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Involvement of C/EBPβ in monocytic differentiation of acute myeloid leukemia cells induced by LW-218, a new synthesized flavonoid

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Our previous study investigated the effects of differentiation inducted by flavonoids derived from a Chinese herb. In this study, we found that LW-218, a new synthesized flavonoid, inhibited proliferation and induced differentiation of acute myeloid leukemia cells. The IC50s of LW-218 in HL-60, U937, K562, and NB4 cell lines were all less than 5 μM, suggesting greater capacity than compounds we have reported. LW-218 induced differentiation effects including morphologic changes, NBT reduction, and both of CD11b and CD14 expression. Results of western blots and siRNA transfection revealed that LW-218 increased the LAP/LIP ratio of C/EBPβ which regulated monocytic differentiation of leukemia cells. Meanwhile, these differentiation effects could be attenuated by silencing PLSCR1 via siRNA transfection. In addition, regulation on LAP/LIP ratio, of C/EBPβ was properly mediated by PLSCR1 which was up-regulated by LW-218. All these results suggested that C/EBPβ was involved in regulation of PKCδ/PLSCR1 pathway during flavonoids-induced differentiation. LW-218 was a prospective differentiation inductor of AML cells and was requisite to proceed further investigation.

Key words: flavonoid, acute myeloid leukemia, differentiation, PLSCR1, C/EBPβ

Leukemias are characterized by the alteration of 2 sets of genes, those that give the malignancy a proliferative advantage and those associated with a block of differentiation[1]. Differentiation therapy has been applied clinically in cure of acute myeloid leukemia (AML). ATRA and arsenic trioxide are most distinguished differentiation inductors and make acute promyelocytic leukemia (APL), an AML subclass, become a highly curable disease[2]. A number of current treatments, such as tyrosine kinase inhibitors, and cytokines, induce differentiation of non-APL AML cells to some extent, but differentiation is not the main goal of these treatments. Forcing expression of certain transcription factors, such as CCAAT/enhancer binding proteins (C/EBPs), has also been useful in inducing differentiation in cell lines, but an effective agent to force expression of these genes in humans is yet to be discovered[3].

69% of anticancer drugs approved between 1940 and 2002 are either natural products or developed based on knowledge gained from natural products[4]. *Scutellaria* baicalensis is one of the most popular and multi-purpose Chinese herb used for treatment of inflammation, hypertension, cardiovascular diseases, and bacterial and viral infections. Many evidences demonstrate that *Scutellaria* also possesses potent anticancer activities and the main antitumor constituents of *Scutellaria* are flavonoids[5]. Flavonoids derived from *Scutellaria* including wogonoside, wogonin, baicalein and baicalin have been shown to inhibit growth of various human cancer cell lines. The doses of 50% inhibition of tumor proliferation are ranging between 20 and 200 μM, depending on the types of tumor cells tested[4]. Many derivatives have been synthetized for screening to achieve much more activities than their precursors.

In our previous studies, several flavonoids derived from *Scutellaria* have been reported to modulate proliferation and differentiation of AML cells through regulating phospholipid scramblase 1 (PLSCR1)[6-10]. PLSCR1 was originally identified as a type II transmembrane protein that mediates the calcium-dependent bidirectional movement of membrane phospholipids. It was also identified as a substrate for several kinases that participate in kinase signaling pathways, including c-Abl, c-Src, and protein kinase Cδ (PKCδ)[11]. The association of PLSCR1 and differentiation caused by flavonoids is yet to be discovered.

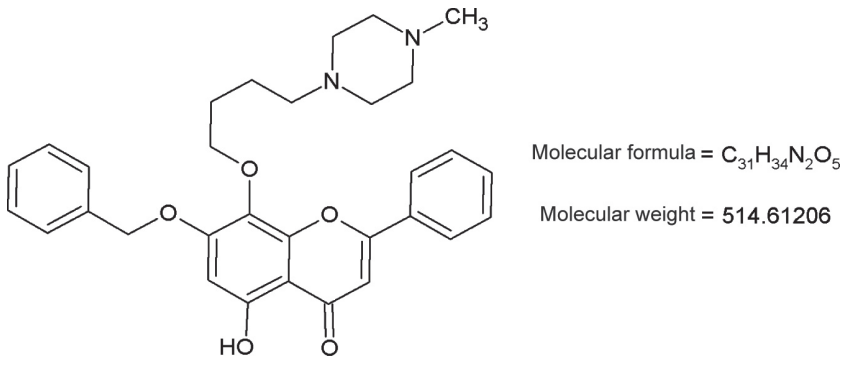


Figure 1. Molecular structure of LW-218 ($C_{31}H_{34}O_2N_5$, MW: 514.6).

LW-218 is a newly synthesized flavonoid with a piperazidine and a benzyl group substitution (Fig. 1). In this study, we investigated its effects of differentiation induction in AML cells and underlying molecular mechanism.

Materials and methods

Medicines and reagents. LW-218 was obtained from Dr. Zhiyu Li (China Pharmaceutical University, China) and was dissolved at a concentration of 0.1 M in DMSO as a stock solution stored at −70°C. ATRA was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO) and dissolved to 20mM as a stock solution in DMSO stored at −20°C. Rottlerin was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO) and dissolved to 0.01Min ethanol stored at −20°C. All drugs in stock solutions were diluted to final concentrations indicated in each assay in culture medium. The final concentration of DMSO did not exceed 0.1% and had no effects on the cell growth and differentiation.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO) and dissolved in PBS to 5 mg/ml as a stock and working solution. Giemsa stain, nitrobluetetrazolium (NBT) and PMA were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO). Phycoerythrin (PE) anti-human CD11b and fluorescein isothiocyanate (FITC) anti-human CD14 antibodies were obtained from eBioscience, Inc. (San Diego, CA).

Primary antibodies for β-actin (1:2000), PKCδ (1:500), p-ERK1/2 (1:500), PLSCR1 (1:500), and C/EBPβ (1:500) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary antibody for ERK1/2 (1:500) was from Bioworld Technology company Ltd. (Dublin, OH). Primary antibody for p-PKCδ (Ser643/676) (1:800) was from Cell Signaling Technology, Inc. (Beverly, MA). IRDye™800-conjugated secondary antibodies were obtained from Rockland, Inc. (Philadelphia, PA) and diluted at the ratio of 1:2000.

Cell culture. All cell lines used in this study were maintained in RPMI1640 (GIBCO, Invitrogen, Carlsbad, CA) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2. Cells were harvested by centrifugation at 500 rpm for 5 min and resuspended in fresh medium every day to make a stock cell suspension. For experiments, cells in logarithmic phase growth were seeded at a concentration of 8×10^4 cells/ml into a 6-well plate (Corning, New York, NY) or incubation flask and treated with LW-218 (0.5μM, 1μM and 2μM,respectively) and ATRA (2 μM).

Giemsa staining. Cells treated with LW-218 at indicated concentrations for 96 h were harvested and collected on the slides, air-dried and fixed in methanol for 10 min at room temperature, and stained with Giemsa for 5min. Then cells were observed under a light microscope (IX51; Olympus, Tokyo, Japan) and images were captured by DP2-BSW software (Olympus, Tokyo, Japan).

NBT reduction assay. Cells treated with LW-218 for 96 h at indicated concentrations were harvested and resuspended in 200μl supersaturated NBT solution containing 100 μg/ml PMA. After the cells were incubated at 37 °C for 30 min, NBT positive cells with blue deposit inside during 300 cells were calculated from three different fields to form a data point. The NBT positive ratio was calculated by the following formula:

$$\text{NBT positive ratio \%} = \left(\frac{\text{NBT positive cell counts}}{300}\right) \times 100\ \%$$

Cell-surface differentiation antigen expression assay. CD11b was used as a marker of granulocytic and monocytic differentiation while CD14 monocytic differentiation[12]. The two cell-surface differentiation antigens were measured by FACScan flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ) with a direct immunofluorescence analysis method as previously described[13]. Briefly, cells were harvested and blocked with 0.5% (w/v) BSA. Then cells were washed and incubated with monoclonal mouse anti-human PE-labeled anti-CD11b and FITC-labeled anti-CD14 for 40 min at 37°C. Fluorescence intensity was analyzed by flow cytometry. Data were based on examination of 10000 cells/sample selected randomly from 5×10^5 cells.

