Salinomycin induces apoptosis and differentiation in human acute promyelocytic leukemia cells

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Abstract. At present, acute promyelocytic leukemia (APL) is the most curable form of acute myeloid leukemia and can be treated using all-*trans* retinoic acid and arsenic trioxide. However, the current treatment of APL is associated with some issues such as drug toxicity, resistance and relapse. Therefore, other strategies are necessary for APL treatment. In the present study, we investigated the effects of salinomycin (SAL) on APL cell lines NB4 and HL-60 and determined its possible mechanisms. We observed that SAL inhibited cell proliferation, as determined by performing Cell Counting Kit-8 (CCK-8) assay, promoted cell apoptosis, as determined based on morphological changes, and increased Annexin V/propidium iodide (PI)-positive apoptotic cell percentage. Treatment with SAL increased Bax/Bcl-2 and cytochrome *c* expression and activated caspase-3 and -9, thus leading to poly(ADP-ribose) polymerase (PARP) cleavage and resulting in cell apoptosis. These results revealed that SAL induced cell apoptosis through activation of the intrinsic apoptosis pathway. The present study is the first to show that SAL induced the differentiation of APL cells, as determined based on mature morphological changes, increased NBT-positive cell and CD11b-positive cell percentages and increased CD11b and C/EBPβ levels. Furthermore, SAL decreased the expression of β-catenin and its targets cyclin D1 and C-myc. Results of immunofluorescence analysis revealed that SAL markedly decreased the β-catenin level in both the nucleus and cytoplasm. Combination treatment with SAL and IWR-1, an inhibitor of Wnt signaling, synergistically triggered SAL-induced differentiation of APL cells. These findings demonstrated that SAL effectively inhibited cell prolifera-

tion accompanied by induction of apoptosis and promotion of cell differentiation by inhibiting Wnt/β-catenin signaling. Collectively, these data revealed that SAL is a potential drug for treatment of APL.

Introduction

Acute promyelocytic leukemia (APL), a distinct subtype of acute myeloid leukemia (AML), is characterized by reciprocal chromosomal translocation of t (15;17), which results in the production of promyelocytic leukemia-retinoic acid receptor α (PML-RARα) fusion protein (1). For decades, APL has been considered as the most malignant form of AML due to its severe bleeding tendency and high early mortality rate (2,3). Notably, at present, APL is the most curable form of AML and can be treated using all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO), which mainly induce cell differentiation and apoptosis (3,4). However, APL treatment is associated with some issues such as ATRA or ATO resistance, relapse, differentiation syndrome and adverse effects (5-7). Therefore, it is necessary to identify other therapeutic strategies for APL treatment.

Salinomycin (SAL), a polyether antibiotic, is widely used as an anticoccidial drug for poultry (8). Recently, Gupta *et al* performed high-throughput screening of 16,000 compounds and found that SAL selectively killed breast cancer stem cells (CSCs) at least 100-times more effectively than conventional chemotherapeutic drug paclitaxel (9). Further studies have indicated that SAL exerts potential anticancer effects against different human cancer cell types, including lung, gastric and prostate cancer, and glioblastoma cells (10-13), without adversely affecting healthy cells (14-16). Accumulating evidence suggests that the presence of CSCs, which have capability of self-renewal and tumor-initiating capacities, are the major cause of drug resistance and relapse after therapy (17). SAL affects the proliferation of various CSCs including those present in breast, gastric and ovarian cancer (9,18,19). A previous study revealed that SAL treatment also reversed multidrug resistance in leukemia stem cells (LSCs) such as KG-1a cells (20). Moreover, SAL was revealed to reverse multi-drug resistance in many cancer cell types (21,22). Collectively, these findings revealed that SAL

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is a potential anticancer drug. However, limited studies have assessed the effect of SAL against leukemia.

SAL is also known as an inhibitor of Wnt/β-catenin signaling (23) which plays key roles in both normal cell development and tumorigenesis (24). Aberrant activation of Wnt/β-catenin signaling is frequently implicated in the pathogenesis of AML. Notably, high β-catenin expression was observed in both AML cell lines and primary blasts (25,26). In addition, recent studies have shown that Wnt/β-catenin signaling was associated with leukemia cell differentiation. Attenuation of Wnt/β-catenin signaling promoted cell differentiation (27), whereas its activation blocked monocyte-macrophage differentiation in AML cell lines (28). Therefore, we hypothesized that SAL induced APL cell differentiation by blocking Wnt/β-catenin signaling.

Since limited studies have assessed the cytotoxicity of SAL against leukemia cells and its effect on leukemia cell differentiation, we investigated the effect of SAL in APL cell lines NB4 and HL-60 in the present study. We found that SAL markedly inhibited cell proliferation and induced the apoptosis and differentiation of APL cell lines NB4 and HL-60. Our results provide a foundation for further exploring the clinical use of SAL.

Materials and methods

Materials. Salinomycin (HY-15597) and IWR-1 (HY-12238) were purchased from MedChem Express (Monmouth Junction, NJ, USA). Cell Counting Kit-8 (CCK-8) reagent was purchased from Sevenseas Futai Biotechnology Co., Ltd. (Shanghai, China). Hoechst 33258 reagent was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Wright Giemsa stain solution was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The phycoerythrin (PE)-conjugated CD11b antibody (1:20; cat. no. 301306) was purchased from BioLegend, Inc., (San Diego, CA, USA). The antibody against CD11b (1:1,000; cat. no. ab133357) was purchased from Abcam (Cambridge, MA, USA). The antibody against LRP6 (1:1,000; cat. no. sc-25317) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against caspase-3 (1:1,000; cat. no. 9665),caspase-9 (1:1,000; cat. no. 9504), cleaved caspase-9 (1:1,000; cat. no. 9509), cytochrome *c* (1:1,000; cat. no. 11940), β-catenin (1:1,000; cat. no. 8480), cyclin D1 (1:1,000; cat. no. 2922) and C-myc (1:1,000; cat. no. 5605) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against Bax (1:500; cat. no. wl01637) and Bcl-2 (1:500; cat. no. wl01158), PARP (1:500; cat. no. WL01932) were purchased from Wanleibio Co., Ltd. (Shenyang, China). Goat anti-rabbit secondary antibody (1:4,000; cat. no. ZB-2301), goat anti-mouse secondary antibody (1:4,000; cat. no. ZB-2305) and anti-β-actin antibody (1:1,000; cat. no. BM0627) were purchased from Zhongshan Golden Bridge Biotechnology; OriGene Technologies (Beijing, China).

