

Hsp90 inhibitor induces KG-1a cell differentiation and apoptosis via Akt/NF- κ B signaling

JIN-HONG QIN^{1,2}, KUN WANG¹, XIN-LU FU³, PENG-JUN ZHOU¹, ZHONG LIU^{1,2}, DAN-DAN XU¹,
YI-FEI WANG^{1,2}, DE-PO YANG⁴, QIU-LING XIE^{1,2} and QIU-YING LIU^{1,2,4}

¹Guangzhou Jinan Biomedicine Research and Development Center, National Engineering Research Center of Genetic Medicine, and ²Guangdong Provincial Key Laboratory of Bioengineering Medicine, Jinan University, Guangzhou, Guangdong 510632; ³Laboratory Animal Center and ⁴School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou, Guangdong 510275, P.R. China

Received December 3, 2016; Accepted May 23, 2017

DOI: 10.3892/or.2017.5797

Abstract. Heat-shock protein 90 (Hsp 90) acts as a molecular chaperone that maintains protein stability and regulates cell proliferation, survival, differentiation and apoptosis. The present study investigated the effect of Hsp90 inhibition on human acute myeloid leukemia (AML) cells using the novel small-molecule inhibitor SNX-2112. We found that SNX-2112 more potently inhibited KG-1a cell growth than the classical Hsp90 inhibitor 17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin as determined by CCK-8 assay. Flow cytometry was used to examine the cell cycle, differentiation, and apoptosis, and western blotting and qRT-PCR were used to analyze the underlying mechanism. The results revealed that low concentrations of SNX-2112 arrested the cells in the G2/M phase and induced their differentiation and apoptosis, possibly by suppressing Akt and inhibitor of κ B kinase, a component of the nuclear factor (NF)- κ B signaling pathway. We also found that SNX-2112 increased the expression of the differentiation transcription factors PU.1 and CCAAT-enhancer-binding protein- α . Thus, SNX-2112 induced KG-1a cell differentiation, cell cycle arrest and apoptosis via modulation of Akt and NF- κ B signaling, suggesting that it is a promising therapeutic agent for the treatment of AML.

Introduction

Acute myeloid leukemia (AML) is a clinically and biologically heterogeneous clonal hematologic disorder that is common and lethal in adults (1). Even with improvements in diagnosis and supportive care, the 5-year survival rate for adults with AML is only 30%. There have been no breakthroughs in AML treatment in the last 40 years, although in the past decade, an increasing number of potential drug targets have been identified (2).

Heat shock protein 90 (Hsp 90) is a chaperone protein required for the folding and stabilization of proteins involved in intracellular signaling such as Akt and the nuclear factor (NF)- κ B signaling pathway component inhibitor of κ B kinase (IKK) α , which regulate cell survival, proliferation and differentiation (3,4). Hsp90 is overexpressed in many types of cancer relative to normal tissues and is therefore considered a potential anticancer drug target (3,5). There are sixteen different Hsp90 inhibitors that have entered clinical testing including first generation Hsp90 inhibitors (geldanamycin and its derivatives) and second generation Hsp90 inhibitors (NVP-AUY922 and SNX-5422). However, there is no FDA approved Hsp90 inhibitor nor standardized assay to ascertain Hsp90 inhibition. The most clinically significant off-target toxicity with the geldanamycin derivatives was hepatotoxicity resulting from the presence of a benzoquinone moiety. In addition to hepatotoxicity, ocular and cardiac toxicities also limited further clinical development of these drugs (6-8). Therefore, development of an Hsp90 inhibitor with better pharmacological properties and a safety profile is important.

Most Hsp90 inhibitors exhibit great anti-acute myeloid leukemia effects. The second-generation Hsp90 inhibitor NVP-AUY922-AG was demonstrated to be cytotoxic in myeloid cell lines and primary AML cells (9), and other Hsp90 inhibitors were tested in phase I clinical trials and have been well tolerated in patients with advanced AML (10). SNX-2112 is a novel inhibitor that competitively binds to the N-terminal ATP-binding site of Hsp90 and has shown anticancer activity *in vitro* and *in vivo* (3,11).

