

Effector mechanisms of sunitinib-induced G1 cell cycle arrest, differentiation, and apoptosis in human acute myeloid leukaemia HL60 and KG-1 cells

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Abstract Acute myeloid leukaemia (AML) is a heterogeneous disease with dismal outcome. Sunitinib is an orally active inhibitor of multiple tyrosine kinase receptors approved for renal cell carcinoma and gastrointestinal stromal tumour that has also been studied for AML in several clinical trials. However, the precise mechanism of sunitinib action against AML remains unclear and requires further investigation. For this purpose, this study was conducted using human AML cell lines (HL60 and KG-1) and AML patients' mononucleated cells. Sunitinib induced G1 phase arrest associated with decreased cyclin D1, cyclin D3, and cyclin-dependent kinase (Cdk)2 and increased p27^{Kip1}, pRb1,

and p130/Rb2 expression and phosphorylated activation of protein kinase C alpha and beta (PKC α/β). Selective PKC α/β inhibitor treatment abolished sunitinib-elicited AML differentiation, suggesting that PKC α/β may underlie sunitinib-induced monocytic differentiation. Furthermore, sunitinib increased pro-apoptotic molecule expression (Bax, Bak, PUMA, Fas, FasL, DR4, and DR5) and decreased anti-apoptotic molecule expression (Bcl-2 and Mcl-1), resulting in caspase-2, caspase-3, caspase-8, and caspase-9 activation and both death receptor and mitochondria-dependent apoptosis. Taken together, these findings provide evidence that sunitinib targets AML cells through both differentiation and apoptosis pathways. More clinical studies are urgently needed to demonstrate its optimal clinical applications in AML.

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Introduction

Acute myeloid leukaemia (AML) is a heterogeneous disorder. With growing understanding of its pathogenesis, cytotoxic chemotherapy with or without follow-up with hematopoietic cell transplantation is the primary treatment for AML. Although much effort has been made concerning AML treatment, the prognosis has not improved substantially in the past decade and still remains dismal [1].

Among all the subtypes of AML, acute promyelocytic leukaemia (APL) is an exception, because with the introduction of all-trans retinoic acid (ATRA), a well-known cell differentiation inducer, to conventional cytotoxic agents, the outcome of patients with APL is greatly superior those with non-promyelocytic AML [2]. Studies have shown that the complete remission rate can reach 90 % when APL patients receive induction and consolidation therapy with regimens composed

of ATRA, arsenic trioxide (ATO), and anthracycline-based chemotherapy [3]. In addition, ATO has emerged as an option for relapse patients because it can resensitize patients to the differentiation effects of ATRA, resulting in secondary remission [4, 5]. Unfortunately, ATRA and ATO are not able to induce non-promyelocytic AML cell differentiation successfully, so the majority of patients with AML do not benefit from these differentiation agents. How to treat non-APL AML successfully with differentiation agents other than ATRA and ATO remains an obstacle and requires further investigation. Several agents have been investigated, such as gefitinib, epigenetic agents, and CCAAT/enhancer binding proteins [6]. Among potential agents, gefitinib is a tyrosine kinase inhibitor targeting epidermal growth factor receptor-tyrosine kinase. The initial positive results from gefitinib suggest that tyrosine kinase inhibitors can potentially induce AML cell differentiation and apoptosis. Sunitinib may be another candidate that harbours this efficacy.

Sunitinib is an orally administered small molecule that inhibits the signal transduction pathways responsible for cell proliferation through blockade of the split-kinase domain family of receptor tyrosine kinases, including vascular endothelial growth factor receptor, platelet-derived growth factor receptor, and the *fms*-related tyrosine kinase (*FLT3*) receptor [7]. It is currently approved for the treatment of metastatic renal cell carcinoma and gastrointestinal stromal tumour refractory to imatinib [8]. Several studies have investigated the potential of sunitinib as an AML treatment. One such study conducted by O'Farrell et al. [9] demonstrates that administration of sunitinib in the MV4:11 orthotopic model of human AML results in sustained inhibition of *FLT3* signalling and prolonged patient survival. A phase I study conducted by Fiedler et al. [10] also demonstrates that sunitinib as a monotherapy may induce partial response in refractory AML, providing preliminary evidence for its clinical application in AML.

Although a study conducted by Nishioka et al. [11] has demonstrated that sunitinib can induce AML cell monocytic differentiation independent of *FLT3* gene status, the molecular mechanisms for sunitinib-induced AML cell differentiation and apoptosis remain largely unclear. Therefore, we conducted the present study to investigate the effector mechanisms of sunitinib-induced human AML cell growth arrest, differentiation, and apoptosis in two AML cell lines (HL60 and KG-1 cells). Patient-derived primary AML blast cells were also studied to strengthen the clinical association of the findings. Our results indicated that sunitinib may act not only as an effective leukaemic differentiation agent but also an apoptosis inducer in AML cells. The results of our study also established a molecular rationale suggesting that sunitinib may have therapeutic potential in patients with AML.

Materials and methods

Reagents

Sunitinib was provided by Pfizer pharmaceutical company (New York, NY). It was dissolved in dimethyl sulfoxide for in vitro studies. Anti-CD11b, anti-CD11c, anti-CD14, anti-CD18, anti-CD54, anti-Fas, and anti-Fas ligand antibodies were purchased from BD Biosciences PharMingen (San Diego, CA). Anti-phospho-protein kinase C (PKC) α/β , anti-cleaved caspase-3, anti-cleaved caspase-8, anti-cleaved caspase-9, and anti-Mcl-1 were purchased from Cell Signalling Technology (Danvers, MA). Anti- β -actin, anti-Bax, anti-Bak, anti-Bcl-2, anti-phospho-PKC α , anti-phospho-PKC β , anti-pRb1, anti-p130/Rb2, anti-cyclin D1, anti-cyclin D3, and anti-Cdk2 were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Anti-DR4, anti-DR5, and anti-p27^{Kip1} were obtained from Abcam (Cambridge, UK). Go6976 (a selective PKC α/β inhibitor) and anti-p53 upregulated modulator of apoptosis (PUMA) antibody were purchased from Calbiochem (La Jolla, CA). Caspase-2, caspase-3, caspase-8, caspase-9, and caspase-12 activity assay kits were purchased from R&D Systems (Minneapolis, MN). A caspase-2 inhibitor (Z-VDVAD-FMK), caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), and caspase-9 inhibitor (Z-LEHD-FMK) were purchased from Kamiya (Thousand Oaks, CA). 1 α ,25-dihydroxycholecalciferol (vitamin D3) was obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

Human myeloid leukaemia cell lines HL60 and KG-1 were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium supplemented with 5 % foetal bovine serum, 2 mM glutamine, and antibiotics (100 unit/ml penicillin and 100 μ g/ml streptomycin) at 37 °C in a 5 % CO₂-humidified atmosphere. The culture medium was changed every 2 days. Viable cells were counted with a haemocytometer using the trypan blue dye exclusion method.

Patients

Primary patient-derived AML blast cells for in vitro studies were obtained from three patients with newly diagnosed AML. Mononuclear cells were separated by Ficoll-Hypaque density-gradient centrifugation. Investigations were carried out with written consent of the patients. The project was approved by the Ethics Committee of Taichung Veterans General Hospital.