Cell lines and culture. The human APL cell lines NB4 and HL-60 obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China) were then maintained in our own laboratory and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; both from Gibco; Life

Technologies, Carlsbad, CA, USA) and penicillin (100 mg/ml) and streptomycin (100 mg/ml) in an environment that contained 5% CO_2 at 37˚C.

Cell viability assay. NB4 or HL-60 cells were seeded into 96-well plates with RPMI-1640 medium supplemented with 10% FBS. For experimental purposes, the cells were seeded at a density of $1x10^4$ cells/well, and then treated with different concentrations of SAL for 24, 48 or 72 h, respectively. Then, 10 μl CCK-8 reagent was added to each well. Following incubation for 2 h, cell viability was assessed by detection of the absorbance at 450 nm using a spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was repeated at least three times.

Hoechst 33258 staining. Cells were treated with SAL for 48 h. Cells were collected and washed twice using phosphate-buffered saline (PBS) and plated onto the glass slides. After being fixed with 4% paraformaldehyde for 20 min, the cells were permeabilized with 0.1% Triton X-100 for 15 min. Subsequently, the cells were stained with Hoechst 33258 reagent for 10 min at 37˚C. The slides were washed three times with PBS. Finally, the nuclear morphological changes were observed under a fluorescence microscope (magnification, x400).

Wright-Giemsa staining. After 72 h of treatment, cells were collected and washed with PBS three times. Then the cells were resuspended in PBS and fixed on slides. The morphological changes of the cells were examined by optical microscopy (magnification, x200 or x1,000) after staining with Wright-Giemsa stain solution.

NBT reduction assay. For the nitroblue tetrazolium (NBT) reduction assay, NB4 and HL-60 cells were treated with SAL (0.6 μM) or ATRA (1 μM, as a positive control) for 3 days. Then each cell suspension was mixed with an equal volume of RPMI-1640 medium containing 1 mg/ml NBT (Sigma-Aldrich, St. Louis, MO, USA) and 200 ng/ml TPA (Sigma-Aldrich; Merck) for 30 min at 37˚C. A total of 200 cells were counted by optical microscope (magnification, x1,000) after staining with or without Wright-Giemsa stain solution

Western blot analysis. For protein analysis, harvested cells were washed with ice-cold phosphate-buffered saline (PBS) three times and lysed in RIPA solution containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF), phosphatase inhibitor NaF and Na3VO3. Protein concentration was measured by BCA method. Equal amounts of extracted total protein (30 or 50 μg) were separated by 10% or 12% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk for 2 h at room temperature, and then incubated with the primary antibodies (1:1,000 or 1:500) overnight at 4˚C. The membranes were then incubated with goat anti-rabbit or goat anti-mouse secondary antibodies (1:4,000) for 1 h at 37˚C. After washing with Tris-buffered saline containing Tween-20 (TBST), the immunoreactive complexes were visualized using an enhanced chemiluminescence system (GE Healthcare, Marlborough, MA, USA). β-actin was used
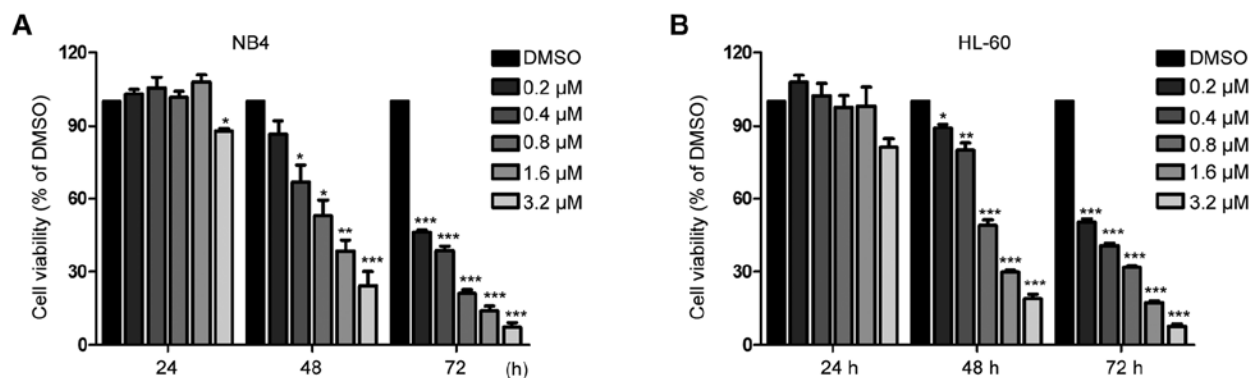
Figure 1. SAL inhibits the proliferation of APL cells. (A) NB4 and (B) HL-60 cells were cultured with SAL at the indicated dosages for 1, 2 and 3 days. Cell viability was detected by CCK-8 assays. Data are expressed as the means ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. the DMSO group, n=3. SAL, salinomycin; APL, acute promyelocytic leukemia; CCK-8, Cell Counting Kit-8; DMSO, dimethyl sulphoxide.

as the internal positive control. Each experiment was repeated at least three times.