A hallmark of AML is that leukemic blast cells are arrested at an early stage of differentiation. It has therefore been suggested that therapies promoting differentiation may be

Correspondence to: Qiu-Ying Liu, Guangzhou Jinan Biomedicine Research and Development Center, Jinan University, 601 West HuangPu Road, Guangzhou, Guangdong 510632, P.R. China
E-mail: qiuying_liu@126.com

Professor Qiu-Ling Xie, Guangdong Provincial Key Laboratory of Bioengineering Medicine, Jinan University, 601 West HuangPu Road, Guangzhou, Guangdong 510632, P.R. China
E-mail: txql@jnu.edu.cn

Abbreviations: AML, acute myeloid leukemia; GM, geldanamycin; 17-AAG, 17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin; ATRA, all-*trans*-retinoic acid; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; Bar, bardoxolone methyl

Key words: Hsp90 inhibitor, SNX-2112, KG-1a cells, differentiation, apoptosis

effective for AML treatment, a concept known as differentiation therapy (12). All-*trans*-retinoic acid (ATRA) and arsenic trioxide used in combination with chemotherapy is the standard treatment for acute promyelocytic leukemia and is a potential paradigm for differentiation therapy in clinical oncology, but not for other subtypes of AML (13,14). Studies have shown that isocitrate dehydrogenase (IDH), glycogen synthase kinase (GSK)-3, and dihydroorotate dehydrogenase inhibition induces AML cell differentiation (15-18), but there are no studies on whether the inhibition of Hsp90 can achieve this effect.

The transcription factor CCAAT/enhancer binding protein α (C/EBP α) is a critical regulator of myeloid development that directs granulocyte and monocyte differentiation. Conditional C/EBP α deficiency in adult mice blocked the transition from common myeloid progenitor (CMP) to granulocyte-monocyte progenitor (GMP), resulting in decreased formation of both granulocytes and monocytes (19). In addition, the suppression of C/EBP α expression may block differentiation, and also stimulate proliferation of transformed cells (20). PU.1 is a member of the E2f transformation-specific sequence family of transcription factors, which is expressed in granulocytes, monocytes and B-lymphoid cells (21), with its level increasing during differentiation. As such, PU.1 mutation, which has been identified in a subset of AML patients, has been linked to leukemogenesis (22). Both C/EBP α and PU.1 are required for differentiation of the granulocytic lineage.

Akt is a serine threonine kinase downstream of phosphoinositide 3-kinase (PI3K) that has many downstream targets associated with cell survival and cell cycle regulation, and also inhibits hematopoietic cell apoptosis (23). Nuclear factor (NF)- κ B regulates various biological processes linked to leukemogenesis, including cell proliferation, differentiation, autophagy and apoptosis, and is constitutively activated in AML cells (24,25).

The present study investigated the potential of the Hsp90 inhibitor SNX-2112 to be used for treatment of AML using human acute leukemia KG-1a cells. We found that SNX-2112 induced cell cycle arrest at the G2/M phase and apoptosis while promoted differentiation and suppressed cell growth, effects that involve modulation of Akt and NF- κ B signaling.

Materials and methods

Cell culture and reagents. Human AML KG-1a cells purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 15% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin in a humidified incubator of 5% CO₂ at 37°C. SNX-2112 was synthesized as previously described (26) with a purity >98.0%, and 10 mM SNX-2112 stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at 4°C. 17-AAG and bardoxolone methyl (BAR; an NF- κ B pathway inhibitor) were obtained from Selleck Chemicals (Houston, TX, USA). RPMI-1640 medium and FBS were purchased from Gibco (Grand Island, NY, USA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), caspase-3, Bcl-x1, Bcl-2, Akt, p-Akt (Thr308), IKK α , IKK β , p65, p-p65, I κ B, and PU.1 were purchased from Cell Signaling Technology (Beverly, MA, USA), and cluster of differentiation (CD)11b was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Cell proliferation assay. Cell proliferation was evaluated using Cell Counting Kit-8 (CCK-8). Briefly, 1.0x10⁴ cells/well were seeded into 96-well plates and treated with SNX-2112 or 17-AAG for 24, 48 or 72 h. A 10- μ l volume of CCK-8 working solution was added to each well for 2 h. The absorbance was assessed at 450 nm on a microplate reader (Bio-Rad, Hercules, CA, USA) and used to determine the drug concentration inhibiting growth by 50% (IC₅₀).

Cell cycle analysis. KG-1a cells were seeded at 1.0x10⁵ cells/ml and treated with SNX-2112 for 48 h. The cells were collected by centrifugation at 500 x g for 5 min; 70% ethanol was then added, followed by 2 washes with phosphate-buffered saline (PBS). Cells were resuspended in 1 ml PBS containing 2.5 μ g/ml ribonuclease and 50 μ g/ml propidium iodide (PI) (Beyotime Institute of Biotechnology, Shanghai, China) and incubated in the dark for 30 min at room temperature before analysis by flow cytometry (BD FACSCalibur, Lake Franklin, NJ, USA; BD Biosciences).

Cell differentiation assay. Cells were seeded at 1.0x10⁵ cells/ml and incubated with SNX-2112 for 48 h. The cells were collected by centrifugation at 500 x g for 5 min and washed with PBS twice. Then, a 20- μ l volume of CD11b was added for 20 min in the dark and analyzed by flow cytometry.