Cell surface CD markers detection

To analyse the cell surface expression of CD markers, cells (2×10^5 cells/ml) were treated with various concentrations of sunitinib for the indicated times. After treatment, cells were washed

with phosphate-buffered saline (PBS) and incubated with phycoerythrin-conjugated or fluorescein isothiocyanate (FITC)-conjugated anti-human CD11b, CD11c, CD14, CD18, and CD54 monoclonal antibodies. Negative control was obtained by staining of the cells with mouse IgG1 and IgG2a followed by incubation at 4 °C for 20 min. Quantitative immunofluorescence measurements were conducted using a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA) and analysed with CELLQuest™ software.

Morphologic investigation

The morphology of HL60 and KG-1 cells was determined by the Wright–Giemsa method. Briefly, cells were harvested and cytocentrifuged onto a microscope slide using a Cytospin (Shandon Southern Instrument Inc.), and the cells were then stained with Giemsa staining solution for 20 min, rinsed with deionised water, air-dried, and observed under a light microscope. The percentage of differentiated cells was determined by counting 200 cells in each treatment.

Nitroblue tetrazolium reduction assay

Cells (2×10^5 cells/ml) were treated with sunitinib for 24 h and then harvested via centrifugation and incubated with an equal volume of 1 % nitroblue tetrazolium (NBT; Sigma-Aldrich, St. Louis, MO) dissolved in PBS containing 200 ng/ml freshly diluted TPA (Sigma-Aldrich, St. Louis, MO) at 37 °C for 30 min in darkness. The reaction was stopped by washing twice with PBS. A light microscope was used to identify NBT-positive cells, which contained blue-black nitroblue diformazan deposits. The percentage of positive cells was determined by counting 200 cells in each sample.

Quantification of apoptosis by annexin V-FITC/propidium iodide labelling

Apoptosis was quantified by detecting surface exposure of phosphatidylserine in apoptotic cells using an annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences, Clontech, Heidelberg, Germany). Cells were seeded in 6-well plates in 2 ml of medium and incubated with sunitinib at the indicated dose for 48 h. Both adherent and floating cells were harvested, treated according to the manufacturer's instructions, and analysed by flow cytometry. The annexin V+/PI- (early stage) and annexin V+/PI+ (late stage) apoptotic cell populations were measured.

Quantification of apoptotic cells by terminal transferase-mediated dUTP-fluorescein nick end labelling assay

Cells were treated with sunitinib for the indicated time periods, washed twice with PBS, fixed in 2 % paraformaldehyde for

20 min, and then permeabilised with 0.1 % Triton X-100/PBS for 30 min at room temperature. After washing with PBS, the terminal transferase-mediated dUTP-fluorescein nick end labelling (TUNEL) assay was performed according to the manufacturer's instructions (Boehringer Mannheim, Germany). Cells were incubated in the TUNEL reaction buffer in a 37 °C humidified chamber for 1 h in the dark, then rinsed twice with PBS and incubated with DAPI (1 mg/ml) at 37 °C for 10 min. The stained cells were visualised using a fluorescence microscope. The fluorescence intensity change was monitored under a fluorescence microscope or analysed by flow cytometry. TUNEL-positive cells were considered apoptotic.

Cell cycle distribution analysis by flow cytometry

Cells were seeded in 6-well plates and treated with sunitinib for the indicated times. The cells were collected, fixed with cold ethanol, and incubated on ice for 30 min. Cells were then resuspended in PI-containing solution (50 µg/ml PI, 0.1 % Na citrate, and 0.1 % Triton X-100) and placed in the dark at 4 °C overnight. Flow cytometric analysis was performed the following day. Each experiment was performed in triplicate.

Protein preparation and western blot analysis

Cells were cultured with sunitinib for the indicated times. After treatment, both adherent and floating cells were harvested, washed twice with ice-cold PBS, and lysed in ice-cold-modified RIPA buffer. After 30-min incubation on ice, cells were centrifuged at $100,000 \times g$ for 30 min at 4 °C, and supernatants were collected. Protein concentration was determined using the Bradford method. For western blot analysis, equal amounts of total protein were loaded onto SDS-polyacrylamide gels, and the proteins were transferred onto a PVDF membrane by electrophoresis (Millipore, Bedford, MA). Immunoblots were analysed using specific primary antibodies. After exposure to a horseradish peroxidase-conjugated secondary antibody for 1 h, proteins were visualised using an enhanced chemiluminescence detection kit (ECL Kits, Amersham Life Science).

Caspase activity assay

Cells lysates obtained from sunitinib-treated (1 µM) or untreated cells were tested for caspase-2, caspase-3, caspase-8, caspase-9, and caspase-12 activity by measuring hydrolysis of a fluorogenic peptide substrate (100 µM) for caspase-2 (VDVAD-AFC), caspase-3 (DEVD-AFC), caspase-8 (IETD-AFC), caspase-9 (LEHD-AFC), and caspase-12 (ATAD-AFC) conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (R&D System Minneapolis, MN). Cleavage of the peptide by caspase enzymatic activity releases the fluorochrome (excitation/emission=405/

505 nm). The level of caspase enzymatic activity in the cell lysate was directly proportional to the fluorescence signal detected with a fluorescent microplate reader (Fluoroskan Ascent, LabSystems, Finland).

Statistical analysis

The figures presented in this text are representative of at least three independent experiments with similar results. The results are expressed as the mean ± standard deviation of three independent experiments. Statistical differences were evaluated by Student's *t* test and considered significant at the following *P* values (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

Results

Sunitinib induces G1 cell cycle arrest and apoptosis in leukaemia cells

To explore the anti-AML effects of sunitinib, human HL60 and KG-1 cell lines were incubated with various concentrations of sunitinib. Cell viability was determined using the trypan blue exclusion method after 24- and 48-h treatment. As shown in Fig. 1a, sunitinib inhibited HL60 and KG-1 cell growth in a dose- and time-dependent manner with IC₅₀ values of 4.5 and 3.9 μM after 48-h administration, respectively. To investigate the precise action responsible for the sunitinib-mediated antiproliferative effect, the cell cycle distribution profile was examined. As shown in Fig. 1b, exposure of HL60 and KG-1 cells to sunitinib for 24 h led to an accumulation of cells in the G0/G1 phase compared with control cells, coupled with a concomitant decrease in the proportion of cells in the S and G2/M phases. Additionally, a hypodiploid DNA content peak, or sub-G1 population, which is indicative of degraded DNA and a hallmark of apoptosis, was observed following 24 h of high-dose treatment and increased continuously after 48-h incubation (Fig. 1b).

Next, co-staining of annexin V and PI was performed. As shown in Fig. 1c, increased annexin V labelling was observed in sunitinib-treated cells, suggesting that HL60 and KG-1 cells underwent apoptosis in response to sunitinib exposure.

