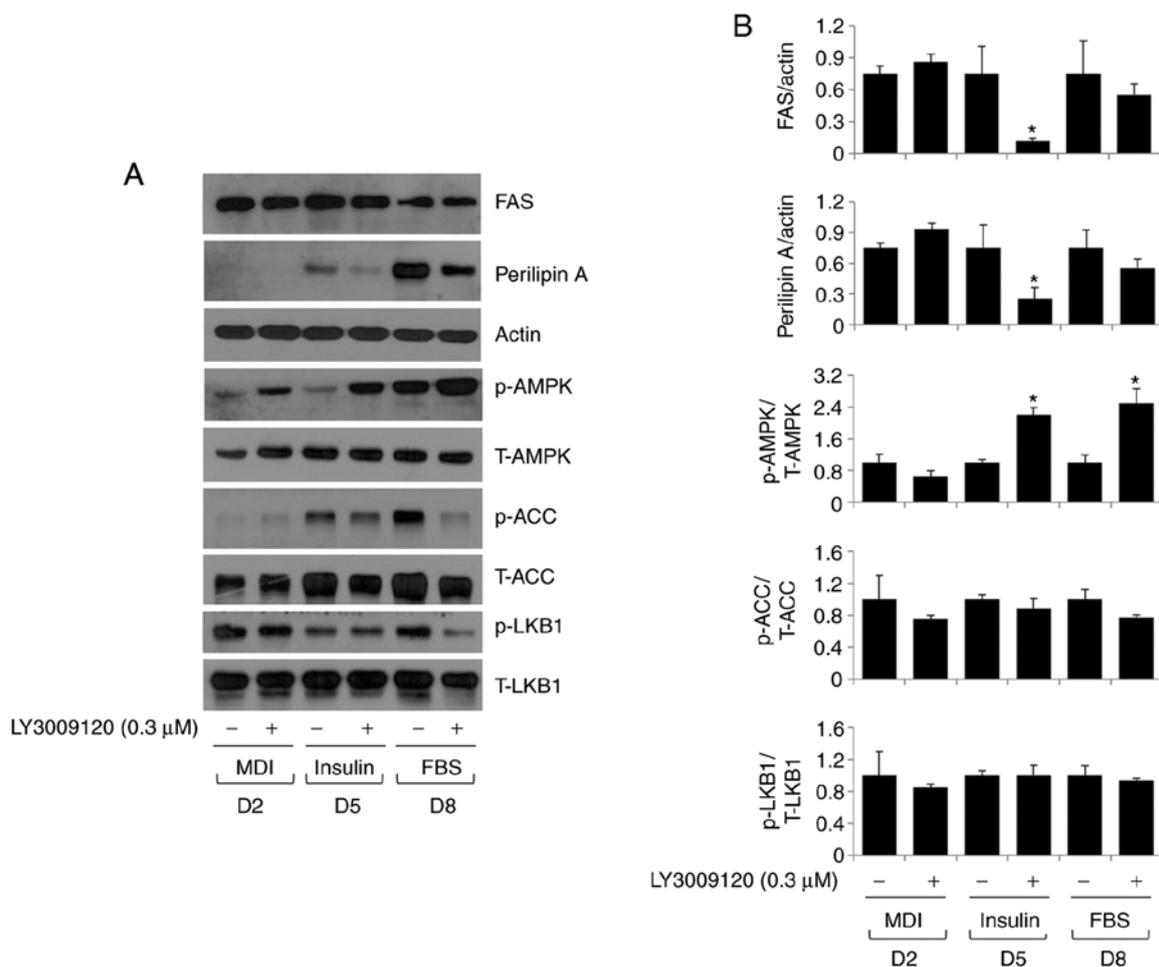


Figure 4. Effect of LY3009120 on the expression and/or phosphorylation of FAS, perilipin A, AMPK, ACC, and LKB1 in differentiating 3T3-L1 cells. (A) 3T3-L1 preadipocytes were induced to differentiate with induction medium in the presence or absence of LY3009120, and harvested on D2, 5, and 8. Proteins were extracted from cells collected at the indicated time points and analyzed by western blot analysis. (B) Densitometry data from western blot experiments performed in triplicate, showing expression levels of FAS, perilipin A, p-/T-AMPK, p-/T-ACC, and p-/T-LKB1 on D2, 5, and 8. Data are expressed as the mean  $\pm$  standard error. \* $P < 0.05$  vs. control at the indicated day. MDI, IBMX, dexamethasone, and insulin; FAS, fatty acid synthase; AMPK, adenosine 3',5'-cyclic monophosphate-activated protein kinase; ACC, acetyl CoA carboxylase; LKB1; liver kinase B1; p-, phosphorylated; T-, total; D, day.

of total AMPK protein, but marginally reduced the levels of total ACC protein. Whether LY3009120 alters the expression and/or phosphorylation of LKB1, the main kinase responsible for AMPK phosphorylation (24), was also determined in the differentiating 3T3-L1 cells. The levels of total LKB1 and the phosphorylation of this protein on days 2 and 5 of 3T3-L1 preadipocyte differentiation were not affected by LY3009120, but were considerably reduced on day 8 due to administration of this drug. Densitometry data from experiments performed in triplicate confirmed the ability of LY3009120 to increase the phosphorylation of AMPK and decrease the phosphorylation and/or total levels of FAS, perilipin A, ACC, and LKB1 during 3T3-L1 preadipocyte differentiation (Fig. 4B).

## Discussion

Preadipocyte differentiation/adipogenesis, excessive levels of which are correlated with the development of obesity, is known to be mediated by Raf kinases. LY3009120 is a pan-Raf kinase inhibitor and demonstrates anticancer activity. In order to establish whether LY3009120 may also serve as an alternative to currently available anti-obesity medications, the