Cell apoptosis assay. Cells were seeded at 1.0x10⁵ cells/ml and incubated with SNX-2112/Bar for 48 h. Samples were prepared according to the instructions provided with the Annexin V-fluorescein isothiocyanate/PI staining kit (Beyotime Institute of Biotechnology) and analyzed by flow cytometry.

Quantitative real-time (qRT)-PCR. KG-1a cells were seeded at 1.0x10⁵ cells/ml, and incubated with DMSO or SNX-2112 for 48 h, and total RNA was immediately extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For analysis of PU.1 and C/EBP α , 2 μ g of total RNA was used to synthesize first-strand DNA with reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was then carried out using Green PCR Master Mix (Shine Co., Shanghai, China), 1 μ l of cDNA, gene-specific primers (Table I), and the Hot Start Fluo-PCR Mix in a 20- μ l reaction under the following cycling conditions: 95°C for 5 min, followed by 35 cycles at 95°C for 10 sec, 57°C for 15 sec, and 72°C for 20 sec. Each sample was prepared in triplicate and transcript levels were quantified using the 2^{- $\Delta\Delta$ Ct} method (27).

Immunofluorescence analysis. KG-1a cells cultured in 6-well plates were treated with DMSO or SNX-2112 (0, 0.25, 0.5 or 1 μ M) for 48 h. The cells were washed 3 times with ice-cold PBS and fixed with 4% paraformaldehyde. The cell membrane was permeabilized by adding 0.1% Triton X-100, and the cells were blocked with 5% bovine serum albumin (HyClone, Logan, UT, USA) and incubated for 2 h at room temperature with an antibody against NF- κ B p65 (1:400) (Cell Signaling Technology), followed by incubation with a fluorophore-conjugated anti-rabbit antibody for 2 h at room temperature. The cells were then stained with PI (Beyotime Institute of Biotechnology) for 15 min, and subsequently washed and examined with an epifluorescence microscope (Zeiss, Jena, Germany).

Table I. Primers used for qRT-PCR.

Gene name		Sequence (5'→3')
GAPDH	F	CGTCTTCACCACCATGGAGA
	R	CGGCCATCACGCCACAGTTT
PU.1	F	GTGCCCTATGACACGGATCT
	R	GAAGCTCTCGAACTCGCTGT
C/EBP α	F	TGGACAAGAACAGCAACGAG
	R	TTGTCACTGGTCAGCTCCAG

F, forward; R, reverse; GAPDH, 3-phosphate dehydrogenase; C/EBP α , CCAAT/enhancer binding protein α .

Western blotting. KG-1a cells were washed twice in ice-cold PBS, lysed in radioimmunoprecipitation buffer for 30 min on ice at 4°C, and centrifuged at 12,000 x g for 15 min. The protein content in the supernatant was determined with the bicinchoninic acid assay. Equivalent amounts (30-50 μ g) of protein were denatured in sodium dodecyl sulfate (SDS) sample buffer and resolved by 10-15% SDS-polyacrylamide gel electrophoresis, and then transferred to an Immobilon polyvinylidene difluoride membrane, which was blocked in 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h and then probed with primary antibodies (1:1,000) overnight at 4°C. The membrane was washed 3 times for 10 min in TBST, and incubated with a secondary antibody (1:6,000-1:8,000) in TBST with 5% skimmed milk at room temperature for 1 h. After washing, immunoreactivity was detected with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). GAPDH served as a loading control.

Clone formation assay. Cells were treated with SNX-2112 or 17-AAG for 2 days, and then washed twice with PBS. The cells were seeded (1×10^3 cells/dish) in 1.5 ml of 0.30-0.35% soft agar (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% FBS and cultured for 12 days at 37°C and 5% CO₂. After 12 days, the colonies were counted under a light microscope.

Statistical analysis. All data were confirmed by at least 3 independent experiments. Data are expressed as the mean \pm SD. Statistical significance was determined by one-way analysis of variance (ANOVA) and covariance calculated using GraphPad 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between 2 groups were detected using two-tailed Student's t-test. P<0.05 and P<0.01 were considered to indicate a statistically significant result.

Results

SNX-2112 inhibits KG-1a cells proliferation. The effects of SNX-2112 and 17-AAG on the viability of KG-1a cells were evaluated using CCK-8 assay. Both compounds showed dose- and time-dependent inhibition of KG-1a cells proliferation after 24, 48 and 72 h. The IC₅₀ values of SNX-2112 were 1.02, 0.29 and 0.22 μ M, respectively. These were lower than the IC₅₀ values of 17-AAG, which were 209.6, 30.27 and 44.75 μ M,

respectively (Fig. 1A and B), indicating that SNX-2112 inhibits cell growth to a greater degree.