Flow cytometric assay. For apoptosis analysis, cells treated with different concentrations of SAL for 48 h were harvested and washed three times with pre-cold PBS. Cells were resuspended and stained with Annexin V-FITC and propidium iodide (PI) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The rate of cell apoptosis was analyzed using a FACSorter (BD Biosciences, San Jose, CA, USA) after incubation for 15 min at room temperature.

For the detection of cell surface differentiation marker CD11b, cells treated with SAL or ATRA for 72 h were washed three times with ice-cold PBS and then incubated with phycoerythrin (PE)-conjugated CD11b antibody at 4˚C for 30 min in the dark. The cells were then washed three times with ice-cold PBS and then analyzed using flow cytometry (BD FACSVantage; BD Biosciences) and CellQuest Pro software version 5.1 (BD Pharmingen; BD Biosciemces, San Diego, CA, USA).

Indirect immunofluorescence assay. The localization of β-catenin was confirmed by indirect immunofluorescence assay. Cells were harvested, centrifuged at 1,000 x g for 5 min at room temperature and washed three times using PBS. Then, cells fixed with 4% paraformaldehyde for 20 min were permeabilized with 0.1% Triton X-100 for 15 min and then blocked with 10% goat serum for 30 min at room temperature. The slides were incubated with the primary antibody β-catenin (1:200; cat. no. 8480) at 4˚C overnight. After being washed three times with PBS, the cells were incubated with secondary antibody goat against rabbit-IgG-FITC (1:200; cat. no. ZF0311; Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies) for 1 h at room temperature. Then nuclei were stained using DAPI (1:10; Beyotime Institute of Biotechnology) for 5 min at room temperature. Finally, the coverslips were viewed using a fluorescence microscope (magnification, x400) (Nikon Corp., Tokyo, Japan).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data were expressed as the means ± standard (SD). One-way analysis of variance followed by the Dunnett's test was performed and Student's t-test was used for comparisons. A value of P<0.05 was considered to indicate a statistically significant result.

Results

Salinomycin inhibits the proliferation of APL cells. We performed CCK-8 assay to determine the viability of NB4 and HL-60 cells which were treated with various concentrations (0-3.2 μM) of SAL for 24, 48 and 72 h. SAL significantly inhibited cell viability in a dose-dependent manner after treatment for 48 and 72 h (Fig. 1). However, treatment with low concentrations (0-1.6 μM) of SAL for 24 h did not exhibit cytotoxicity against NB4 (Fig. 1A) and HL-60 (Fig. 1B) cells. Therefore, we selected a time-point of 48 h to investigate the effect of SAL on cell apoptosis in subsequent experiments.