Western blots. Cells were harvested, washed with ice-cold PBS, and lysed with ice-cold lysisbuffer (50mMTris-HCl, 150mMNaCl, 3% nonidet P-40, 1mM EDTA, and protease inhibitor phenylmethanesulfonyl fluoride (PMSF), NaF, Leupeptin and DTT) on ice for 1h. Cell lysates were centrifuged at 12500rpm (5430R; Eppendorf, Hamburg, Germany) for 25min at 4°C and protein in the supernatant was quantified with BCA protein assay kit with a Varioskan multimode microplate spectrofluorometer and spectrophotometer (Thermo, Waltham, MA). Protein extracts were equally loaded on 10% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 1% (w/v) BSA in PBS at 37°C for 90min and incubated with primary antibody diluted at the indicated ratio in PBST (PBS and 1% Tween-20) at 37°C for 90min and then at 4°C overnight. The membranes were washed with PBST three times, each time for 10min, and then were incubated with IRDye^{TM}800-conjugated secondary antibody for 1 h at 37°C. After washing the membranes with PBST three times, each time for 10min, detection was performed by the Odyssey Infrared Imaging System (LI-COR; Lincoln, NE) and blot band intensity was quantified by Odyssey software. All blots were stripped and reprobed with polyclonal anti-β-actin antibody to ascertain equal loading of protein.

siRNA transfection. PLSCR1 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and transfection was performed using Lipofectamine 2000TM reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions provided by the vendor. The cells were then treated with LW-218 or vehicle and harvested for western blots and flow cytometryassays.

Statistical evaluation. All results are presented as mean ± S.E.M. from triplicate experiments performed in parallel, unless otherwise indicated. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by the Bonferroni post-test for multiple-group comparisons. The significance of differences is indicated as *P<0.05, **P<0.01.

Results

LW-218 inhibited growth of HL-60, U937, K562, and NB4 cells. The effects of LW-218 on the growth of HL-60, U937, K562, and NB4 cell lines at various concentrations (1–32 μM) were tested by MTT assay at 24, 48, 72, and 96 h time points