SNX-2112 induces cells cycle arrest at the G2/M phase. To determine whether cell growth inhibition by SNX-2112 is associated with cell cycle arrest, the cell cycle distribution was analyzed using flow cytometry. KG-1a cells treated with SNX-2112 were arrested in the G2/M phase after 48 h (Fig. 1C); the proportion of arrested cells was 22.5, 41.2 and 28.7% at concentrations of 0.25, 0.5 and 1 μ M, respectively, as compared to 17% in the control group (Fig. 1D).

SNX-2112 induces differentiation and decreases colony formation. To determine whether KG-1a cells differentiation was affected by SNX-2112 treatment, we analyzed the expression of the differentiation marker CD11b by flow cytometry at 24 and 48 h. CD11b expression was increased by treatment with SNX-2112 from 3.32 to 19.18% at 24 h (data not shown), and from 4.99 to 50.8% at 48 h (Fig. 2A and B).

Since the aim of AML differentiation therapy is to suppress the growth of AML cells, we performed a colony formation assay to determine whether SNX-2112 induced irreversible growth arrest. KG-1a cells were exposed to the drug for 2 days, and after washing, an equal number of viable cells were plated on soft agar. A marked decrease in the number of colonies formed was observed upon treatment with SNX-2112 (Fig. 2C), indicating that SNX-2112 can cause KG-1a cells growth arrest, and that SNX-2112 was more potent than 17-AAG.

SNX-2112 induces KG-1a cells apoptosis. To determine whether SNX-2112 inhibits KG-1a cells growth by inducing apoptosis, the treated cells were labeled with Annexin V/PI and analyzed by flow cytometry. Annexin V⁺/PI⁻ and Annexin V⁺/PI⁺ cells were designated as early apoptotic and necrotic cells, respectively. The number of apoptotic cells was increased from 3.93% in the control cells to 14.82, 29.5 and 35.2% by treatment with 0.25, 0.5 and 1 μ M SNX-2112, respectively, for 48 h (Fig. 3A and B). Moreover, the expression of the apoptosis-related protein cleaved caspase-3 was increased in the presence of SNX-2112, although that of B cell lymphoma (Bcl)-2 and Bcl-2-associated X protein were unaltered (Fig. 3C).

SNX-2112 inhibits Akt and NF- κ B signaling. Hsp90 inhibition has been demonstrated to block PI3K and IKK signaling pathways (9). We therefore investigated whether SNX-2112 treatment affected the expression of Akt and IKK by western blotting. Previous studies revealed that Hsp90 was overexpressed in many types of cancer (3,5). Our results revealed that Hsp90 was highly expressed in KG-1a cells, and Hsp90 client proteins IKK α , IKK β and Akt expression levels were downregulated upon treatment with SNX-2112 for 48 h (Fig. 4A). Consistent with previous studies (28), NF- κ B pathway inhibitor Bar, inhibited KG-1a cell proliferation and induced apoptosis (Fig. 4B-D). Bar is an IKK β -inhibitor which inhibited the expression of downstream protein p65 but did not directly interfere with the expression of IKK after treatment with Bar (0.5 μ M) for 48 h (Fig. 4E), as Bar could possibly block the upstream kinases that have been implicated in the activation of IKK (29). SNX-2112 treatment resulted in the upregulation of NF- κ B