present study investigated its effect on adipogenesis in differentiating 3T3-L1 cells. It was demonstrated that LY3009120 had an anti-adipogenic effect on these cells by controlling the expression and phosphorylation of C/EBP- $\alpha$ , PPAR- $\gamma$ , STAT-3, FAS, ACC, perilipin A, and AMPK.

It has previously been shown that C-Raf kinase is phosphorylated in insulin-treated 3T3-L1 cells, and its hyperphosphorylation is crucial for hormone-induced 3T3-L1 cell differentiation (19). The present study revealed that the three Raf kinase isoforms, C-Raf, A-Raf, and B-Raf, were expressed and phosphorylated in differentiating 3T3-L1 cells. Notably, at the concentrations assessed, LY3009120 did not appreciably influence the expression and phosphorylation of these Raf kinases, but markedly reduced lipid accumulation and TG content in the differentiating 3T3-L1 cells, particularly when administered at a concentration of 0.3  $\mu$ M, with no cytotoxic or apoptotic effects. These results suggested that LY3009120 exerts a potent anti-adipogenic effect through a Raf-independent mechanism. At present, the mechanism underlying the restriction of adipogenesis in differentiating 3T3-L1 cells by LY3009120 at 0.3  $\mu$ M in the absence of pan-Raf kinase inhibition remains to be fully elucidated. It has been shown that LY3009120 inhibits A-Raf,

B-Raf, and C-Raf kinase in A375 melanoma cells, with  $IC_{50}$  values of 44, 31-47, and 42 nM, respectively (22). It is suggested that the inhibitory effect of LY3009120 on Raf kinases (and its inhibitory concentration) differs between cell types; therefore, a concentration  $>0.3 \mu\text{M}$  may be required to inhibit all Raf kinases in differentiating 3T3-L1 cells. However, the administration of LY3009120 at a concentration  $>0.3 \mu\text{M}$  ( $0.4 \mu\text{M}$  in the present study) was cytotoxic to these cells. Taken together,  $0.3 \mu\text{M}$  LY3009120 may be used to induce a Raf-independent anti-adipogenic effect with no cytotoxicity.