Sunitinib regulates G1 phase regulatory molecules

A recent study demonstrated that sunitinib treatment consistently caused cell cycle arrest in the G0/G1 phase and dephosphorylation of the retinoblastoma protein (pRb1) in papillary thyroid cancer cell lines [12]. Therefore, we next analysed the effect of sunitinib on G0/G1-specific regulatory molecules, specifically the G1 cyclins, Cdks, and Cdk inhibitors. As shown in Fig. 2, exposure to sunitinib for 24 h markedly increased the expression of p27^{Kip1}, pRb1, and

Fig. 1 Sunitinib induces G1 cell cycle arrest and apoptosis in leukaemia cells. **a** Antiproliferative effect of sunitinib. HL60 and KG-1 cells were treated with the indicated concentrations of sunitinib for 24, 48, and 72 h. Cell viability was determined by trypan blue dye exclusion assay. Cells treated with dimethyl sulfoxide were used as control. **b** Sunitinib causes G1 cell cycle arrest. The proportion of HL60 and KG-1 cells in different cell cycle phases was analysed by flow cytometry after sunitinib treatment for 24 and 48 h. **c** Induction of apoptosis by sunitinib. HL60 and KG-1 cells were treated with 6 μM sunitinib for 48 h. Apoptosis was examined by flow cytometry with annexin-V and propidium iodide staining. Bars indicate mean, and error bars indicate standard deviation of three independent experiments

p130/Rb2 but decreased the expression of cyclin D1, cyclin D3, and Cdk2. However, the expression of Cdk4, Cdk6, cyclin D2, and cyclin E were not affected by sunitinib in either tested AML cell line (data not shown).

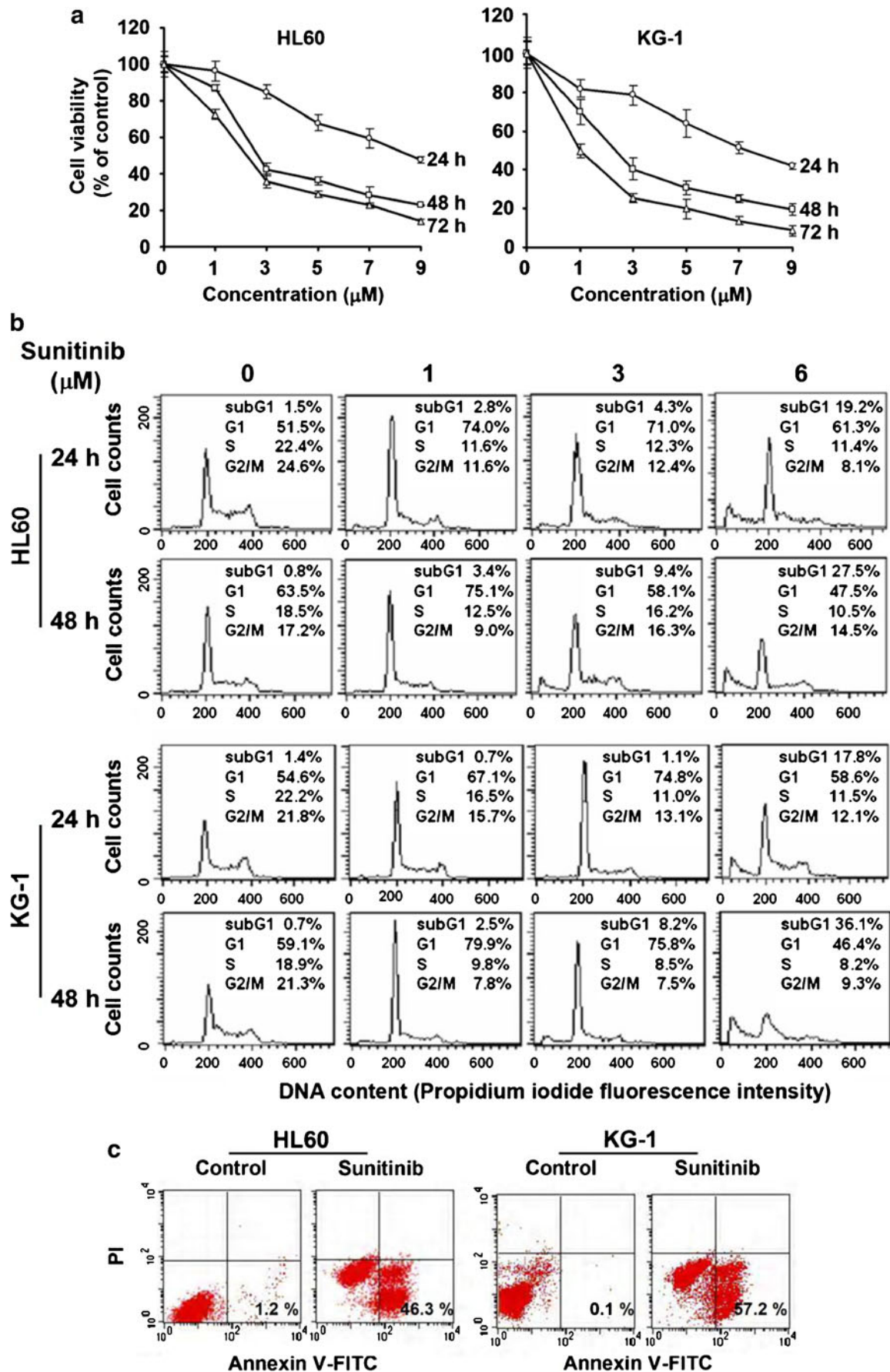
Sunitinib induces caspase-dependent apoptosis

Because caspase plays a pivotal role in apoptosis [13], we next examined the effect of sunitinib on caspase activity using a fluorogenic substrate assay kit. Sunitinib stimulated the activation of caspase-2, caspase-3, caspase-8, and caspase-9 at 48 h (Fig. 3a). Caspase-12 activity, however, did not change in sunitinib-treated HL60 and KG-1 cells. To further confirm activation of caspase-3, caspase-8, and caspase-9, specific antibodies were used to detect the proteolytically activated fragments of each caspase (Fig. 3b). As expected, the active fragments of caspase-3, caspase-8, and caspase-9 were observed after sunitinib treatment for 48 h. Moreover, pretreatment with inhibitors of caspase-2, caspase-3, caspase-8, and caspase-9 significantly prevented sunitinib-induced apoptosis as demonstrated by a reduction in the sub-G1 population (Fig. 3c), which suggested that both the mitochondria- and death receptor-induced caspase activation pathways were involved in sunitinib-induced apoptosis.

Sunitinib regulates apoptosis-related molecules

Because Bcl-2 family proteins are key factors in controlling the mitochondria-dependent apoptotic pathway [14], we further examined the expression levels of anti- and pro-apoptotic molecules in sunitinib-treated and untreated AML cells. As shown in Fig. 4a, decreased Bcl-2 and Mcl-1 (anti-apoptotic proteins) and increased Bax, Bak, and PUMA (pro-apoptotic proteins) were observed in sunitinib-treated AML cells compared with controls; however, the expression of Bcl-xL did not change after sunitinib treatment (data not shown).