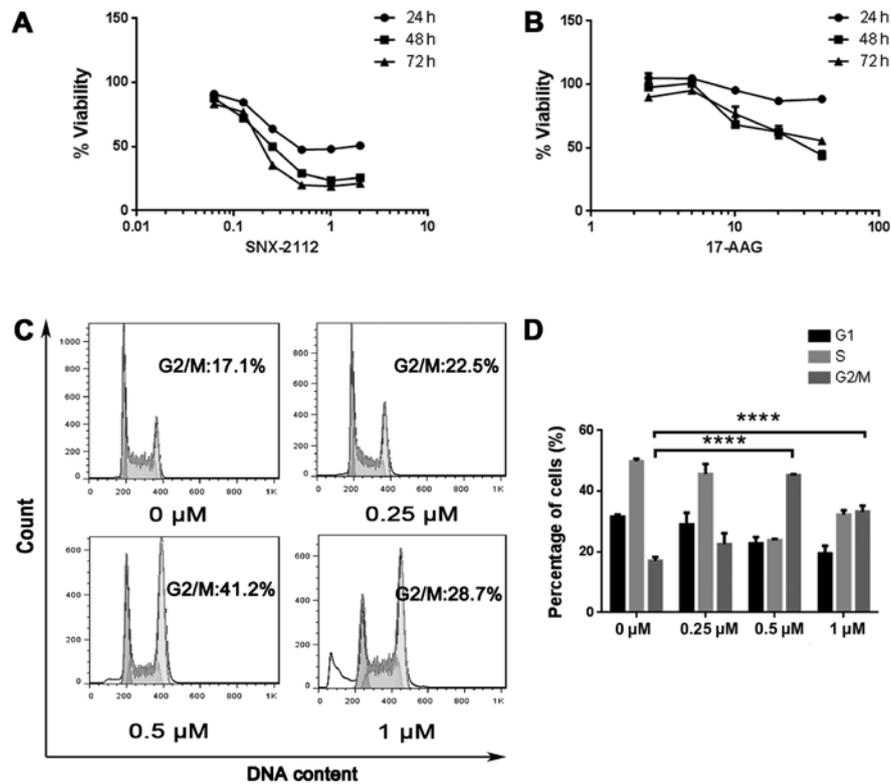


Figure 1. SNX-2112 inhibits proliferation and induces cell cycle arrest in KG-1a cells. (A and B) KG-1a cells were cultured in the presence of SNX-2112 or 17-AAG for 24, 48 or 72 h, and the cell proliferation was assessed using CCK-8 assay. (C and D) KG-1a cells were arrested at the G2/M phase with SNX-2112 treatment. Cells were treated with indicated concentrations of SNX-2112 and analyzed after 48 h by PI staining and flow cytometry. Data are presented as the mean \pm SEM (n=3/group); ****P<0.0001 compared to the corresponding control.

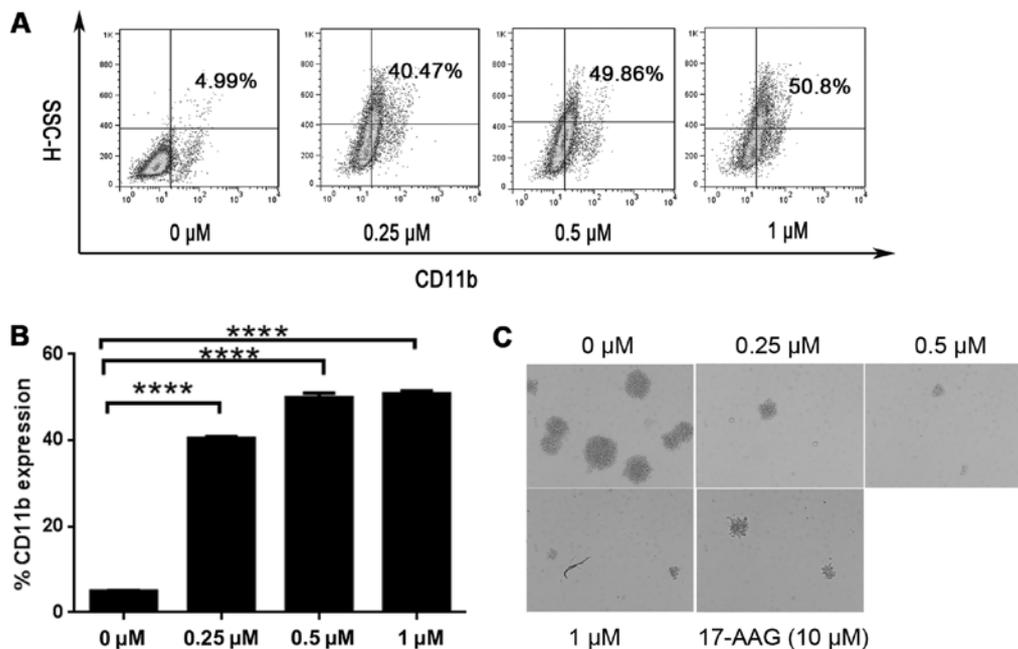


Figure 2. SNX-2112 induces differentiation and decreases colony formation in KG-1a cells (A and B) KG-1a cells were treated with indicated concentrations of SNX-2112 and analyzed after 48 h by flow cytometry. Representative bar graphs reveal the expression of the differentiation marker CD11b. Data are presented as the mean \pm SEM (n=3/group); ****P<0.0001 compared to the corresponding control. (C) SNX-2112 suppresses clonal growth of KG-1a cells, as determined by the colony formation assay.

inhibitors (i.e., I κ B) and downregulation of p65 and p-p65 after 48 h (Fig. 4F and G). Since C/EBP α and PU.1 play key roles in myeloid differentiation and NF- κ B has been demonstrated to

regulate PU.1 (30-32), we evaluated their expression after 48 h by qRT-PCR. The levels of both transcripts were increased by >2-fold in the presence of SNX-2112 (Fig. 5A and B). The