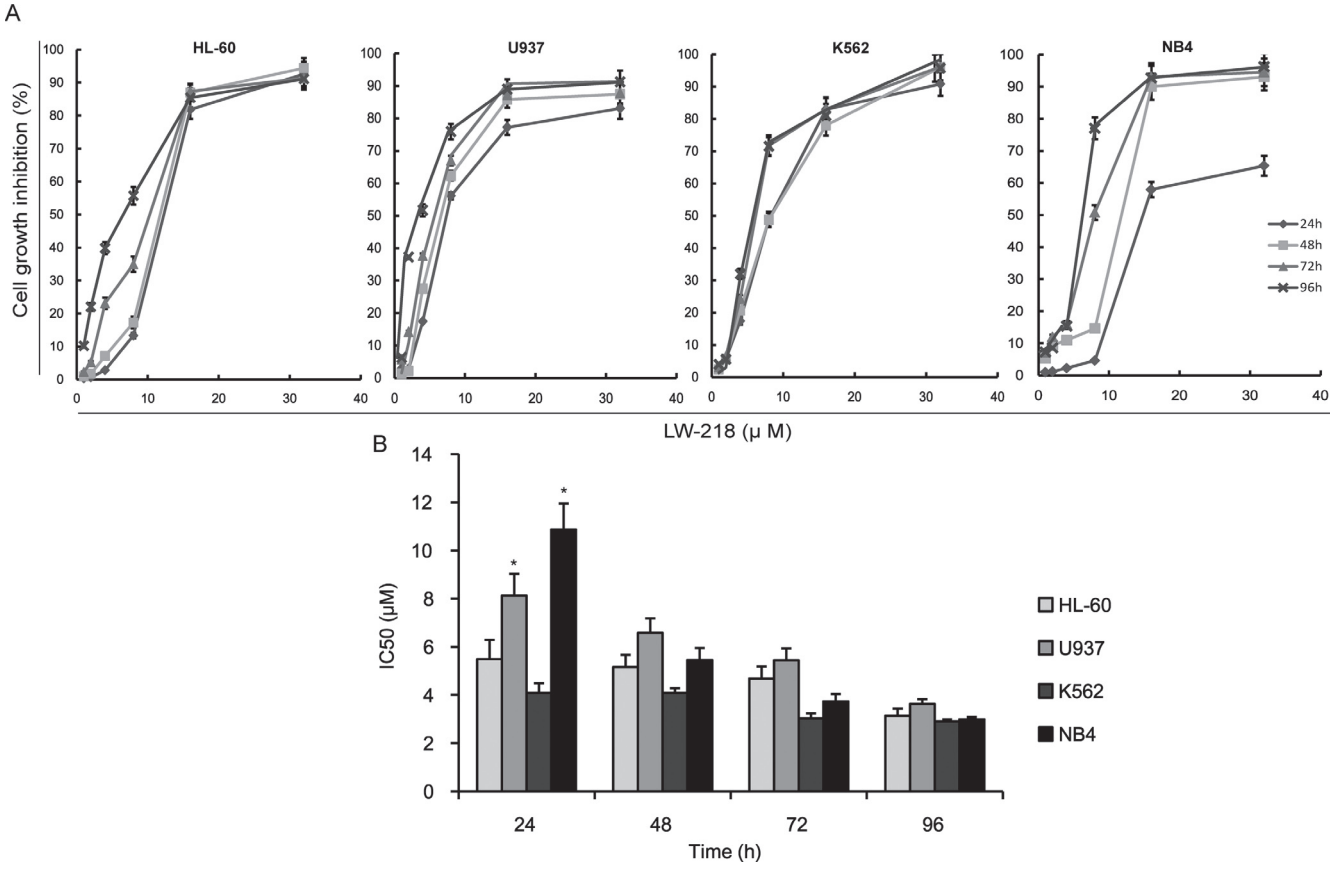
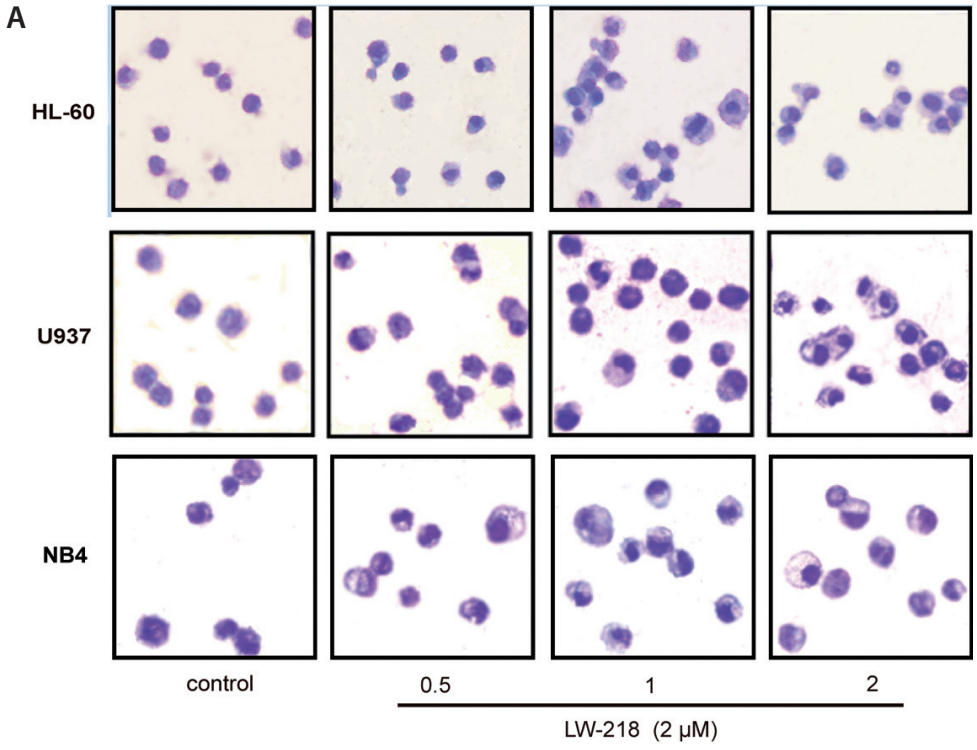
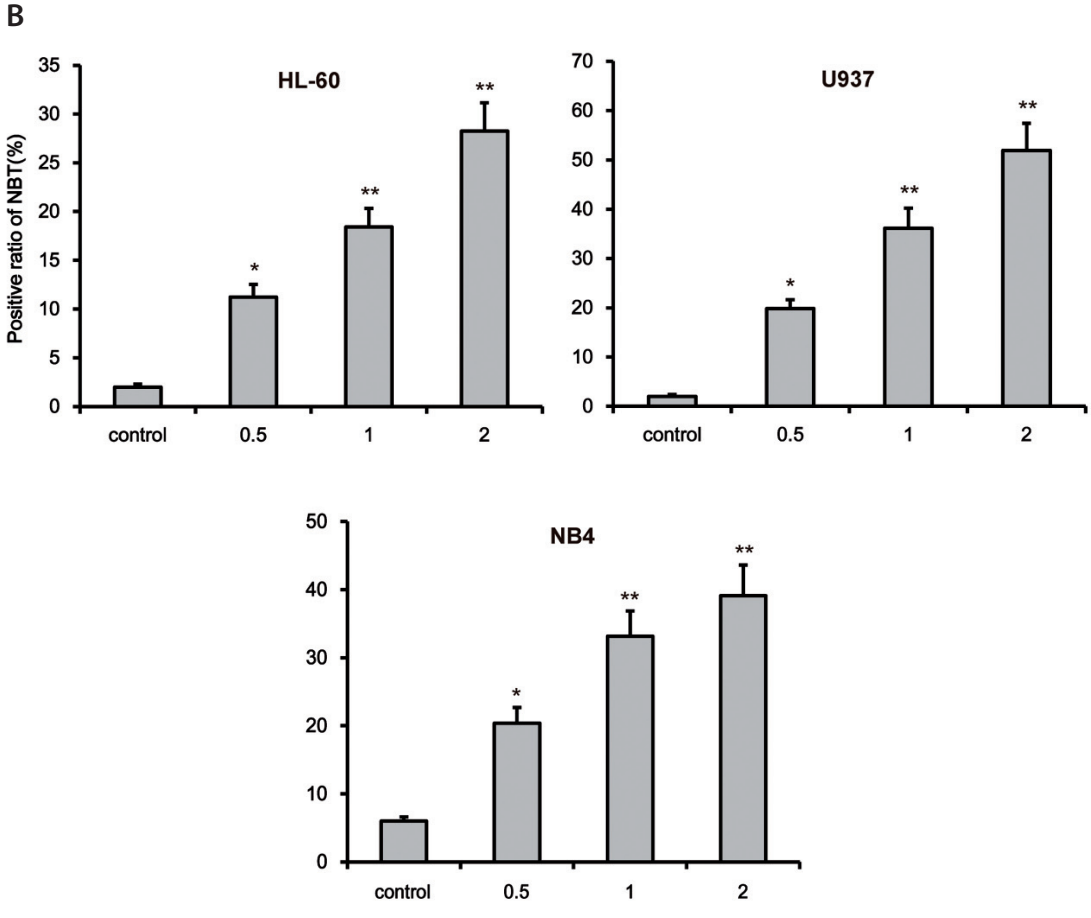
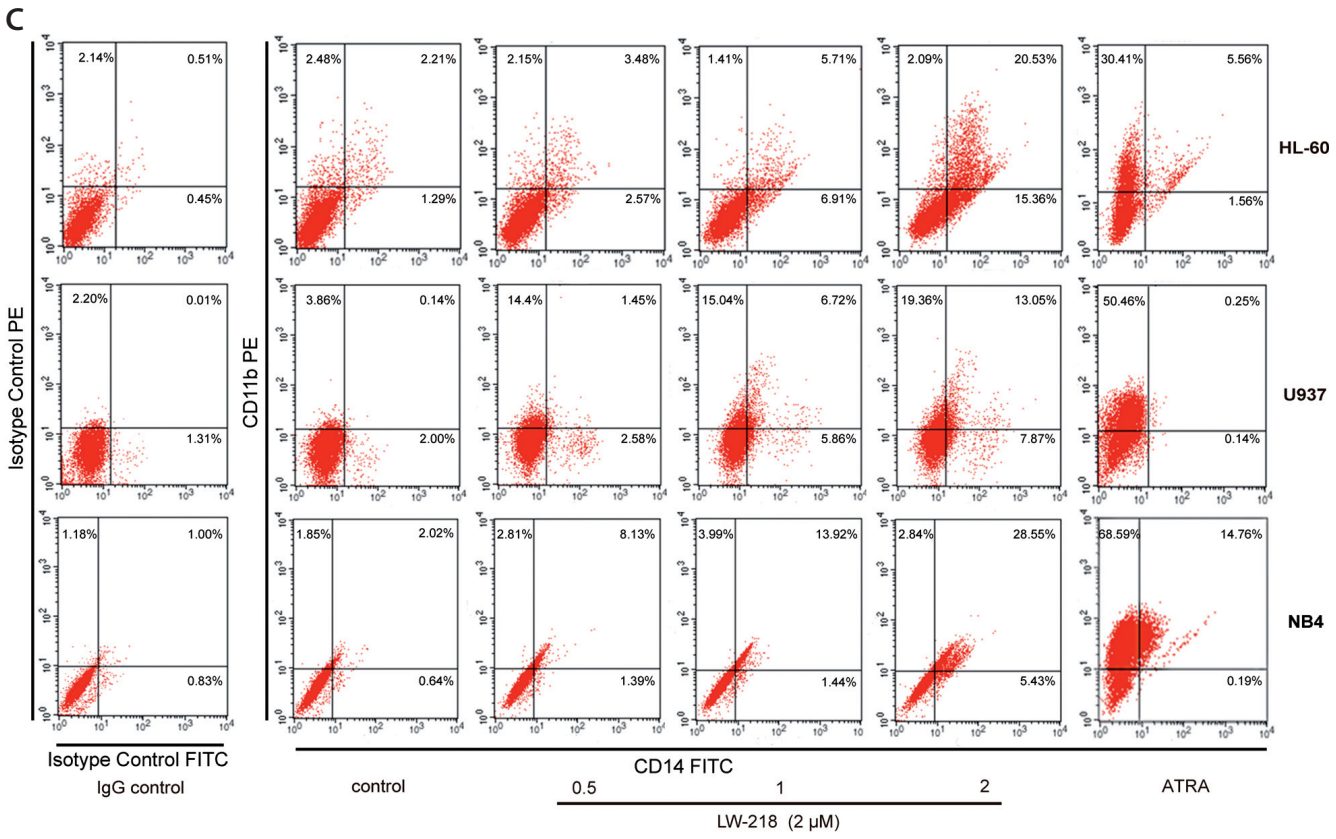


Figure 2. Effects of LW-218 on growth of NB4, U937, HL-60, and K562 cells. Cells were treated by various concentrations of LW-218 for 24, 48, 72, and 96 h. Then growth inhibition was evaluated by MTT assay. (A) Growth inhibition of four cell lines. (B) The IC50s of four cell lines at 96 h time point. (mean ± S.E.M., n = 3).