Salinomycin exerts a pro-apoptotic effect on NB4 and HL-60 cells. To examine whether SAL-induced cell death of NB4 and HL-60 cells was mediated by apoptosis induction, we performed Annexin V-FITC and PI staining and flow cytometric analysis. As shown in Fig. 2A, SAL treatment increased the percentage of apoptotic NB4 and HL-60 cells in a dose-dependent manner. The percentage of apoptotic NB4 cells increased from 6.01% among control cells [treated with dimethyl sulphoxide (DMSO)] to 32.30, 61.90 and 76.22% among cells treated with 0.8, 1.6 and 3.2 μM SAL, respectively. The apoptotic HL-60 cells increased from 3.16% among control cells (treated with DMSO) to 37.45, 64.46 and 85.75% among cells treated with 0.8, 1.6 and 3.2 μM SAL, respectively. Results of Hoechst 33258 staining revealed altered morphology of NB4 and HL-60 cells treated with 1.6 μM SAL for 48 h. SAL-treated cells exhibited typical morphological changes associated with apoptosis, such as nuclear fragmentation and condensation (Fig. 2B). To further explore the mechanism of apoptosis induced by SAL in NB4 and HL-60 cells we examined the expression levels of apoptosis-associated proteins including Bcl-2, Bax, caspase-3, -8 and -9, cleaved PARP and cytochrome *c*. As shown in Fig. 2C, SAL increased the expression level of Bax while it decreased the expression level of Bcl-2 and deregulated the ratio of Bax/Bcl-2 in NB4 and HL-60 cells. Furthermore, the increased expression levels of cleaved caspase-3, cleaved caspase-9
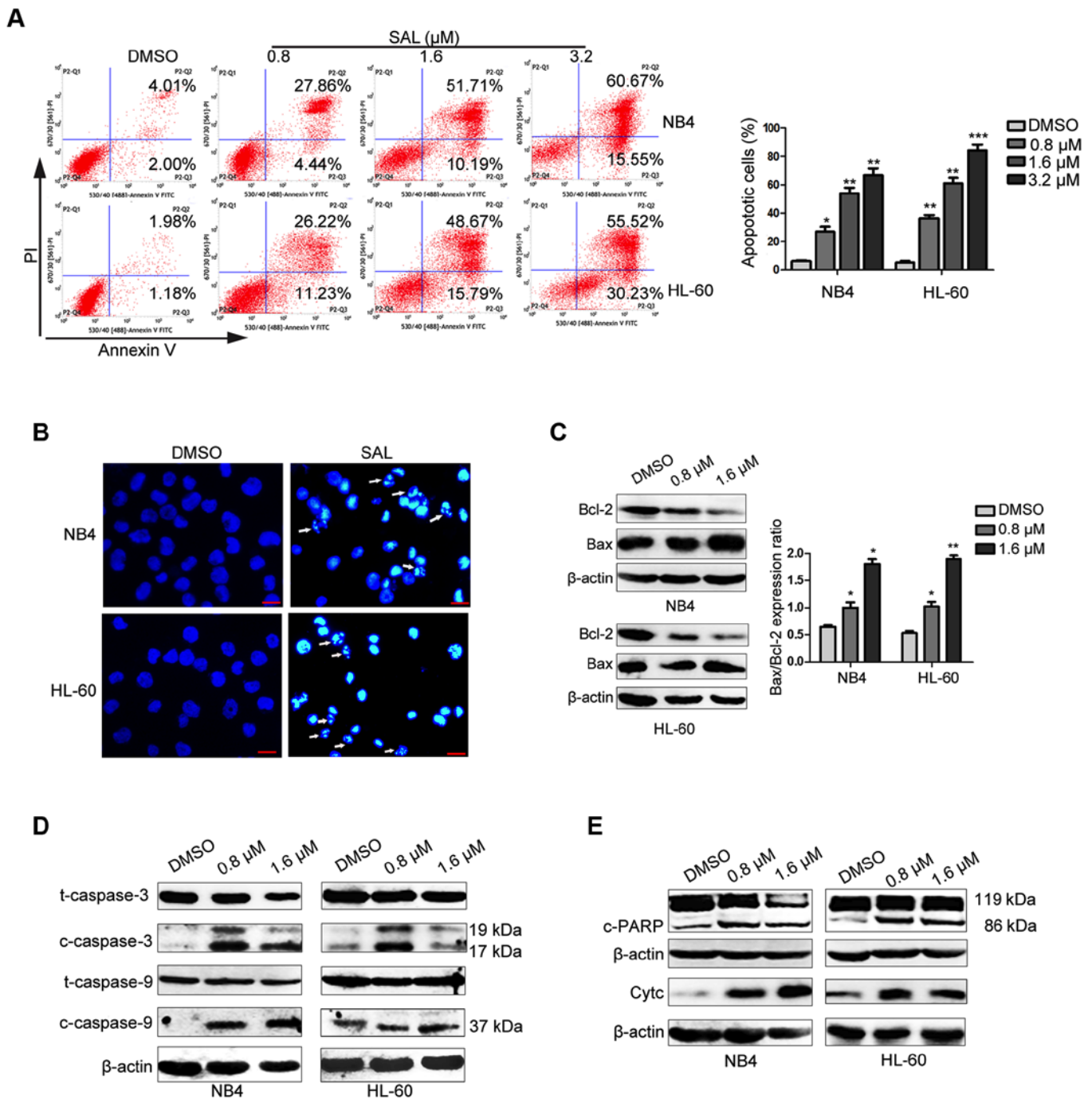
Figure 2. SAL has a pro-apoptotic effect on NB4 and HL-60 cells. (A) Cell apoptosis was assessed by Annexin V-FITC/propidium iodide (PI) staining assay by flow cytometry. The images display a representative experiment from three independent experiments. (B) Apoptotic cell morphology was assessed by Hoechst 33258 staining after treatment with the DMSO control or SAL (1.6 μM) for 48 h (magnification, x400). (C-E) NB4 and HL-60 cells were treated with indicated concentrations of SAL for 48 h. (C) Then, the apoptosis-related protein levels of Bax and Bcl-2 were assessed by western blotting with the β-actin protein as an internal control. The relative protein expression levels of Bax/Bcl-2 ratio was quantified. Data are expressed as the means ± SD of three independent experiments. (D) Caspase-3, cleaved-caspase-3, caspase-9 and cleaved caspase-9 levels were assessed by western blotting. (E) PARP and cytochrome *c* levels were assessed by western blotting. *P<0.05, **P<0.01, ***P<0.001 vs. the DMSO group, n=3. SAL, salinomycin; DMSO, dimethyl sulphoxide.

(Fig. 2D) and cleaved PARP and cytochrome *c* (Fig. 2E) were observed after SAL treatment. However, cleaved caspase-8 was not detected after SAL treatment (data not shown). Collectively, these data indicated that SAL effectively induced the apoptosis of APL cells.

Salinomycin induces the differentiation of APL cells. Targeting Wnt/β-catenin signaling using 6-benzylthioinosine was