Next, the regulation of death receptor superfamily proteins by sunitinib in AML cells was investigated. As shown in Fig. 4b, Fas, Fas-L, DR4, and DR5 expression significantly increased after sunitinib treatment. In contrast, tumour necrosis receptors I and II were not affected by sunitinib (data not shown). These results indicated that both the Bcl-2 family and



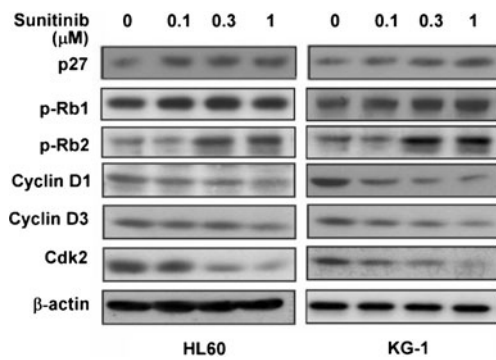


Fig. 2 Sunitinib-regulated G1 phase regulatory molecules. As a dose-dependent experiment, HL60 and KG-1 cells were treated with different concentrations of sunitinib for 24 h. Cell lysates from untreated or sunitinib-treated HL60 and KG-1 cells was then extracted. G1 phase regulatory proteins p27^{Kip1}, pRb1, p130/Rb2, cyclin D1, cyclin D3, and Cdk2 were analysed by western blot. β -actin was used as a loading control

the death receptor superfamily proteins were regulated during sunitinib-induced apoptosis in HL60 and KG-1 cells.

Sunitinib induces monocytic differentiation in AML cells

Sunitinib has been shown to induce monocytic differentiation of HL60 and MOLM13 human AML cells [11]. Therefore, the potential of sunitinib to induce cell differentiation in HL60 and KG-1 cells was investigated. Data from flow cytometric analyses demonstrated that the cell surface levels of differentiation markers CD14 and CD11b dose-dependently increased in sunitinib-treated cells compared with controls (Fig. 5a). Next, the cell surface markers of HL60 and KG-1 cells, including CD11b, CD11c, CD14, CD18, and CD54 were examined after 1 μ M sunitinib treatment; vitamin D3 treatment was used as a positive control. As shown in Fig. 5b, the proportion of cells expressing CD11b, CD11c, CD14, CD18, and CD54 increased after sunitinib and vitamin D3 treatments compared with untreated group.

Furthermore, sunitinib-induced AML cell differentiation was characterised by Wright–Giemsa staining. As shown in Fig. 5c, control HL60 and KG-1 cells were predominantly myeloblasts with round and regular cell margins and large nuclei. However, sunitinib-treated HL60 and KG-1 cells contained bilobate nuclei, decreased cell size, and increased cytoplasm-to-nuclear ratio, which is morphologically consistent with monocytic differentiation. Approximately 76.5 \pm 6.3 % of HL60 cells and 87.2 \pm 7.5 % of KG-1 cells in the 1 μ M sunitinib-treated group displayed evidence of monocytic differentiation, which was significantly higher than those treated with vehicle control (Fig. 5d). NBT assay data further confirmed that sunitinib induced HL60 and KG-1 monocytic differentiation. As shown in Fig. 5d, an increase of cytoplasmic black granules, indicating myeloid cell

differentiation, was observed in both HL60 and KG-1 cells after 24-h sunitinib treatment (Fig. 5e). In each experiment, 200 cells were counted three times. On average, approximately 48.5 \pm 5.6 % of HL60 cells and 57.3 \pm 5.9 % of KG-1 cells were positive for NBT staining following 1 μ M sunitinib treatment, which was significantly higher than that in the control group (Fig. 5f).

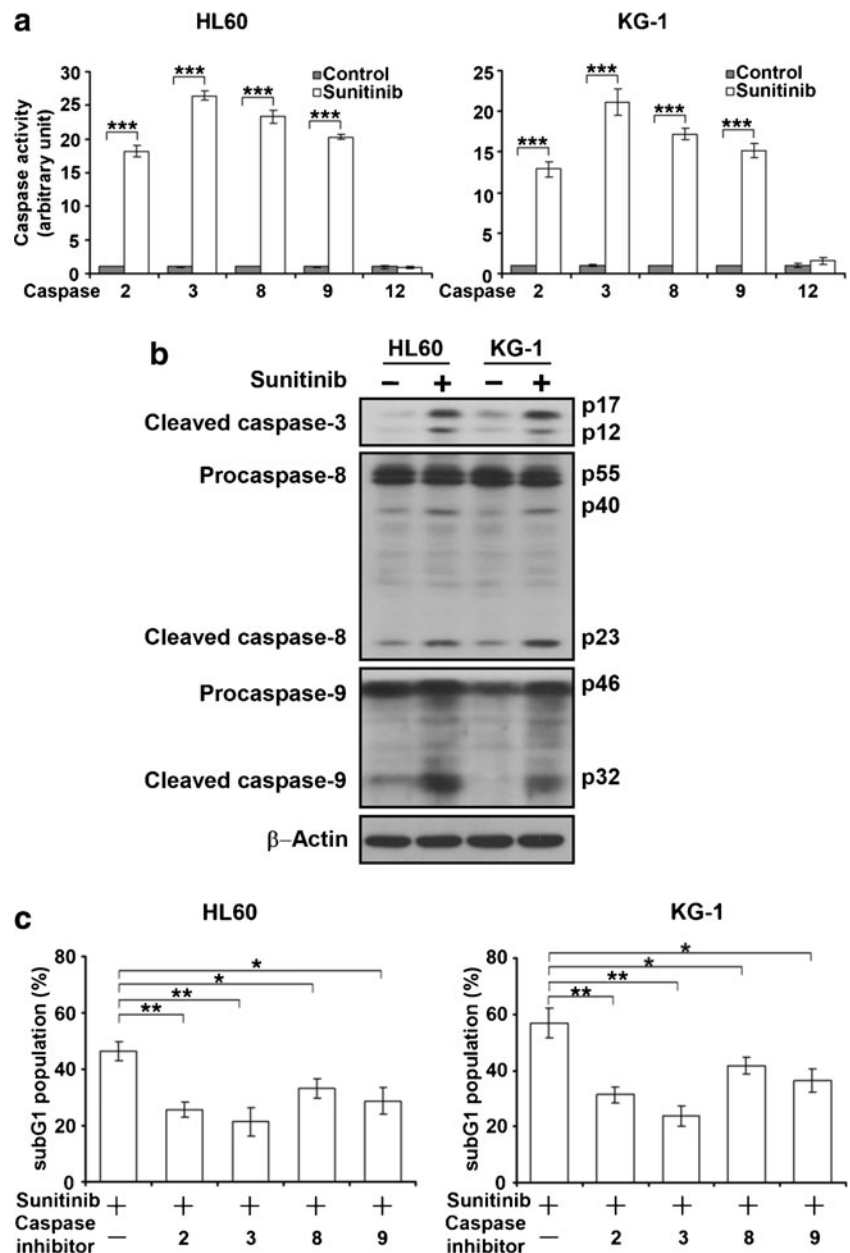
Association of sunitinib-induced AML cell differentiation with PKC

A number of intracellular signal transduction proteins, including mitogen-activated protein kinase, Janus family kinases, signal transducer and activator of transcription, phosphatidylinositol-3-kinase, Smads, and PKC, regulate the process of myeloid differentiation [15]. To gain insights into the signalling molecules involved in sunitinib-induced differentiation, we applied pharmacological inhibitors of different signalling pathways to examine their effect on differentiation. The results demonstrated that the selective PKC α/β inhibitor Go6976 partially but significantly reduced the sunitinib-stimulated surface expression of CD14 and CD11b (Fig. 6a) in HL60 and KG-1 cells, suggesting that inhibition of PKC could block sunitinib-induced cell differentiation. Results from western blot analysis revealed that phosphorylated PKC α and PKC β increased in a dose-dependent manner after 2-h sunitinib treatment (Fig. 6b). By contrast, the expression of total PKC α , PKC β , PKC δ , and phosphorylated PKC δ were not affected by sunitinib (data not shown). These results indicated that sunitinib-induced differentiation of AML cells may be associated with the PKC α/β signalling pathway.