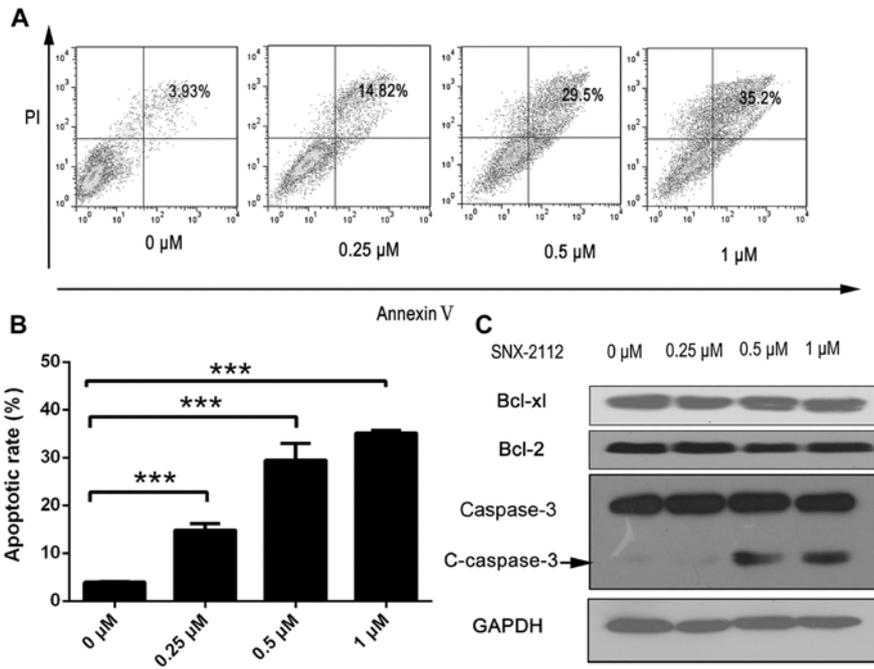


Figure 3. SNX-2112 induces KG-1a cell apoptosis. (A and B) KG-1a cells were treated with indicated concentrations of SNX-2112 and Annexin V/PI staining was detected after 48 h by flow cytometry. Data are presented as the mean ± SEM (n=3/group); ***P<0.001, the corresponding control. (C) KG-1a cells were treated with indicated concentrations of SNX-2112 for 48 h and Bcl-x1, Bcl-2, caspase-3 expressions were analyzed by western blotting.

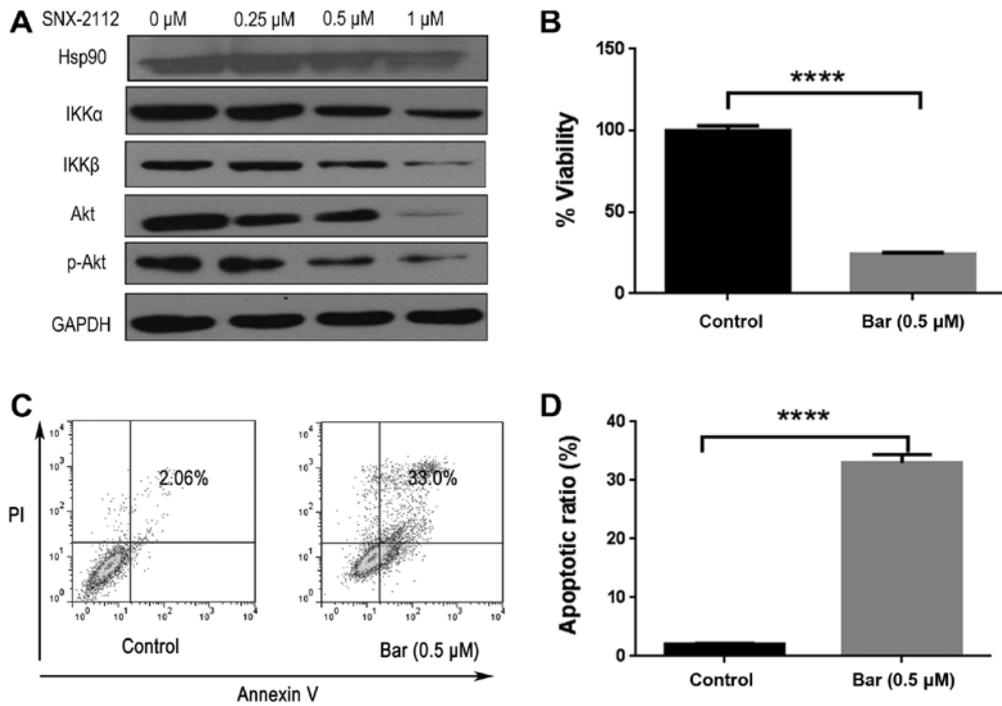


Figure 4. SNX-2112 inhibits Akt and NF-κB signaling. (A) KG-1a cells were treated with indicated concentrations of SNX-2112 for 48 h. Hsp90, IKKα, IKKβ, Akt and p-Akt were analyzed by western blotting relative to that of GAPDH. (B) KG-1a cells were treated with an NF-κB pathway inhibitor (Bar, 0.5 μM) or left untreated (control) for 48 h, and cell proliferation was assessed with CCK-8 assay. (C and D) KG-1a cells were treated with Bar (0.5 μM) or a vehicle for 48 h and Annexin V/PI staining was detected after 48 h by flow cytometry. Data are presented as the mean ± SEM (n=3/group); ****P<0.0001 compared to the corresponding control.

increase in PU.1 expression was confirmed by immunofluorescence analysis (Fig. 5C). These results indicate that inhibition of Hsp90 by SNX-2112 suppresses the expression of Akt and IKK and induces that of C/EBPα and PU.1 via inhibition of NF-κB signaling in KG-1a cells.