revealed to induce the differentiation of leukemia cells (27). In addition, shRNA-mediated downregulation of β-catenin promoted ATRA-induced differentiation of HL-60 cells (29). Therefore, we determined whether SAL, a Wnt signaling inhibitor, also induced the differentiation of leukemia cells. For this, NB4 and HL-60 cells were incubated with SAL (0.6 μM) or ATRA (1 μM, positive control) for 72 h, and cell differentiation was evaluated based on morphological changes by performing
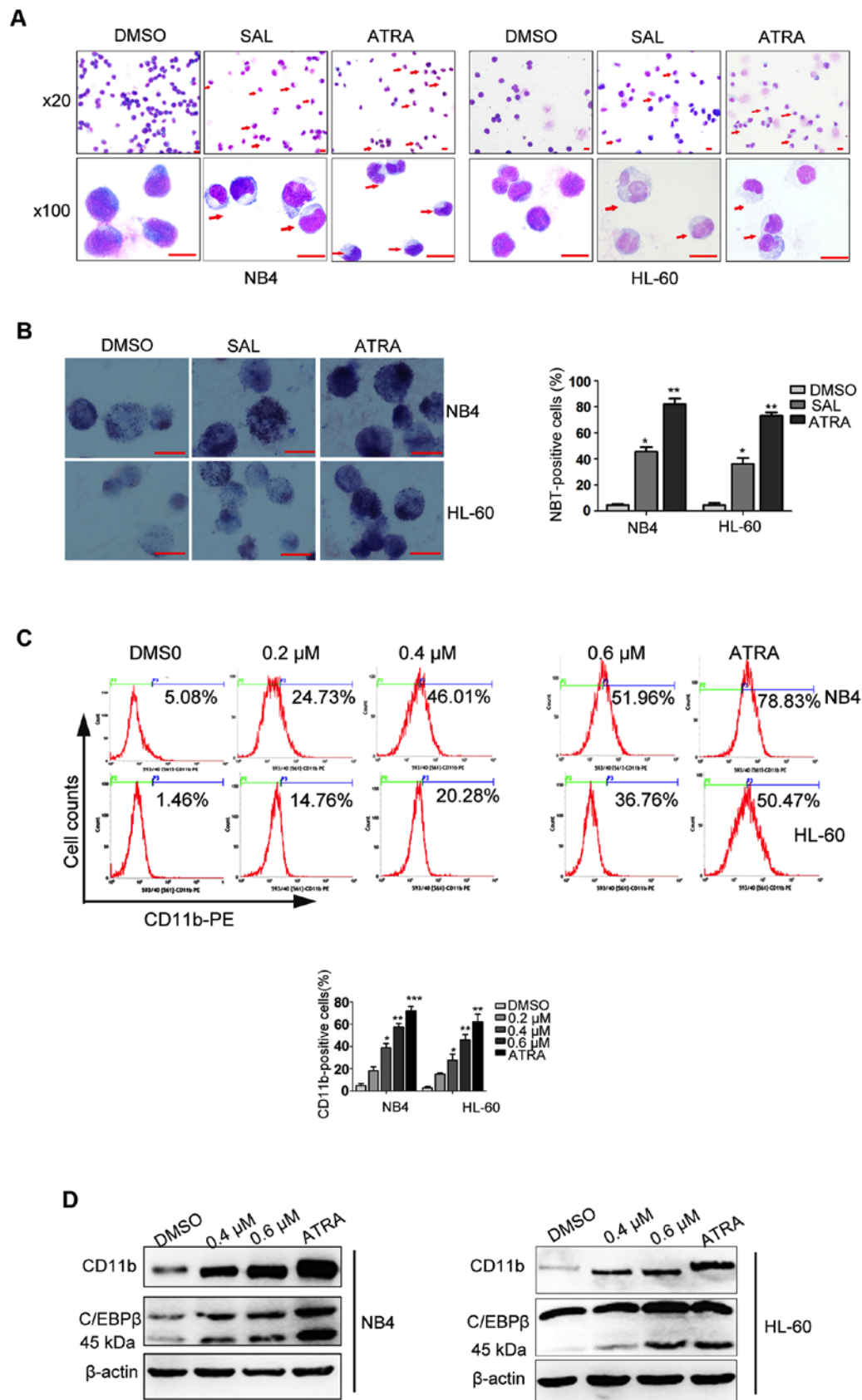
Figure 3. SAL induces cell differentiation in NB4 and HL-60 cells. (A and B) NB4 and HL-60 cells were treated with SAL (0.6 μM) or ATRA (1 μM, as a positive control) for three days. (A) Then, cell morphology was examined by Wright's staining under a light microscope (magnification, x20 and x100). (B) Differentiation was also assessed by NBT reduction test. A total of 200 cells were counted under a microscope to determine the percentage of NBT-positive cells. Data is expressed as the means ± SD of three independent experiments. (C) NB4 and HL-60 cells were treated with 0 (DMSO), 0.2, 0.4 and 0.6 μM SAL or 1 μM ATRA for 72 h, and the percent of differentiated cells was determined by assessing CD11b expression and analyzed by flow cytometry. (D) NB4 and HL-60 cells were treated with 0 (DMSO), 0.4 and 0.6 μM SAL or 1 μM ATRA for 72 h, and the expression of differentiation marker CD11b and C/EBPβ were examined by western blot analysis. Each experiment was repeated at least three times. *P<0.05, **P<0.01, ***P<0.001 vs. the DMSO group, n=3. SAL, salinomycin; ATRA, all-*trans* retinoic acid; NBT, nitroblue tetrazolium; C/EBPβ, CCAAT/enhancer binding protein β; DMSO, dimethyl sulphoxide.
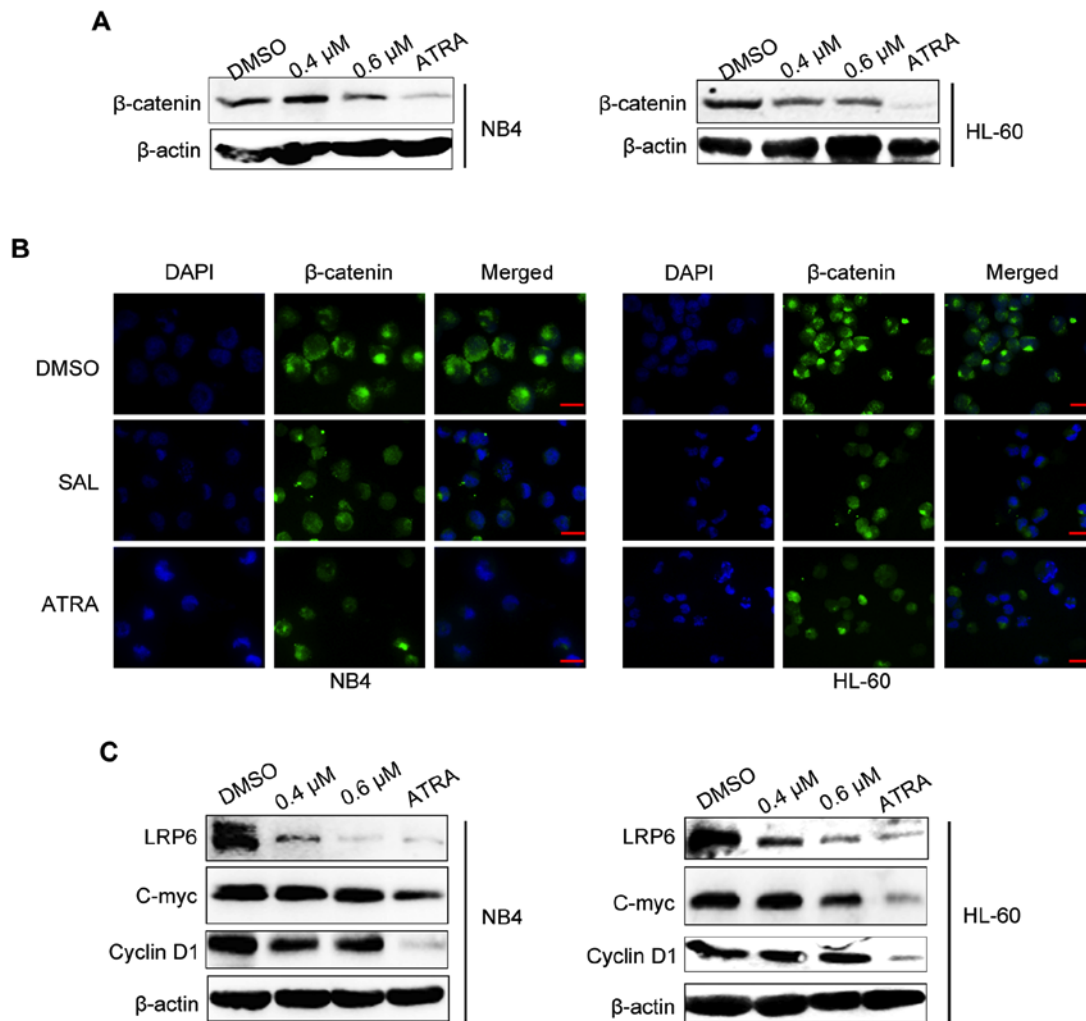
Figure 4. SAL suppresses canonical Wnt signaling in NB4 and HL-60 cells. (A) NB4 and HL-60 cells were treated with different concentrations of SAL or ATRA for 72 h. β-catenin protein levels were determined by western blot analysis. (B) The expression level and subcellular localization of β-catenin were examined by immunofluorescence microscopy as described in Materials and methods (magnification, x40). (C) The levels of LRP6, C-myc, cyclin D1 were examined by western blotting. Each experiment was repeated at least three times. SAL, salinomycin; ATRA, all-*trans* retinoic acid; DMSO, dimethyl sulphoxide.