Sunitinib induces monocytic differentiation and cell death in patient-derived AML blast cells

To evaluate the potential value of sunitinib as a therapeutic agent in AML, the effects of sunitinib on the differentiation and viability of freshly isolated patient-derived AML blasts were examined. Monocytic differentiation was induced by sunitinib treatment (0.3 and 1 μ M) in two (identified as AML FAB-M2) of three patients' AML blasts, as indicated by the induction of the monocytic differentiation markers CD14 and CD11b (Fig. 7a). The levels of CD14 and CD11b differentiation antigens were unchanged in the blasts isolated from an AML FAB-M5 patient, indicating that sunitinib did not induce differentiation in this sample. Moreover, sunitinib exhibited a strongly cytotoxic effect on the blasts from all three tested patients. Almost all AML blasts died after treatment with 6 μ M sunitinib for 48 h. At 3 μ M sunitinib treatment, AML blasts decreased to approximately 25 \pm 3, 35 \pm 6, and 20 \pm 5 % of the number in untreated cultures from patients 1, 2, and 3, respectively (Fig. 7b).

Fig. 3 Sunitinib induces caspase-dependent apoptosis. **a** Sunitinib induced caspase activation. Cell lysates were obtained from HL60 and KG-1 cells treated with 6 μ M sunitinib for 48 h to examine caspase-2, caspase-3, caspase-8, caspase-9, and caspase-12 activities by fluorometric caspase assay kits, and **b** the cleaved caspase-3, caspase-8, and caspase-9 were determined by western blot analysis. **c** Caspase inhibitors prevented sunitinib-mediated apoptosis. HL60 and KG-1 cells in (a) were pretreated with caspase inhibitors. Apoptotic cells were determined by TUNEL assay. Data are presented as mean \pm standard deviation of three independent experiments (* P < 0.05; ** P < 0.01; *** P < 0.001)



Discussion

The “two-hit” hypothesis for leukaemogenesis suggests that leukaemia is the result of two classes of mutations. The class I mutation impairs hematopoietic differentiation, and the class II mutation confers a proliferative and/or survival advantage on hematopoietic progenitors [16]. Therefore, induction of differentiation and apoptosis is a potential strategy for AML treatment. In this study, we report that sunitinib inhibited proliferation of human AML cell lines HL60 and KG1 with IC_{50} values of 4.5 and 3.9 μ M, respectively, after 48-h treatment. Additionally, sunitinib induced cell cycle G0/G1 phase arrest, monocytic differentiation, and apoptosis in HL60 and KG-1 cells. These sunitinib-

mediated actions were independent of p53 status, because the p53-null HL60 cells [17] exhibited a response to sunitinib treatment comparable to that of KG-1 cells, which have a functional p53. Furthermore, sunitinib also induced monocytic differentiation and cytotoxicity in primary patient-derived AML blasts.

In mammalian cells, a subset of the G1 to S phase transition proteins, D-type cyclins, Rb family proteins, and Cdk inhibitors (including INK4 and Cip/Kip family proteins), trigger this cell cycle progression. D-type cyclins activate their Cdk-4/6 catalytic partners in mid- to late-G1 phase, while cyclin E appears later in G1 and activates its Cdk-2 kinase subunit near the G1/S checkpoint [18]. Furthermore, the mammalian Cdk inhibitor p27^{Kip1} functionally

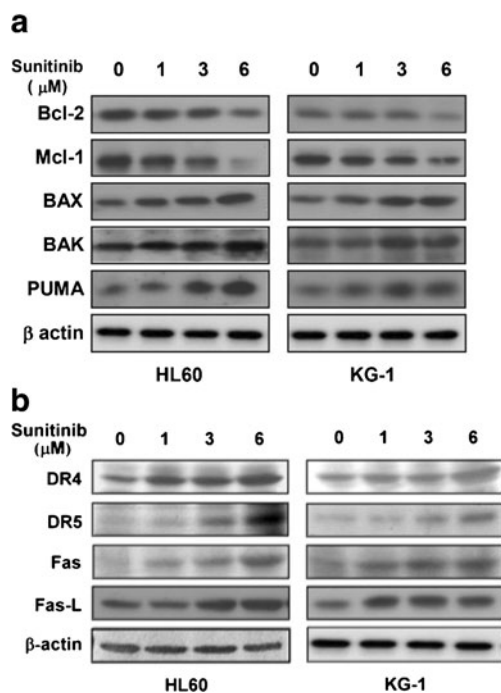


Fig. 4 Sunitinib regulates apoptosis-related molecules. **a** Bcl-2 family proteins. After treatment with increasing concentrations of sunitinib for 48 h, cell lysates were extracted from HL60 and KG-1 cells. Expression of Bcl-2 family proteins Bcl-2, Mcl-1, Bax, Bak, and PUMA were analysed by western blot. **b** Death receptor superfamily proteins Fas, Fas-L, DR4, and DR5 were also analysed by western blot in the cell lysates extracted in (a). β -actin was used as the loading control

links to G0/G1-S progression control, while overexpression of p27^{Kip1} induces cell cycle arrest in the G0/G1 phase [19]. The retinoblastoma (Rb) protein family, including pRb1, p130/Rb2, and p107, are additional negative regulators responsible for controlling G0/G1 phase in the cell cycle by their expression pattern, timing of association with E2Fs, and the cell cycle arrest elicited by their overexpression [20]. A recent report conducted by Yang et al. [21] demonstrated that sunitinib induced cell growth arrest in medulloblastoma via downregulation of cyclin E, cyclin D2, and cyclin D3 and upregulation of p21^{Cip1}. In our study, we found that sunitinib induced G0/G1 phase arrest in AML cells, which may be associated with downregulation of cyclin D1, cyclin D3, and Cdk2 proteins and upregulation of p27^{Kip1}, pRb1, and p130/Rb2. These results suggest that the sunitinib-mediated cell cycle perturbation in AML cells may be the result of a multifaceted attack on multiple target molecules that are critically involved in G0/G1 phase arrest in the cell cycle.