A
HL-60
U937
NB4
control
0.5
1
2
LW-218 (2 μM)


B
HL-60
U937
NB4
Positive ratio of NBT(%)
control
0.5
1
2
*
**



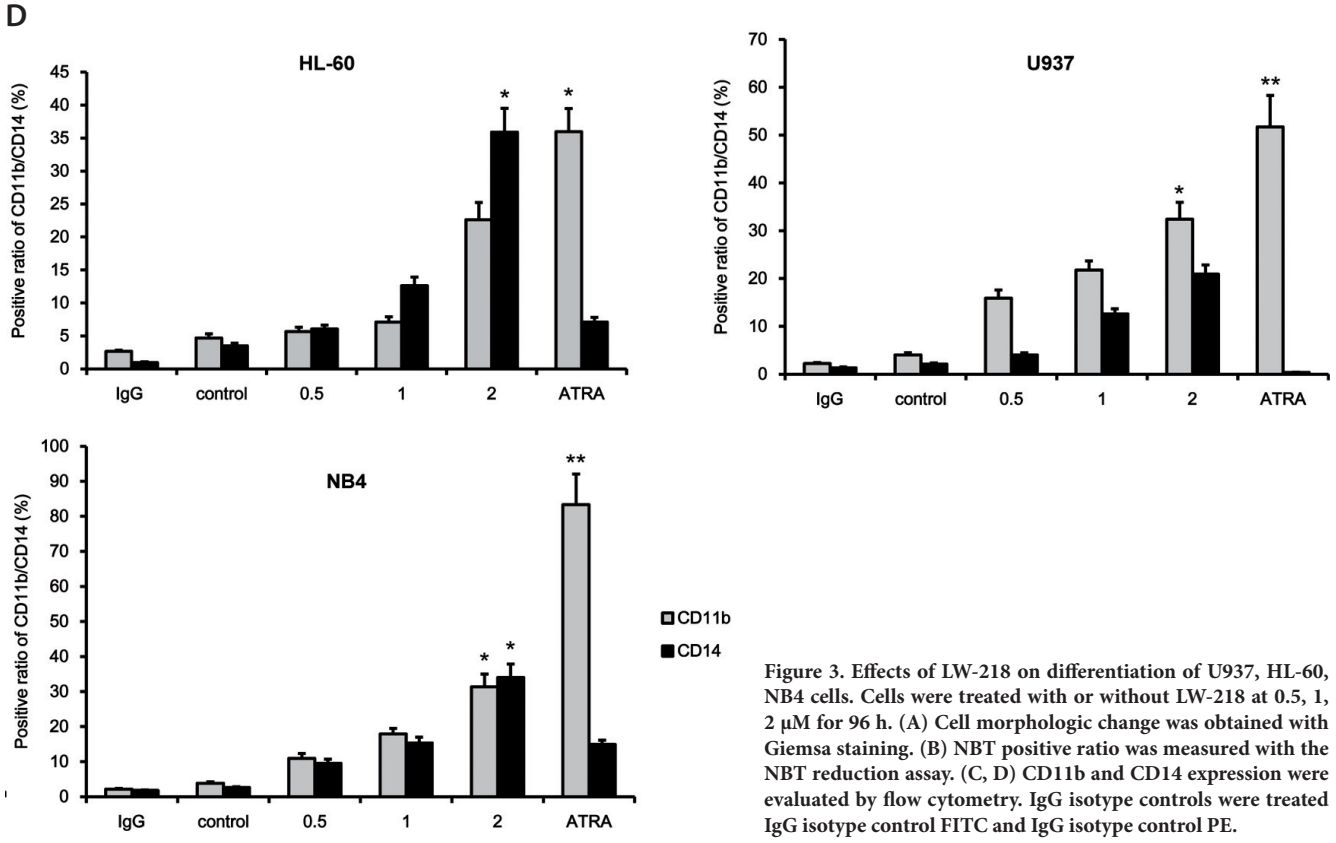


Figure 3. Effects of LW-218 on differentiation of U937, HL-60, NB4 cells. Cells were treated with or without LW-218 at 0.5, 1, 2 μM for 96 h. (A) Cell morphologic change was obtained with Giemsa staining. (B) NBT positive ratio was measured with the NBT reduction assay. (C, D) CD11b and CD14 expression were evaluated by flow cytometry. IgG isotype controls were treated IgG isotype control FITC and IgG isotype control PE.

(Fig. 2A). The continued growth of the cell lines at progressively reduced rates at increasing LW-218 concentrations suggests a cytostatic rather than acute toxic effect. IC50s in HL-60, U937, K562, and NB4 cells at 96 h time point were 3.134±1.13 μM, 3.624±0.56 μM, 2.897±0.32 μM, and 2.993±0.17 μM, respectively (Fig. 2B).

LW-218 induced differentiation of HL-60, U937, and NB4 cells. To evaluate the differentiation induction effects of LW-218 on AML cell lines, Giemsa stain, NBT reduction assay, and flow cytometry for CD11b and CD14 expression were performed. Based on the growth-inhibition results on AML cell lines, HL-60, U937, and NB4 cells were treated with different concentrations of LW-218 for 96 h. Cells were all characterized by a decreased nuclear/cytoplasmic ratio and ruffled plasma membranes and adherence to the bottom of the culture flasks, untreated control cells lacked these characteristics (Fig. 3A). Consistent with the cytological changes of terminal differentiation, the NBT reduction abilities of HL-60, U937, and NB4 cells were significantly increased compared with that of control cells (Fig. 3B). Furthermore, CD11b, a marker for both granulocytic and monocytic differentiation, and CD14, is a specific marker of monocytic differentiation, were all increased in LW-218-treated cells (Fig. 3C, D). CD14 expression was not increased in ATRA-treated cells, which differed from the situation in LW-218-treated cells.

LW-218 increased phosphorylation of PKCδ and expression of PLSCR1. PLSCR1 has been recognized to play potential roles in hematopoiesis and leukemogenesis. The expression of PLSCR1 protein in several myeloid leukemia cell lines and primary leukemia cells is markedly increased in response to some differentiation inducing agents such as ATRA, PMA and interferon (IFN), and is required for these agents triggered leukemic cell differentiation[14,15]. We assessed PLSCR1 expression of U937 cells when they were treated by 2 μM LW-218 for 24, 48, 72, and 96 h (Fig. 4A, B). Results showed PLSCR1 was up-regulated at 24 h and kept increasing until 96 h time point. PKCδ is the upstream kinase and its activation regulates expression of PLSCR1, so we evaluated phosphorylation level of PKCδ at 3, 6, 9, and 12 h. In Fig. 4C-D, The level of phosphorylated PKCδ was increased, which lasted from 3 h until 12 h time point. In addition, the enhancement of PLSCR1 induced by LW-218 was in a concentration dependent manner (Fig. 4E, F).

LW-218 increased LAP/LIP ratio. The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors plays an important role in controlling cell proliferation and differentiation. C/EBPα and C/EBPβ have been reported to be key regulators of granulocytic and monocytic differentiation, respectively [16,17]. After treatment of LW-218, the C/EBPα expression was barely changed in U937 cells (Fig. S2). But LW-218 showed a marked influence on C/EBPβ. Translation of the CEBPβ mRNA results in the synthesis of two main isoforms: liver-enriched activating protein (LAP), a positively acting 44 kDa isoform and liver-enriched inhibitory protein (LIP), an inhibitory 20 kDa isoform[18]. LAP/LIP ratio has been suggested to be an important determinant of regulation of gene expression by C/EBPβ. After U937 cells were treated by LW-218 for 48 h, LAP and LIP expression were both up-regulated, but LAP/LIP ratio was increased significantly (Fig. 4G, H).

PKCδ inhibitor reversed LW-218-induced up-regulation of PLSCR1 and LAP/LIP ratio, and attenuated differentiation effects. To investigate the role of PKCδ in PLSCR1, LAP/LIP ratio, and cell differentiation, rottlerin, a compound reported to be a PKCδ-selective inhibitor[19], was used to block activation of PKCδ. In Fig. 5A-D, results of western blots showed that rottlerin abolished phosphorylation of PKCδ and up-regulation of PLSCR1 in U937 cells, indicating that PKCδ activation played a role in LW-218 increasing PLSCR1 expression. Treatment by rottlerin increased LIP expression compared with control cells, while LW-218 showed no effect on LAP/LIP ratio (Fig. 5E, F). In addition, rottlerin also attenuated activity of NBT reduction and CD11b/CD14 expression in LW-218-treated U937 cells (Fig. 5G-I). It suggested that LW-218 exerted effects of differentiation in AML cells by biological functions of PLSCR1 and C/EBPβ whose expression was mediated by PKCδ.