Wright-Giemsa staining. Morphological analysis revealed that undifferentiated control (DMSO-treated) cells were predominantly promyelocytes with round and large nuclei, whereas cells treated with SAL or ATRA displayed morphological features of cell differentiation, such as a smaller nucleus pattern, cytoplasmic enlargement, lower nuclear/cytoplasmic ratio (Fig. 3A). The percentage of mature NB4 cells increased from 1.5% among control cells (treated with DMSO) to 39.5 and 74.5% among cells treated with 0.6 μM SAL or 1 μM ATRA, respectively. The percentage of mature HL-60 cells increased from 4.0% among control cells (treated with DMSO) to 30.5 and 63.0% among cells treated with 0.6 μM SAL or 1 μM ATRA, respectively. These morphological data were further confirmed by the results of NBT testing. NBT-positive cells significantly increased after treatment with SAL for 72 h (Fig. 3B). Cell differentiation was further confirmed by detecting the expression of CD11b, a surface myeloid differentiation marker, by performing flow cytometric and western blot analyses. As shown in Fig. 3C, SAL or ATRA treatment significantly increased the percentage of CD11b-positive cells in a dose-dependent manner. Results of western blot analysis

also revealed that CD11b expression increased after SAL or ATRA treatment. Previous studies have demonstrated that CCAAT/enhancer binding protein β (C/EBPβ) plays a crucial role in myeloid differentiation (30). In the present study, we found that SAL also enhanced C/EBPβ expression (Fig. 3D). Thus, these results revealed that SAL effectively induced leukemic-cell differentiation.