Downregulation of cyclin D1 proteins and upregulation of p27^{Kip1}, pRb1, and p130/Rb2 may also play an important role in monocytic differentiation of AML cells after sunitinib treatment. Studies have shown that Rb family proteins and Cdk inhibitors such as p21^{Cip1} and p27^{Kip1} can also induce embryonic, neural, or hematopoietic stem cell

Fig. 5 Sunitinib induces monocytic differentiation in AML cells. **a** HL60 and KG-1 cells were treated with various concentrations of sunitinib (0, 0.1, 0.3, and 1 μ M) for 24 h. Expression of CD14 and CD11b was analysed by flow cytometry. **b** Expression of cell surface markers. Cells were treated with 1 μ M sunitinib or 30 nM vitamin D3 (Vit D3) for 24 h. The expressions of surface markers were examined by flow cytometry. Untreated cells are shown in *white histogram*. Sunitinib-treated cells are shown in *gray histogram*. Vit D3-treated cells are shown in *black histogram*. **c** Morphological analysis. Monocytic differentiation of HL60 and KG-1 cells treated with various concentrations of sunitinib for 24 h were investigated with Wright–Giemsa staining. Cells with cytoplasm enlargement, nuclear deformation, and the presence of large bilobate nuclei were defined as demonstrating monocytic differentiation. **d** Quantification of differentiated cells after Wright–Giemsa staining. Two hundred cells in (c) were counted to quantify differentiated cells. **e** Monocytic differentiation identified by nitroblue tetrazolium (NBT) reduction. Cells treated in (c) were analysed by NBT reduction assay. Cells demonstrating monocytic differentiation were identified by their intracellular blue formazan deposits. **f** Two hundred cells in (e) were counted to quantify differentiated cells. Cells treated with dimethyl sulfoxide were used as negative control. Bars indicate mean, and error bars indicate standard deviation of three independent experiments (***) $P < 0.001$

differentiation [18]. Additionally, mice lacking p27^{Kip1} demonstrate differentiation defects [22]. In the hematopoietic cell system, accumulation of p27^{Kip1} also plays a crucial role in erythroid differentiation coupled with G1 arrest [23, 24]. Moreover, upregulation of pRb1 and Rb2/p130 is specifically observed during differentiation in many cellular systems, such as muscle cells [25], adipocytes [26], myeloid cells and regenerating liver [27], osteoblasts [28], keratinocytes [29], astrocytomas and normal astrocytes [30], murine neuroblastoma cells [31], and human neuronal cells [32]. In the present study, we found that p27^{Kip1}, pRb1, and p130/Rb2 expression increased during sunitinib-induced monocytic differentiation; however, further studies are needed to address the exact role of p27^{Kip1}, pRb1, and p130/Rb2 in sunitinib-induced monocyte differentiation. Taking these findings together, we hypothesise that decreased expression of cyclin D1 and increased expression of p27^{Kip1}, pRb1, and p130/Rb2 are not only responsible for G1 phase arrest but also monocytic differentiation in AML cells treated by sunitinib.

This study also revealed that PKC α/β may be important molecules involved in sunitinib-induced monocytic differentiation of AML cells. In the hematopoietic system, PKC activation during monocytic differentiation has been shown [33]. PKC α and PKC β have also been shown to play an important role in TPA-induced differentiation in U937 cells [34]. Overexpression of PKC- α or PKC- δ promotes TPA-induced macrophage differentiation of 32Dcl3 cells [35]. Additionally, overexpression of PKC α leads to monocytic differentiation in U937 cells [36]. Moreover, G-CSF-induced HL60 cell differentiation is blocked by PKC inhibitors [37]. Our results demonstrated that sunitinib enhanced phosphorylated PKC α and PKC δ activities (Fig. 6b) and sunitinib-induced monocytic differentiation could be partially blocked by the PKC α/δ inhibitor Go6976 (Fig. 6a),