Silencing PLSCR1 by siRNA up-regulated LIP expression and abolished LW-218-induced cell differentiation effects. To investigate the association between PLSCR1 and C/EBPβ, we used siRNA of PLSCR1 to silence its expression. In Fig. 6A-D, after PLSCR1 siRNA was transfected in U937 cells, the LIP was up-regulated markedly while LAP was unchanged, moreover, treatment of LW-218 showed no effect on the expression and relative ratio of LAP and LIP. These results indicated that PLSCR1 might be involved in the inhibition of LIP and play a role of mediator of LW-218-induced up-regulation of LAP. In addition, silencing PLSCR1 attenuated CD11b and CD14 expression of U937 cells which were caused by treatment of LW-218 (Fig. 6E, F), indicating the importance of PLSCR1 in differentiation effects of LW-218.

Discussion

Differentiation therapy has been proven to be clinically effective for APL. More agents of differentiation therapy are needed to be discovered for non-APL AML. Previous reports suggested that flavonoids have potential activities of inducing differentiation of AML cells. In this study about LW-218, a new synthesized flavonoid, we assessed growth inhibition of LW-218 on HL-60 (M2 of AML), U937 (M5 of AML), and NB4 cells (M3 of AML considered as APL), three AML cell lines, and K562, a chronic myelogenous leukemia (CML) cell line. LW-218 showed marked growth inhibitory effects in all of four cell lines, IC50s of 24 h, 48 h, 72 h, and 96 h were all less than 10 μM. More than 10 μM of LW-218 displayed acute toxic effects on cells. But LW-218 with doses less than 10 μM showed time-dependent anti-growth effects. We chose doses under IC50s to evaluate the differentiation effects and found that LW-218 had activities of monocytic differentiation effects, different from ATRA which is a granulocytic differentiation

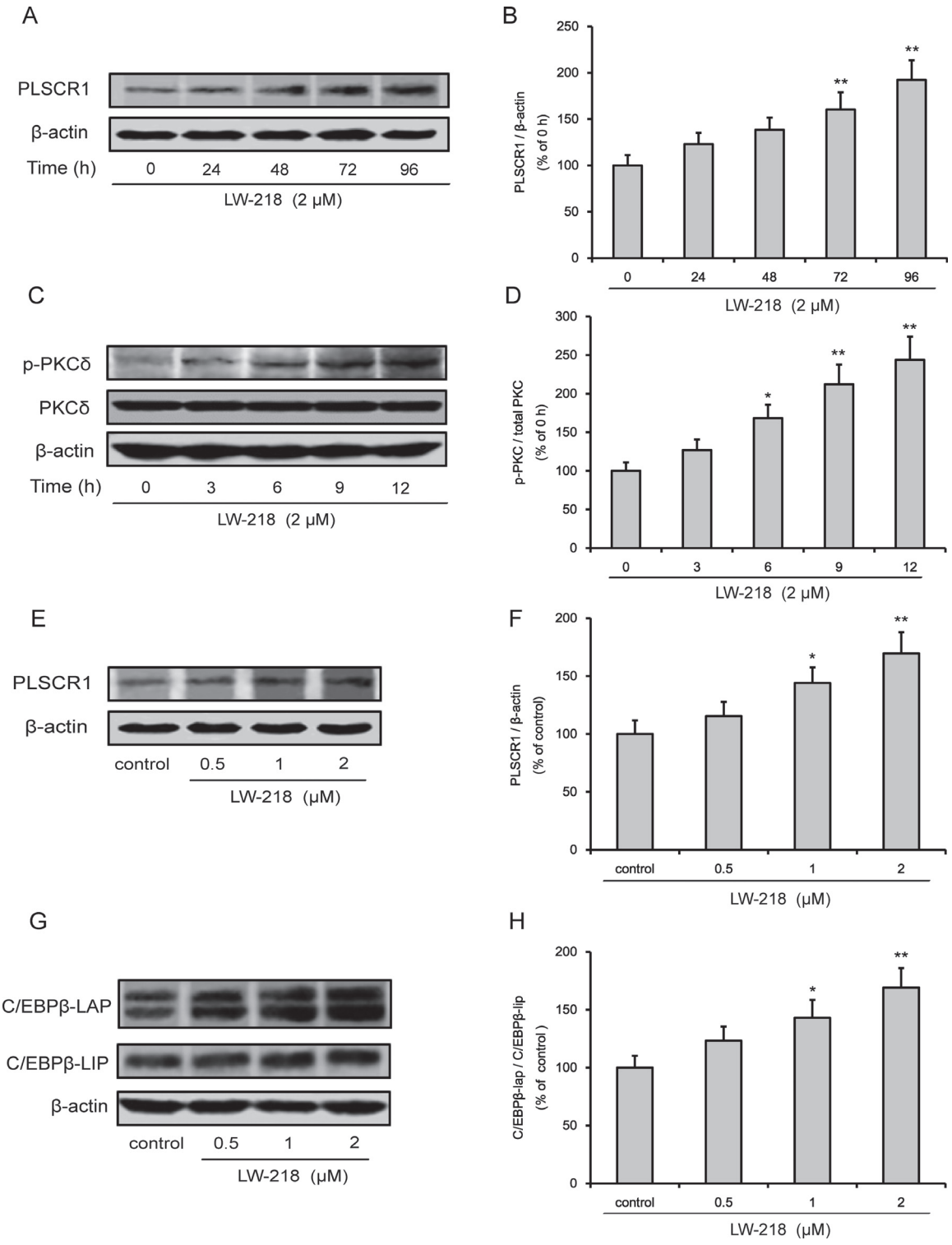
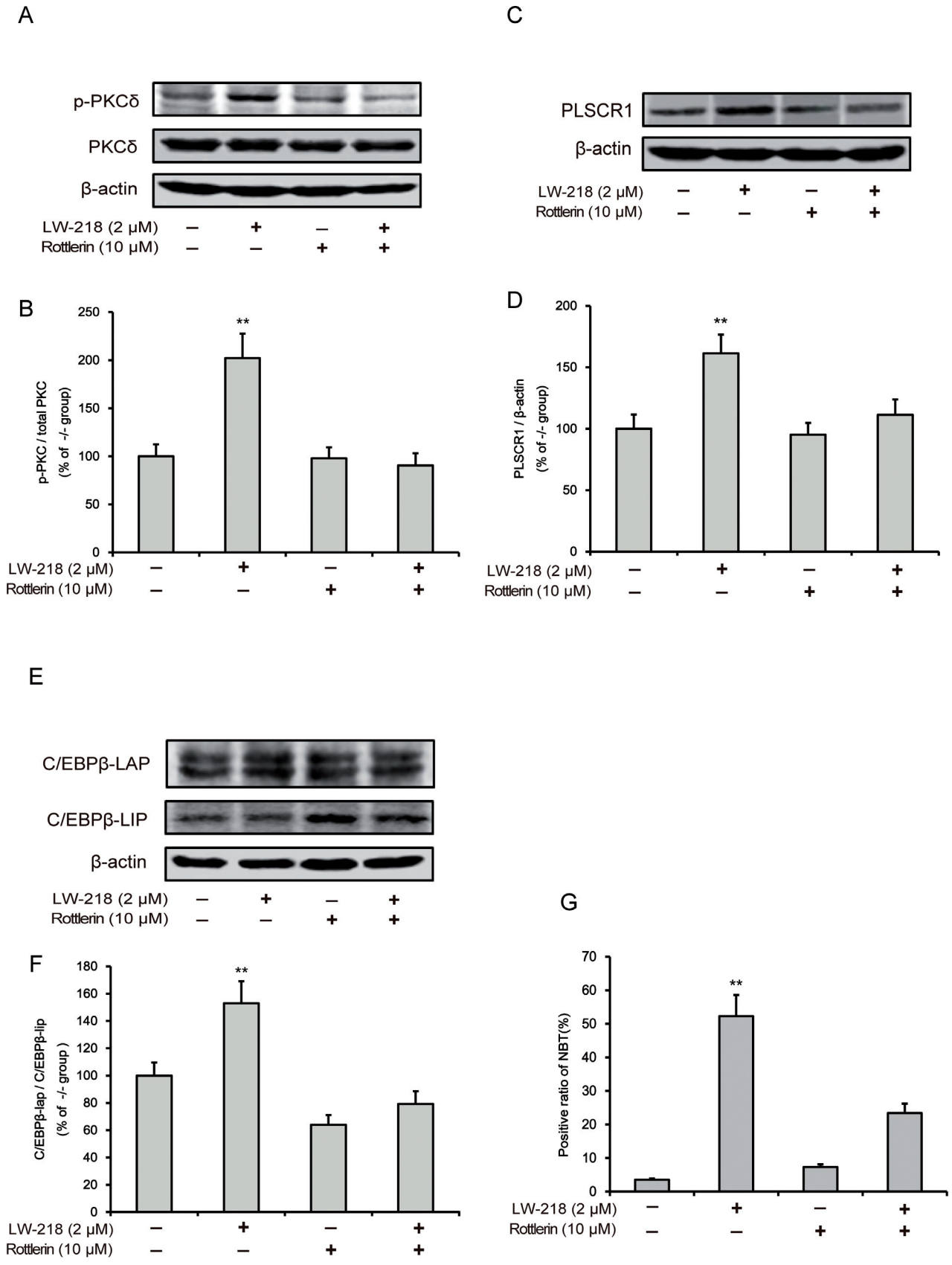