Salinomycin inhibits Wnt/β-catenin signaling. Since activation of canonical Wnt signaling resulted in low ability of cell differentiation (28), we explored whether canonical Wnt signaling was involved in SAL-induced cell differentiation. β-catenin is the central molecule involved in canonical Wnt signaling, therefore we evaluated β-catenin expression in NB4 and HL-60 cells treated with SAL (0.4 and 0.6 μM) or ATRA (1 μM) for three days by performing western blotting. We found that the total β-catenin level was decreased after SAL or ATRA treatment for 72 h (Fig. 4A). It has been revealed that after stabilization and accumulation, β-catenin translocates into the nucleus and binds transcription factors belonging to T-cell factor/lymphoid enhancer factor (TCF/LEF) family to
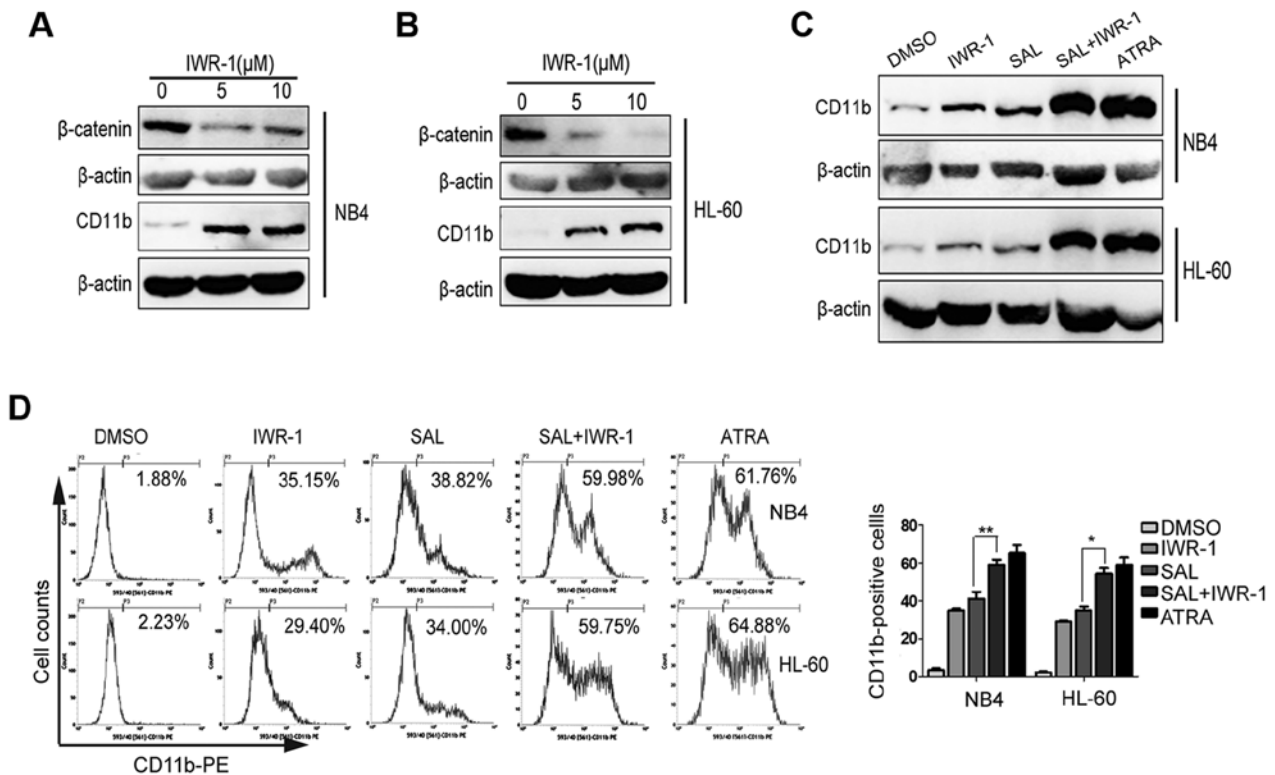
Figure 5. IWR-1 enhances cell differentiation induced by SAL. (A and B) IWR-1 induced cell differentiation. (A) NB4 and (B) HL-60 cells were treated with 0 (DMSO), 5 and 10 μM IWR-1 for three days, and the expression of β-catenin and differentiation marker CD11b was assessed by western blotting. (C and D) NB4 and HL-60 cells were treated for three days with IWR-1 (10 μM), SAL (0.4 μM) or both or ATRA (1 μM, as a positive control), and then the cell surface marker CD11b was determined using (C) western blotting and (D) flow cytometry. Each experiment was repeated at least three times. SAL, salinomycin; ATRA, all-*trans* retinoic acid; DMSO, dimethyl sulphoxide. *P<0.05, **P<0.01 vs. the DMSO group.

stimulate the expression of target genes such as cyclin D1 and C-myc (31). Therefore, we investigated the subcellular localization of β-catenin using immunofluorescence assay. As shown in Fig. 4B, β-catenin preferentially accumulated in the nucleus of the control (DMSO-treated) cells. SAL or ATRA treatment decreased β-catenin levels in both the nucleus and cytoplasm. Furthermore, the expression of LRP6, C-myc and cyclin D1 also decreased after treatment with SAL or ATRA for 72 h (Fig. 4C). These data indicated that Wnt/β-catenin signaling was involved in SAL or ATRA induced-cell differentiation.

IWR-1, a Wnt inhibitor, promotes salinomycin-induced cell differentiation. NB4 and HL-60 were treated with Wnt inhibitor IWR-1 (5 or 10 μM) for 72 h. We observed that IWR-1 treatment decreased β-catenin expression in NB4 (Fig. 5A) and HL-60 (Fig. 5B) cells. Next, we evaluated whether IWR-1 also induced cell differentiation. As shown in Fig. 5A and B, IWR-1 treatment promoted cell differentiation, as indicated by increased CD11b expression. To further determine whether SAL-induced differentiation of leukemic-cells were involved the canonical Wnt signaling, we investigated the effect of combined treatment with SAL and IWR-1 on NB4 and HL-60 cells. For this, NB4 and HL-60 cells were treated with IWR-1 (10 μM), SAL (0.4 μM) or both or with ATRA (1 μM) for three days. We observed that compared with SAL treatment alone, the combination treatment with IWR-1 and SAL enhanced CD11b expression as determined by performing flow cytometric and western blot analyses (Fig. 5C and D). These results indicated that combined treatment with SAL and

IWR-1 increased cell differentiation and that SAL induced cell differentiation by suppressing Wnt signaling.

Discussion

Although prognosis of patients with APL has significantly improved since the introduction of ATRA and ATO, the current treatment of APL is associated with some issues such as drug toxicity, resistance and relapse. Therefore, it is necessary to determine novel alternative therapeutic strategies to overcome these issues and to improve the outcome of patients with APL. It was determined that SAL is a potential agent for the elimination of LSCs (20). In addition, it was revealed that SAL exerts non-toxic effects on normal peripheral blood cells (14,16). Therefore, SAL may be a potential drug for the treatment of leukemia and we thus further investigated its effect on apoptosis and differentiation in APL cells in this study.