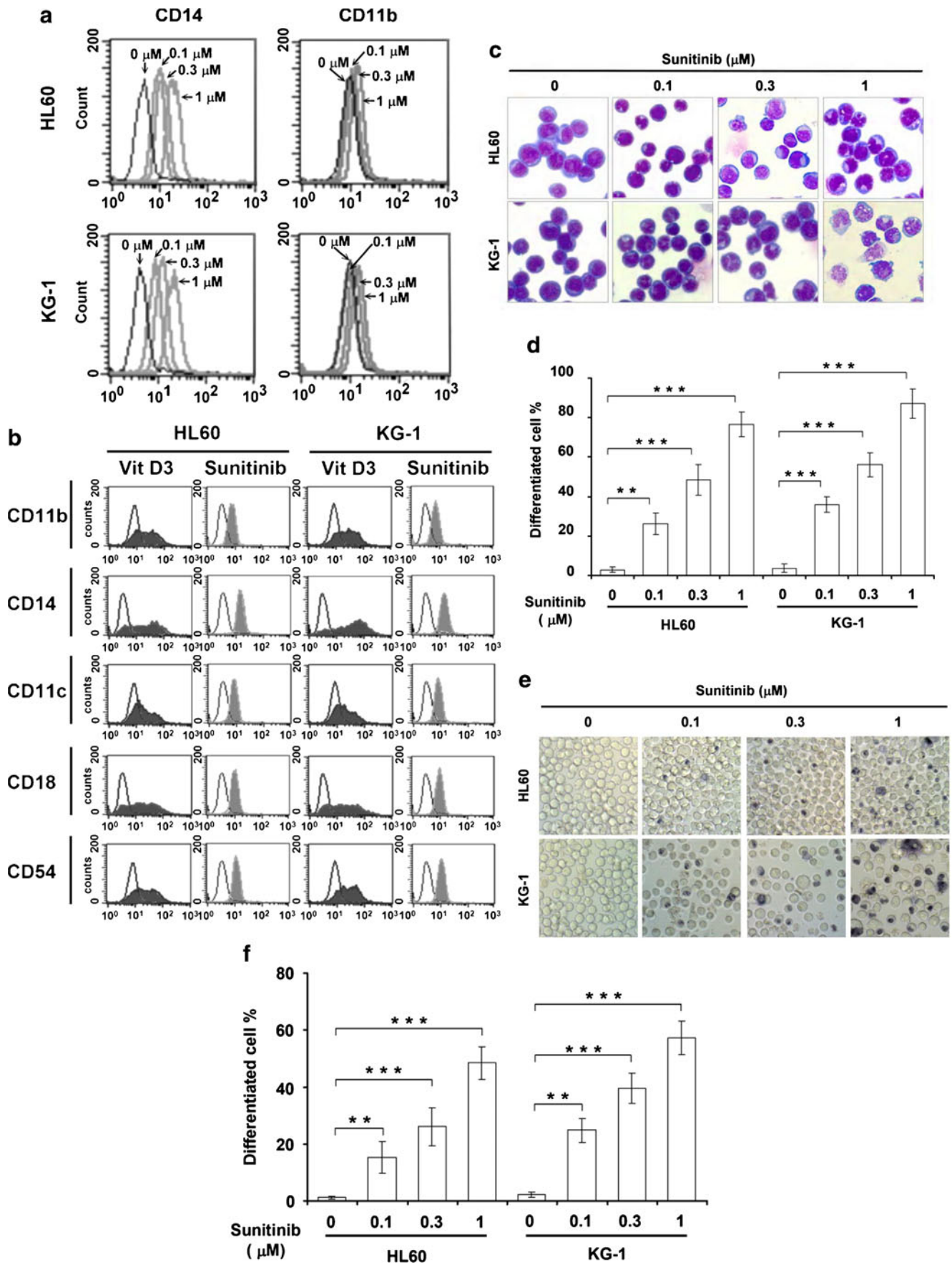
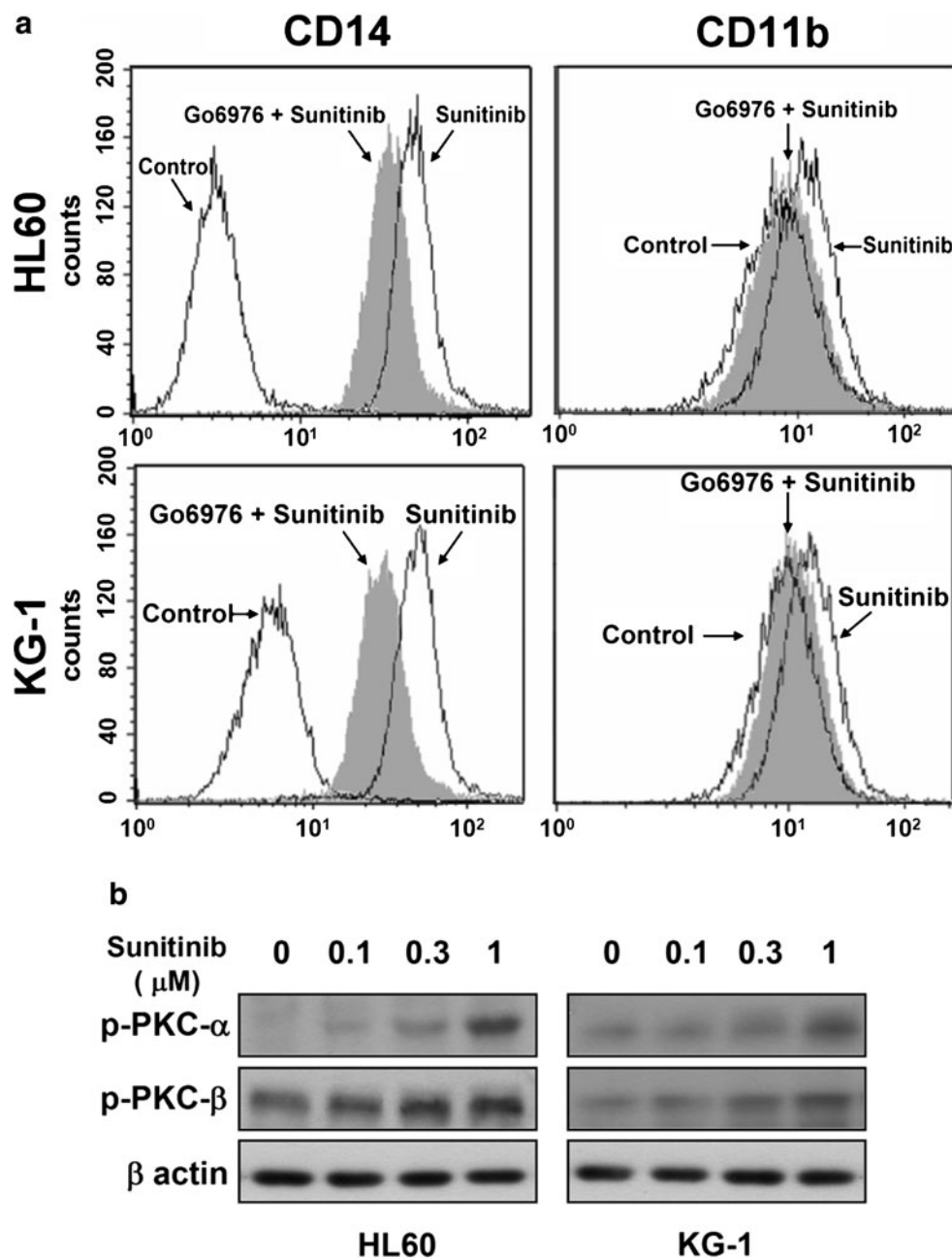


Fig. 6 Sunitinib-induced AML cell differentiation is PKC related. **a** The PKC α inhibitor Go6976 reversed sunitinib-induced cell differentiation. HL60 and KG-1 cells were pretreated with 0.1 μ M Go6976 for 2 h, and then 1 μ M sunitinib was added for another 24 h. After treatment, expression of CD11b and CD14 was measured using flow cytometry. **b** Regulation of PKC α/β by sunitinib. AML cells were treated with various concentrations of sunitinib for 2 h. The levels of phosphorylated PKC α and PKC β proteins were analysed by western blot. β -actin was used as the loading control



suggesting that PKC α/δ may play an important role in sunitinib-induced monocytic differentiation. However, these results cannot exclude the possibility that other molecules are involved in the sunitinib-induced monocytic differentiation of AML cells. A study conducted by Nishioka et al. [11] demonstrated that sunitinib-induced differentiation was accompanied by upregulation of C/EBP- α and downregulation of c-Myc in MOLM13 and HL60 cells. However, in our study, sunitinib did not affect the expression of either C/EBP- α or c-Myc in KG-1 and HL60 cells (data not shown).

The mechanisms underlying sunitinib-induced AML cell apoptosis were also investigated in this study. Sunitinib has been reported to inhibit cell proliferation and induce apoptosis

in various cancer cell lines including transformed Ba/F3 cells expressing FLT3 K663Q [38], renal cell carcinoma cells [39], FLT3-mutated MV4-11 and MOLM13 leukaemia cells [40], and human bladder T24 cancer cells [41]. However, the precise mechanisms of sunitinib-induced apoptotic death in AML cells have not been extensively explored. In the current study, sunitinib-induced apoptosis in human AML cells was confirmed by an increased proportion of AML cells in sub-G1 phase and translocation of phosphatidylserine to the cell surface. Many molecules participate in cell apoptosis regulation, among which caspases are the central effectors promoting apoptosis in response to apoptosis-inducing signals from both intrinsic (mitochondrial disruption and endoplasmic reticulum