Figure 4. Effects of LW-218 on phosphorylation of PKCδ and expression of PLSCR1 and C/EBPβ. (A) PLSCR1 expression was assessed by western blots when U937 cells were treated with LW-218 for 0, 24, 48, 72, and 96 h. (B) Bars shown are mean ± SEM tested PLSCR1/β-actin fold change relative to the vehicle-treated group. (C) PKCδ and p-PKCδ expression were measured by western blots when U937 cells were treated with LW-218 for 0, 3, 6, 9, and 12 h. (D) Bars shown are mean ± SEM tested p-PKCδ/total PKCδ fold change relative to the vehicle-treated group. (E) PLSCR1 expression was assessed by western blots when U937 cells were treated with LW-218 at 0.5, 1, 2 μM for 96 h. (F) Bars shown are mean ± SEM PLSCR1/β-actin fold change relative to the vehicle-treated group. (G) The isoforms LAP and LIP expression was assessed by western blots when U937 cells were treated with LW-218 at 0.5, 1, 2 μM for 48 h. (H) Bars shown are mean ± SEM LAP/LIP fold change relative to the vehicle-treated group.


A
p-PKCδ
PKCδ
β-actin
LW-218 (2 μM) – + – +
Rottlerin (10 μM) – – + +
B
p-PKC / total PKC (% of -/- group)
250
200
150
100
50
0
**
LW-218 (2 μM) – + – +
Rottlerin (10 μM) – – + +
C
PLSCR1
β-actin
LW-218 (2 μM) – + – +
Rottlerin (10 μM) – – + +
D
PLSCR1 / β-actin (% of -/- group)
200
150
100
50
0
**
LW-218 (2 μM) – + – +
Rottlerin (10 μM) – – + +
E
C/EBPβ-LAP
C/EBPβ-LIP
β-actin
LW-218 (2 μM) – + – +
Rottlerin (10 μM) – – + +
F
C/EBPβ-lap / C/EBPβ-lip (% of -/- group)
180
160
140
120
100
80
60
40
20
0
**
LW-218 (2 μM) – + – +
Rottlerin (10 μM) – – + +
G
Positive ratio of NBT(%)
70
60
50
40
30
20
10
0
**
LW-218 (2 μM) – + – +
Rottlerin (10 μM) – – + +

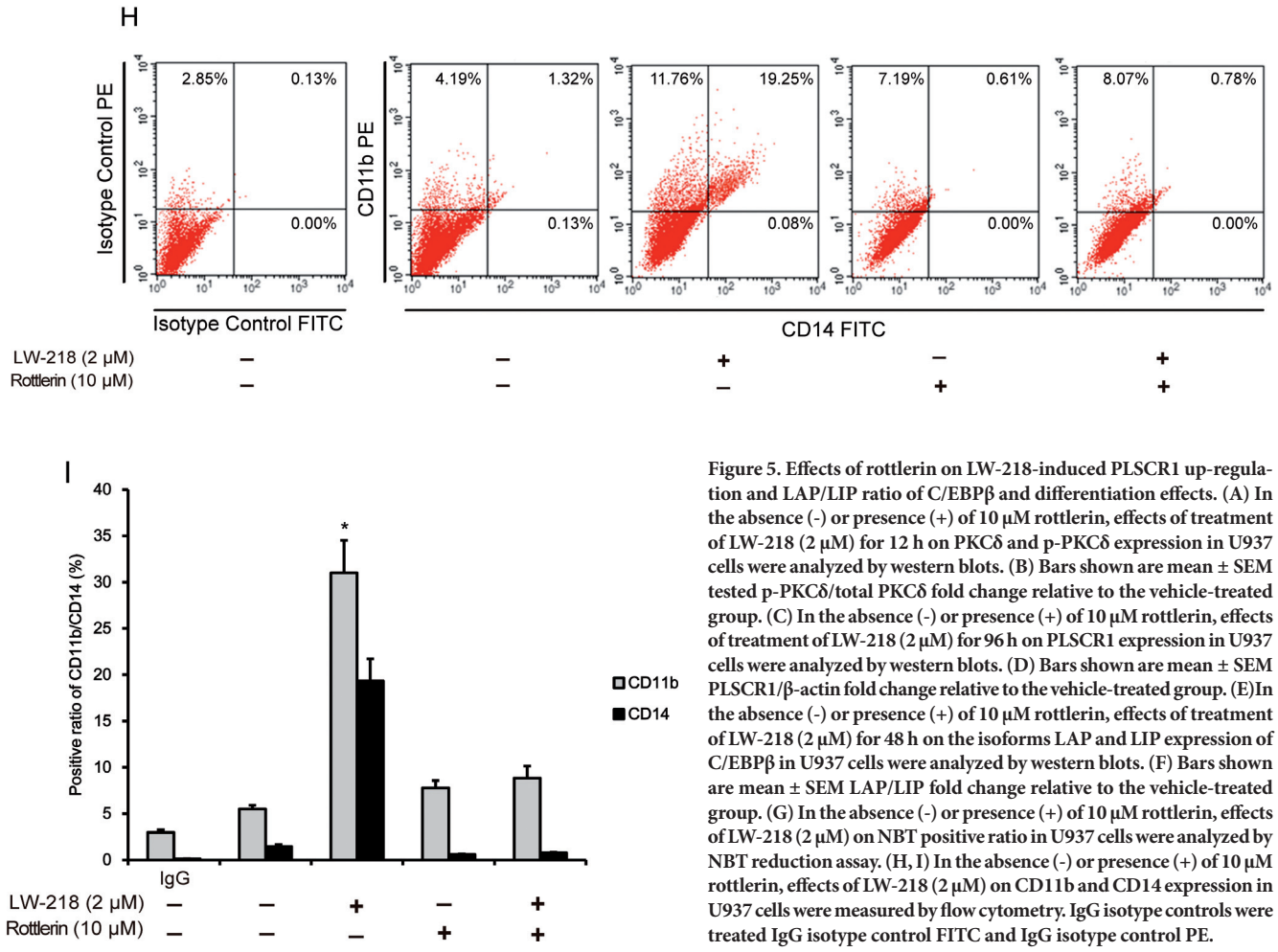


Figure 5. Effects of rottlerin on LW-218-induced PLSCR1 up-regulation and LAP/LIP ratio of C/EBPβ and differentiation effects. (A) In the absence (-) or presence (+) of 10 μM rottlerin, effects of treatment of LW-218 (2 μM) for 12 h on PKCδ and p-PKCδ expression in U937 cells were analyzed by western blots. (B) Bars shown are mean ± SEM tested p-PKCδ/total PKCδ fold change relative to the vehicle-treated group. (C) In the absence (-) or presence (+) of 10 μM rottlerin, effects of treatment of LW-218 (2 μM) for 96 h on PLSCR1 expression in U937 cells were analyzed by western blots. (D) Bars shown are mean ± SEM PLSCR1/β-actin fold change relative to the vehicle-treated group. (E)In the absence (-) or presence (+) of 10 μM rottlerin, effects of treatment of LW-218 (2 μM) for 48 h on the isoforms LAP and LIP expression of C/EBPβ in U937 cells were analyzed by western blots. (F) Bars shown are mean ± SEM LAP/LIP fold change relative to the vehicle-treated group. (G) In the absence (-) or presence (+) of 10 μM rottlerin, effects of LW-218 (2 μM) on NBT positive ratio in U937 cells were analyzed by NBT reduction assay. (H, I) In the absence (-) or presence (+) of 10 μM rottlerin, effects of LW-218 (2 μM) on CD11b and CD14 expression in U937 cells were measured by flow cytometry. IgG isotype controls were treated IgG isotype control FITC and IgG isotype control PE.