Discussion

Hsp90 is overexpressed in most cancers; pharmacological inhibition of this protein can induce apoptosis in hematologic and other types of tumors (9). In the present study, we

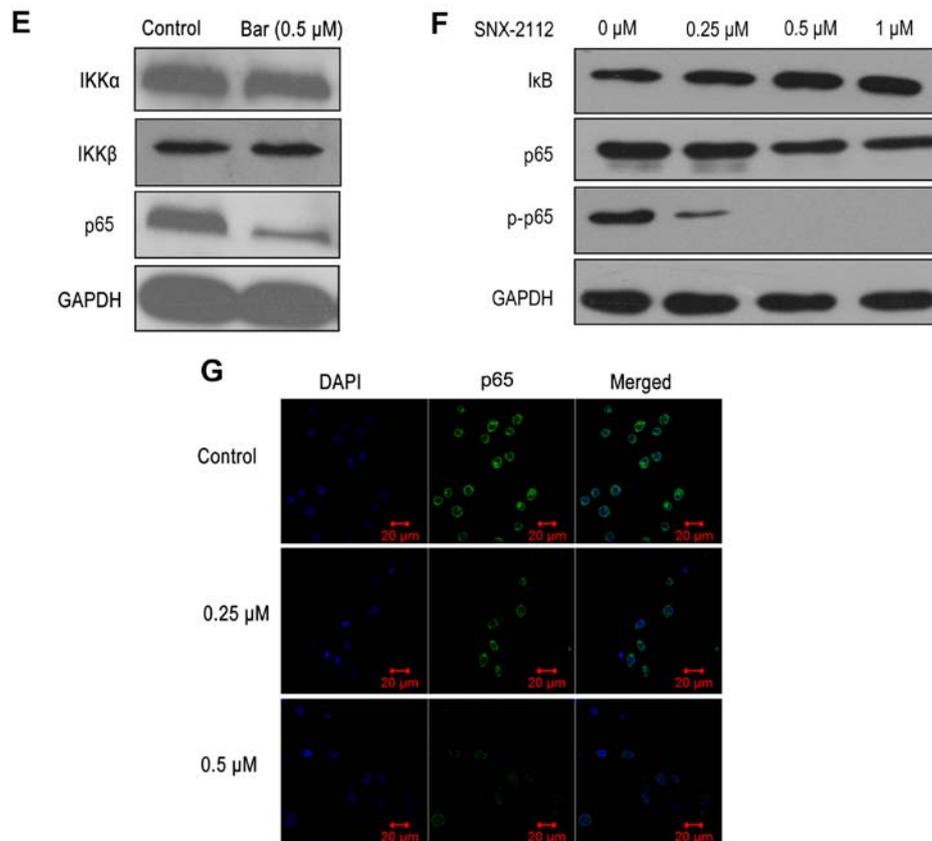


Figure 4. Continued. SNX-2112 inhibits Akt and NF- κ B signaling. (E) KG-1a cells were treated with Bar (0.5 μ M) or left untreated (control) for 48 h. IKK α , IKK β and p65 levels were analyzed by western blotting relative to that of GAPDH. (F) KG-1a cells were treated with indicated concentrations of SNX-2112 for 48 h. I κ B, p65 and p-p65 levels were analyzed by western blotting relative to that of GAPDH. (G) KG-1a cells were treated with indicated concentrations of SNX-2112 for 48 h and p65 expression was detected by immunofluorescence labeling. Nuclear cytoplasmic expression of NF- κ B p65 was decreased by SNX-2112 treatment.