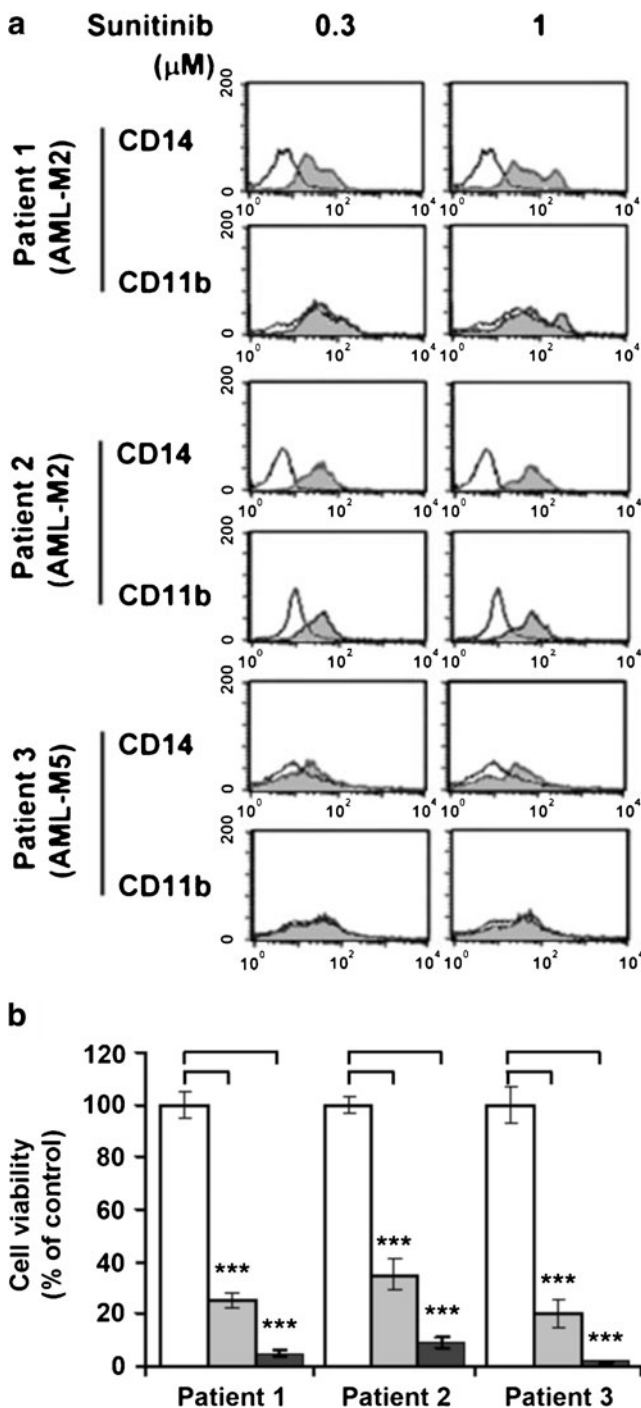


Fig. 7 Sunitinib induces monocytic differentiation of patient-derived AML blast cells. Mononucleated cells were freshly isolated from three AML patients and then incubated with different concentrations of sunitinib for 24 h. **a** Expressions of cell surface markers CD14 and CD11b were measured using flow cytometry. Untreated control group is shown in *white histogram*; sunitinib-treated group is shown in *gray histogram*. **b** AML blast cells were treated with 3 and 6 μM sunitinib for 48 h. Cell viability was determined by trypan blue dye exclusion method. *White bar* indicates untreated control; *gray bar* indicates cells after 3 μM sunitinib treatment; and *black bar* indicates cells after 6 μM sunitinib treatment (*** $P < 0.001$)

stress) and extrinsic (cell surface death receptor) pathways. Caspase activation during sunitinib-induced apoptosis has been reported in several cancer systems including cervical cancer cells [42], medulloblastoma [21], human urothelial carcinoma TCC-SUP and 5637 cell lines [43], 4 T1 breast cancer cells [44], gastrointestinal stromal T1 cells [45], and hormone refractory prostate cancer [46]. In our study, caspase-2, caspase-3, caspase-8, and caspase-9, were activated by sunitinib-induced apoptosis in AML cells. Treatment with caspase inhibitors conferred partial protection against this effect, suggesting that both mitochondrial and death receptor-ligand signalling pathways may be involved in sunitinib-induced AML cell apoptosis.

Bcl-2 family proteins have been well documented as playing a crucial role in mitochondrial apoptotic pathway regulation. Cell response to apoptotic stimuli critically depends on the balance between pro- and anti-apoptotic Bcl-2 family proteins. Anti-apoptotic proteins in the Bcl-2 family include Bcl-2, Bcl-xL, and Mcl-1, which prevent cell death. On the contrary, pro-apoptotic molecules in Bcl-2 family proteins include Bax, Bak, PUMA, and Bad [14]. Recently, a study conducted by Yoon et al. [47] demonstrated that sunitinib induced cell apoptosis in human bladder cancer HTB5 cells by increasing Bax and Bad pro-apoptotic protein expression. In addition, Bcl-2 downregulation is also observed during sunitinib-induced apoptosis in T24 cells [41]. Ding et al. [48] also demonstrate that sunitinib-induced cell death in both colon cancer SW620 cells and lung cancer 95-D cells is associated with decreased Mcl-1 expression.

In the present study, Bcl-2 and Mcl-1 expression decreased while Bax, Bak, and PUMA increased in sunitinib-induced apoptotic AML cells. These results indicated that mitochondria-triggered apoptosis in sunitinib-treated AML cells may be attributable to downregulation of anti-apoptotic Bcl-2 family members and upregulation of pro-apoptotic Bcl-2 family members. We also found that sunitinib activated caspase-8 and increased Fas, FasL, DR4, and DR5 protein expression. These results provided the first evidence that extrinsic apoptotic pathway (death receptor/caspase-8 system) activation could be an essential mechanism responsible for sunitinib-induced apoptosis in AML cells. However, how these anti- and pro-apoptotic molecules are regulated in sunitinib-treated AML cells remains unclear. The precise mechanisms underlying sunitinib-induced apoptosis still require further investigation.

To characterise the potential clinical application of sunitinib in AML, we examined its cytotoxic and differentiation capacities in patient-derived myeloid blast cells. The results in myeloid blast cells from patients 1 and 2 were basically consistent with those in AML cell lines, suggesting that sunitinib could possibly benefit patients with non-APL AML. However, monocytic differentiation did not significantly increase in myeloid blast cells from patient 3 after

sunitinib treatment. One possible explanation for this inconsistent finding is that since patient 3 was diagnosed with acute monocytic leukaemia, sunitinib could not further differentiate the monocytic blast cells. However, more patient-derived myeloid blast cells are still needed to confirm this speculation.

In conclusion, our study provided evidence that the multitarget tyrosine kinase receptor inhibitor sunitinib induced G0/G1 phase arrest, monocytic differentiation, and apoptosis in human AML cell lines HL60 and KG-1. Increased p27^{Kip1}, pRb1, and p130/Rb2 expression and decreased cyclin D1, cyclin D3, and Cdk2 expression may be responsible for both G0/G1 phase arrest in the cell cycle and monocytic differentiation. PKC α and PKC β may also play a crucial role on sunitinib-induced monocytic differentiation. Moreover, caspase cascade activation (caspase-2, caspase-3, caspase-8, and caspase-9), pro-apoptotic molecule (Bax, Bak, PUMA, DR4, and FasL) upregulation, and anti-apoptotic molecule (Bcl-2 and Mcl-1) downregulation demonstrated that sunitinib-induced apoptosis in AML cells occurred via both intrinsic and extrinsic apoptotic pathways. Well-designed clinical trials are urgently needed to determine the most optimal clinical setting for sunitinib use in patients with non-APL AML.

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Author contributions C-L Teng and S-L Hsu performed the research and wrote the paper, W-L Hwang, C-T Yu and G-Y Hwang designed research and revised the paper, and H-C Liu and J-R Tsai performed the experiments.

Conflict of interest The authors have no conflicting financial interests.

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