inducer. In study about molecular mechanism, we found that C/EBPβ, a regulator of monocytic differentiation, was modulated by LW-218 through increasing LAP/LIP ratio. Our results showed PKCδ and PLSCR1 were still required in LW-218-induced cell differentiation (Fig. 4). In addition, data about the influence of PKCδ/PLSCR1 pathway on C/EBPβ showed that either PKCδ inhibition or PLSCR1 silencing could markedly up-regulated expression of LIP, the inhibitory isoform of C/EBPβ, while treatment of LW-218 showed no effects on LAP expression (Fig. 5-6).

PLSCR1 has been reported to play an important role in leukemia cells differentiation induced by inductors such as ATRA and PMA. However, few evidences suggested the association between PLSCR1 and monocytic differentiation. In our previous reports, wogonoside, another flavonoid compound, has ability of trafficking PLSCR1 into the nucleus and facilitated its binding to the inositol 1,4,5-trisphosphate receptor 1 (IP3R1) promoter[8]. IP3R1 is known to play a key role in IP3-mediated mobilization of intracellular calcium ion Ca^{2+} stores from the endoplasmic reticulum in a variety of cells and tissues, and it is also essential for cell growth and differentiation. Mechanism of monocytic differentiation induced by flavonoid compounds has not been investigated. In this study, we found that the influence of PKCδ/PLSCR1 pathway on LAP/LIP ratio, expression of two isoforms of CEBPβ, might explain the monocytic differentiation effects of LW-218, suggesting a novel mechanism of flavonoid compounds with differentiation induction effects. The interaction of PKCδ/PLSCR1 pathway and LAP/LIP ratio was probably performed via its inhibition on LAP. The formation of different isoforms of C/EBPβ is mainly based on alternative translation. This procedure depends on many factors including weak Kozak consensus sequences around LAP or LIP, an ex-frame upstream open reading frame (uORF) which is essential for LIP expression, and the modulation of the translation machinery by translation initiation factors (especially eIF-2α and eIF-4E)[20,21]. The mechanisms of regulation of LAP/LIP by PLSCR1 or LW-218 are unknown and need further investigation.

PLSCR1 induction in leukemia cells has been reported to induce granulocytic differentiation and increase cyclin-

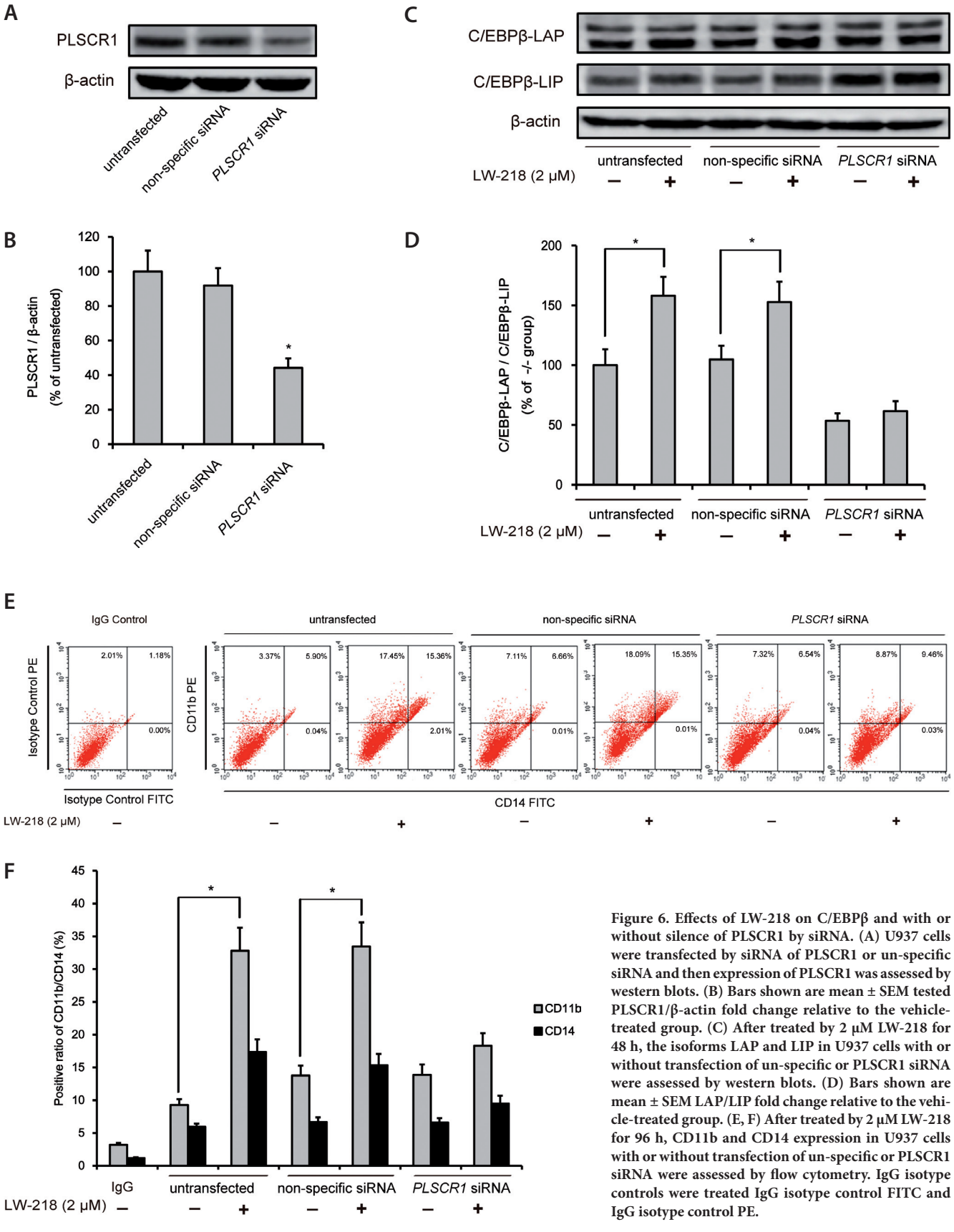


Figure 6. Effects of LW-218 on C/EBPβ and with or without silence of PLSCR1 by siRNA. (A) U937 cells were transfected by siRNA of PLSCR1 or un-specific siRNA and then expression of PLSCR1 was assessed by western blots. (B) Bars shown are mean ± SEM tested PLSCR1/β-actin fold change relative to the vehicle-treated group. (C) After treated by 2 μM LW-218 for 48 h, the isoforms LAP and LIP in U937 cells with or without transfection of un-specific or PLSCR1 siRNA were assessed by western blots. (D) Bars shown are mean ± SEM LAP/LIP fold change relative to the vehicle-treated group. (E, F) After treated by 2 μM LW-218 for 96 h, CD11b and CD14 expression in U937 cells with or without transfection of un-specific or PLSCR1 siRNA were assessed by flow cytometry. IgG isotype controls were treated IgG isotype control FITC and IgG isotype control PE.