It is well established that the expression and activities of C/EBP- $\alpha$ , PPAR- $\gamma$ , and STAT-3/5 are critical for 3T3-L1 cell adipogenesis (6-10,25,26). In the present study, LY3009120 decreased not only the levels of C/EBP- $\alpha$  and PPAR- $\gamma$ , but also the phosphorylation of STAT-3 in differentiating 3T3-L1 cells. Therefore, it is likely that the anti-adipogenic effect of LY3009120 may be linked to the reduced expression/phosphorylation of these adipogenic transcription factors. Adipocyte-specific proteins, including FAS and perilipin A, are also involved in adipocyte differentiation. It has been reported that FAS is a lipogenic enzyme involved in fatty acid synthesis (11), and perilipin A is an LD-associated protein that binds and stabilizes newly formed LDs during the differentiation of adipocytes (13,14,27). As LY3009120 was shown to lower levels of FAS and perilipin A in differentiating 3T3-L1 cells, it is possible that the attenuation of lipid accumulation and TG content induced by this drug is attributable to the downregulation of these proteins.

Another observation in the present study was the regulation of AMPK phosphorylation at T172, indicating activation, by LY3009120 in differentiating 3T3-L1 cells. AMPK is a key regulator of fat and energy metabolism (28,29), and notably, there have been several reports indicating that its activation inhibits adipogenesis (30-32). There is also evidence that the activation of AMPK induces the phosphorylation (at S79) of its downstream effector ACC, which lacks the enzymatic activity required to synthesize fatty acids (12). ACC is a multi-subunit lipogenic enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA for the biosynthesis of fatty acids (33). In addition, there is accumulating evidence that the activation of AMPK inhibits ATP-consuming anabolic processes but activates ATP-producing catabolic processes (34), in part via the phosphorylation of ACC (12). In the present study, LY3009120 demonstrated the ability to increase the phosphorylation of AMPK and decrease the phosphorylation and expression of ACC in differentiating 3T3-L1 cells. These results suggested that the anti-adipogenic and lipid-lowering effects of this drug are further mediated through the activation of AMPK and downregulation of ACC, which may lead to the inhibition of ATP-consuming anabolic processes, including fatty acid synthesis. In addition, its suppressive effect on the phosphorylation and expression of ACC is unlikely to be AMPK-dependent. LKB1 is the kinase principally responsible for AMPK phosphorylation (24,35,36), and a previous study demonstrated the regulation of AMPK by LKB1 in adipocytes (37). However, the present study showed that the phosphorylation and expression of LKB1 in differentiating 3T3-L1 cells were not markedly altered by LY3009120, being only marginally decreased on days 2 and 8 of differentiation. This indicated that promotion of the phosphorylation

of AMPK by LY3009120 occurs independently of LKB1. The phosphorylation of AMPK is also controlled by other kinases, including CAMKK2, also known as CAMKKb (38,39), and other mechanisms entirely, including changes in the intracellular AMP/ATP ratio (40). Therefore, future investigations are required to examine whether LY3009120 alters intracellular ATP levels (and the AMP/ATP ratio) and/or the expression and activity of CAMKK2 in differentiating 3T3-L1 cells, which is likely to improve current understanding of the activation of AMPK by this pan-Raf kinase inhibitor.

In conclusion, the present study is the first, to the best of our knowledge, to show that LY3009120 has an anti-adipogenic effect on 3T3-L1 cells, which may be mediated via control of the expression and/or phosphorylation of C/EBP- $\alpha$ , PPAR- $\gamma$ , STAT-3, FAS, ACC, perilipin A, and AMPK. Although important issues remain to be elucidated, for example, whether this effect can be reproduced in animal models of obesity, the present findings reveal that LY3009120 demonstrates potential as a treatment for obesity.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

SMY and YKP performed experiments. TYL, BCJ, JK, and YHL designed the work and analysed the data. TYL and BCJ wrote the paper. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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