We firstly evaluated the effect of SAL on cell viability and found that SAL significantly inhibited the growth of NB4 and HL-60 cells (Fig. 1), which was consistent with the result of previous studies assessing the effect of SAL on leukemia cell proliferation (14,16). Next, we investigated whether SAL-induced cell death was accompanied by induction of cell apoptosis. Both flow cytometric analysis and morphological changes revealed that SAL effectively induced APL cells apoptosis (Fig. 2A and B). Apoptosis is regulated by two central apoptotic pathways: the extrinsic pathway (death receptor-mediated pathway) and the intrinsic pathway (mitochondrial-mediated pathway). The extrinsic pathway is

activated via ligation of death receptors on the cell surface membrane leading to activation of caspase-8, followed by caspase-3. The intrinsic pathway is mediated by different apoptotic stimuli. Most intrinsic signals induce depolarization of the mitochondrial membrane and the release of cytochrome *c* into the cytoplasm. The release of cytochrome *c* activates caspase-9. This results in activation of caspase-3, and commitment to cell death. This pathway is regulated by the B-cell lymphoma 2 family of proteins comprised of 25 pro- and anti-apoptotic members such as Bcl-2 and Bax (32). To determine the apoptotic pathway induced by SAL in NB4 and HL-60 cells, we further evaluated Bcl-2, Bax, cytochrome *c*, caspase-3, -8 and -9 and PARP expression. We found that the expression of Bax/Bcl-2, cytochrome *c*, cleaved caspase-9, cleaved caspase-3 and cleaved PARP increased following SAL treatment (Fig. 2). However, cleaved caspase-8 was not observed in our study. These results revealed that SAL induced APL cell apoptosis through the intrinsic pathway. Studies have revealed that inhibition of Wnt/β-catenin signaling induces apoptosis of leukemic cells (14,33). To determine whether β-catenin signaling is involved in SAL-induced apoptosis, we detected the levels of some Wnt-related proteins. We found that the expression of LRP6, β-catenin and C-myc were also reduced after treatment with 0.8 and 1.6 μM SAL (inducing apoptosis; data not shown). Thus, Wnt/β-catenin signaling was also involved in SAL-induced apoptosis.

Since APL is characterized by the accumulation of cells blocked in the promyelocytic stage, targeting cell differentiation is an effective therapy for APL. However, little information is available on role of SAL in modulating leukemia cell differentiation. Therefore, we investigated the potential of SAL to induce the differentiation of APL cell lines. We found that cells treated with SAL exhibited typical morphological changes associated with differentiation. Moreover, SAL treatment markedly increased the percentage of NBT-positive and CD11b-positive cells and protein levels of CD11b and C/EBPβ (Fig. 3). These results indicated that SAL effectively induced leukemia cell differentiation.

Deregulation of Wnt signaling plays a critical role in the pathogenesis of various types of cancers including AML (34). Moreover, recent studies have revealed that Wnt/β-catenin signaling is associated with leukemia cell differentiation (27-29). Therefore, we hypothesized that cell differentiation induced by SAL involves the inhibition of Wnt/β-catenin signaling. β-catenin is at the core of Wnt/β-catenin signaling. In the absence of Wnts, cytoplasmic β-catenin is targeted for ubiquitination and proteasomal degradation and is maintained at a low level. However, the presence of Wnts which bind to Frizzled (Fzd) receptors and lipoprotein receptor-related protein 5/6 (LRP5/6) leads to the formation of the Wnt/Fzd/LRP5/6 complex on the cell surface. This leads to stabilization of cytosolic β-catenin, which then translocates into the nucleus to bind to transcription factors of the TCF/LEF family and stimulates the expression of target genes such as cyclin D1 and C-myc (31,35,36). Results of western blot analysis performed in the present study revealed that SAL blocked β-catenin, C-myc and cyclin D1 expression (Fig. 4). Immunofluorescence analysis revealed that the β-catenin level was decreased in both the nucleus and cytoplasm of SAL- or ATRA-treated NB4 and HL-60 cells. These results indicated

that SAL blocked Wnt/β-catenin signaling in NB4 and HL-60 cells. This was consistent with a previous study which revealed that SAL inhibited LRP6, a co-receptor for Wnt ligands and activated Wnt/β-catenin signaling, thus inhibiting Wnt/β-catenin signaling in breast and prostate cancer cells (37) . The present study revealed that the LRP6 level was also reduced in SAL-treated NB4 and HL-60 cells (Fig. 4C). To further confirm whether cell differentiation induced by SAL was associated with blocking Wnt/β-catenin signaling, we further determined the effect of IWR-1, another Wnt inhibitor (38), on NB4 and HL-60 cells. We found that IWR-1 also enhanced CD11b expression (Fig. 5B). Moreover, compared with SAL treatment alone, the combination treatment with SAL and IWR-1 synergistically triggered the differentiation of NB4 and HL-60 cells (Fig. 5C and D). Collectively, these results indicated that SAL induced leukemia cell differentiation by inhibiting Wnt/β-catenin signaling.

Autophagy is a well-known cellular process that plays an important role in the regulation of leukemia cell differentiation. It was previously reported that a high β-catenin level inhibited autophagy, thus decreasing the differentiation of AML cells (39), and autophagy was upregulated during ATRA-mediated APL cell differentiation (40). Recent studies have revealed that autophagy plays a vital role in regulating PML-RARα degradation by p62/SQSTM1 and APL cell differentiation (41). Notably, a recent study revealed that SAL upregulated p62/SQSTM1 expression and activated an autophagic response in AML cell lines (16). Thus, these findings indicated that autophagy may be involved in SAL-induced cell differentiation. However, additional studies are needed to investigate the effect of autophagy on SAL-mediated cell differentiation. In addition, a previous study demonstrated that SAL activated the Toll-like receptor pathway in AML cells (16). Activation of Toll-like receptor pathways has been revealed to promote differentiation and growth inhibition in AML cells (42). Therefore, cell differentiation induced by SAL may be related to Toll-like receptor pathways.

In summary, we found that SAL effectively inhibited the proliferation and induced the apoptosis of NB4 and HL-60 cells. To the best of our knowledge, this is the first study to reveal that SAL induced the differentiation of APL cells, possibly by blocking Wnt/β-catenin signaling. Our results provide a foundation to broaden the clinical application of SAL which may be a promising agent for treatment of APL or other AML types. Further studies are warranted to investigate the combination of ATRA and SAL on APL cells.

Acknowledgements

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YZ and BZL conceived and designed the study. YZ, LL, SFY, MC, LWL, ZLS, CLX, LGG and TX performed the experiments. YZ wrote the paper. YZ, LZ and BZL reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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