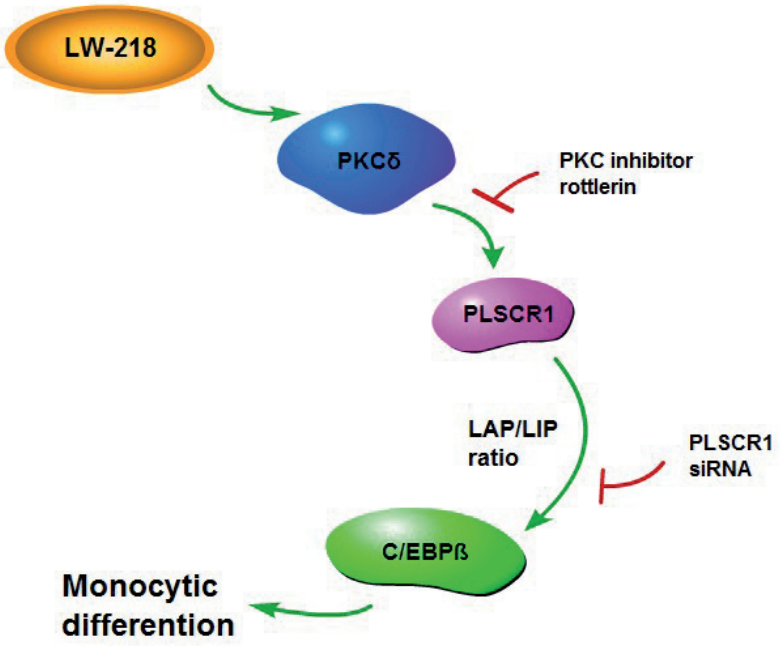


Figure 7. LW-218 induces AML cells to monocytic differentiate via PKCδ/PLSCR1 pathway involving C/EBPβ regulation.

dependent kinase inhibitors p27Kip1 and p21Cip1 proteins, together with down-regulation of S phase kinase-associated protein 2 (SKP2). Additionally, PLSCR1 induction significantly decreased c-Myc protein and anti-apoptotic Bcl-2 protein. But PLSCR1 failed to change protein levels of AML1, PU.1 and C/EBPα proteins, several transcriptional factors critical for leukemic cell differentiation[22]. No evidences showed how PLSCR1 participate in monocytic differentiation of leukemia cells. In our study, we focused to mechanism of LW-218 on AML cells and preliminary showed the association of PLSCR1 and C/EBPβ in cell monocytic differentiation.

Moreover, in Fig.3, LW-218 could markedly increase the expression of CD11b and CD14 in all AML cells to a similar degree. However, U937 cells exerted a higher NBT positive ratio increase and a better time-dependent reduction effect on cell growth than the other cells. In addition, we had studied the mechanism of all these three AML cell lines during the research (data not shown), suggesting the protein changes of U937 cells were the most remarkable among these cell lines. Taken together, U937 cells are the most sensitive and suitable AML cells to reflect the effect of LW-218 to induced proliferation inhibition and differentiation.

Besides the new finding about mechanism of differentiation induction by flavonoid, we also observed that LW-218 showed similar inhibition effects on leukemia cell lines including HL-60, U937, NB4, three AML cell lines, and K562, a CML cell line, with close IC50s which were 3.134, 3.624, 2.993, and 2.897, respectively. We suggested that LW-218 might be a broad-spectrum anti-leukemia compound. To support this point, more data about other leukemia cell lines and primary blasts would be included in future study.

In conclusion, our results not only represented that LW-218 inhibited growth and induced differentiation of leukemia cells, but also provided novel insights into the mechanisms of C/EBPβ and PLSCR1 in leukemia cell differentiation (Fig. 7).

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Supplemental information

Involvement of C/EBPβ in monocytic differentiation of acute myeloid leukemia cells induced by LW-218, a new synthesized flavonoid

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Section 1: Growth-inhibitory effects of LW-218 on PBMC

Materials and methods

Medicines and Reagents

Lymphocyte separation media was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China)

Cell culture

Peripheral blood mononuclear cell (PBMC) was obtained from healthy donor and isolated by using discontinuous density gradient centrifugation method with lymphocyte separation media and used as a normal cell control.

Results

Growth-inhibitory effects of LW-218 on PBMC

MTT assay showed that the concentration of LW-218 we used to perform our research (0.5 μM, 1 μM and 2 μM) incubated for 96 h had no obvious growth inhibitory effects on PBMC (Fig. S1). The cell growth inhibition ratio increased with the concentration of LW-218 rose. Besides, the cell growth inhibition of the same concentration of LW-218 shows a significant difference between PBMC and U937 cells over 2 μM.

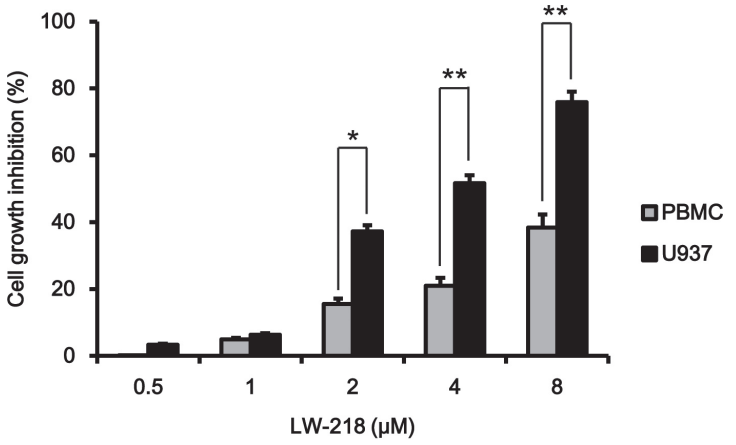

Figure S1. Growth inhibition effects of LW-218 on PBMC and U937 cells at 96 h. Cells were treated by 0.5, 1, 2, 4, 8 μM of LW-218 for 96 h. Then growth inhibition was evaluated by MTT assay.

Section 2: Effects of LW-218 on expression of C/EBPα.

Materials and methods

Medicines and Reagents

C/EBPα (1:500) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Western blots

Western blots assay was implemented as detailed in section "Materials and methods" in the original study.

Results

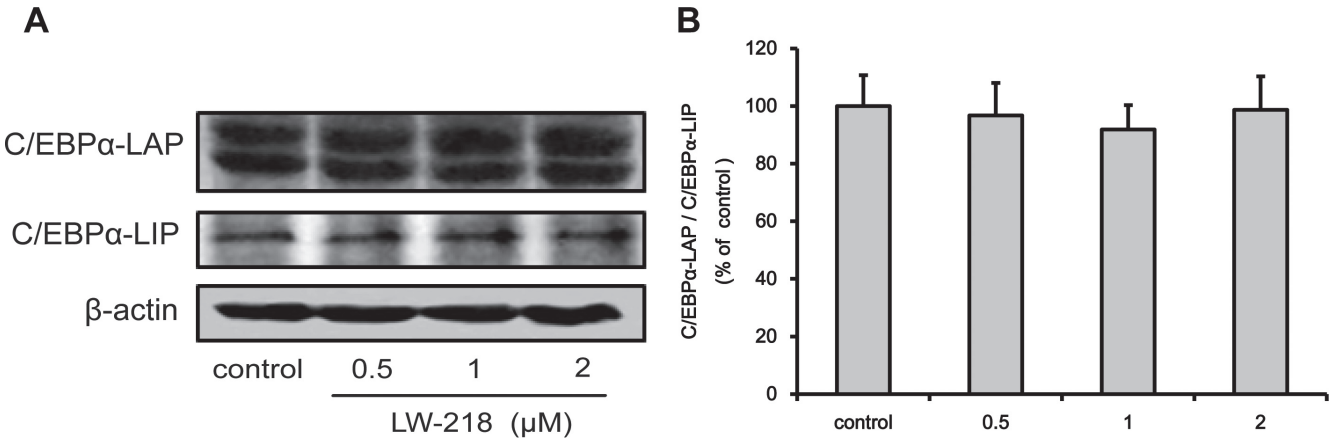

Figure S2. Effects of LW-218 on expression of C/EBPα. (A) The isoforms LAP and LIP of C/EBPα expression was assessed by western blots when U937 cells were treated with LW-218 at 0.5, 1, 2 μM for 48 h. (B) Bars shown are mean ± SEM LAP/LIP fold change relative to the vehicle-treated group.