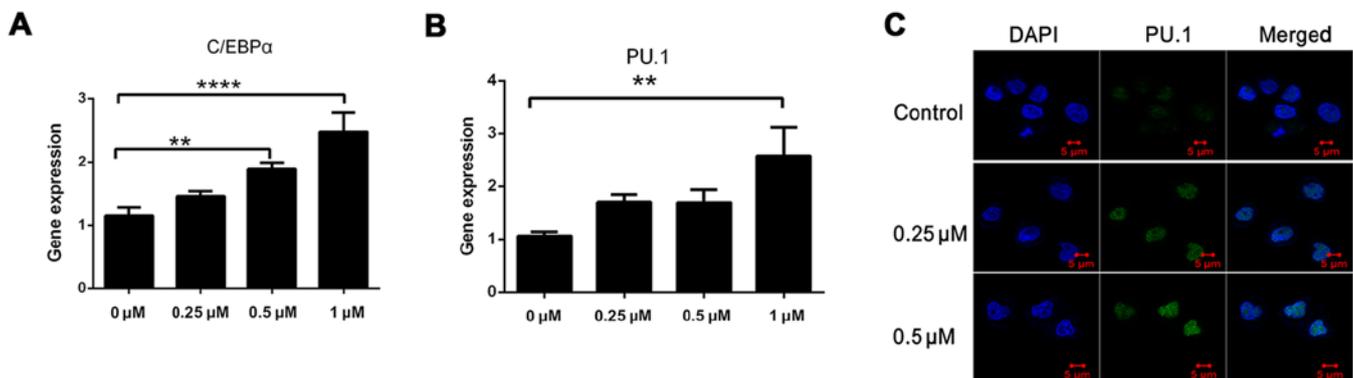


Figure 5. SNX-2112 upregulates C/EBP α and PU.1. (A and B) SNX-2112 upregulates C/EBP α and PU.1. Cells were treated with indicated concentrations of SNX-2112 for 48 h, and C/EBP α and PU.1 expression was evaluated by qRT-PCR. Data are presented as the mean \pm SEM (n=3/group). **P<0.01 and ****P<0.0001 compared to the corresponding control. (C) SNX-2112 treatment increases PU.1 expression. Cells were treated with indicated concentrations of SNX-2112 for 48 h, and PU.1 was detected by immunofluorescence labeling. PU.1 immunoreactivity was significantly decreased in the nucleus.

demonstrated that the Hsp90 inhibitor SNX-2112 suppressed the proliferation of KG-1a cells and induced their arrest in the G2/M phase and their differentiation and apoptosis in a time- and dose-dependent manner. Previous studies have revealed that SNX-2112 affects the activity and expression of Hsp90 client proteins (11), and our results revealed that SNX-2112 treatment resulted in the downregulation of the Hsp90-associated proteins Akt and IKK α as well as cleavage

of caspase-3. We previously demonstrated that SNX-2112 suppressed K562 cell proliferation (33). Other studies have reported that it also inhibits the growth of multiple myeloma and other hematologic tumors (34). In the present study SNX-2112 had more potent effects than the classical Hsp90 inhibitor 17-AAG in KG-1a human acute leukemia cells.

AML is characterized by uncontrolled proliferation of myeloid progenitors that have decreased capacity for

differentiation into mature granulocytes or macrophages (35). Differentiation therapy is a promising approach for the treatment of AML. Small-molecule inhibitors of mutant IDH2 (15) or IDH1 (16) can induce tumor cell differentiation in a subset (15%) of AML patients with *IDH1/2* mutations; GSK-3 inhibition sensitizes AML cells to differentiation induced by an ATRA analog (17), with inhibition of dihydroorotate dehydrogenase having a similar effect (18). It has been reported that co-treatment with tubacin and 17-AAG decreased the viability of primary AML cells (36), whereas 17-AAG suppressed the growth of lymphoma stem cells (37). However, few studies have investigated the effect of HSP90 inhibition on AML. In the present study, we found that the Hsp90 inhibitor SNX-2112 promoted the differentiation of KG-1a cells.

Aberrant expression of C/EBP α and PU.1 contributes to the development of AML. PU.1 is a critical transcription factor during early granulopoiesis; PU.1 deficiency impairs hematopoietic development and can lead to leukemia (38). But PU.1 knockdown inhibited the proliferation of human AML U937 cells (39). In contrast, C/EBP α expression is required for the differentiation of myeloid lineage cells, and C/EBP α overexpression in primary human CD34⁺ cells led to granulocytic differentiation (40). In the present study, we found that C/EBP α and PU.1 were upregulated by SNX-2112 treatment, indicating that this drug induces AML cell differentiation by increasing C/EBP α and PU.1 levels.

The Hsp90 inhibitor NVP-AUY922-AG has been reported to induce apoptosis of AML cells by inhibiting the PI3K and IKK signaling pathways (9). Our findings indicate that Bar blocks KG-1a cell proliferation and induces apoptosis. Since p65 is constitutively active in AML (25), we speculated that IKK signaling is activated in KG-1a cells. SNX-2112 treatment decreased IKK expression as well as p65 expression, phosphorylation, and nuclear translocation. It has been previously shown that p65 regulates PU.1 expression to induce myeloid differentiation (32). Additionally, PU.1 expression is suppressed as a result of C/EBP α inhibition (20). We propose that SNX-2112 exerts its effects on KG-1a cells by inhibiting NF- κ B signaling and modulating the expression of PU.1 and C/EBP α , although additional studies are warranted to clarify the detailed molecular mechanisms. Nonetheless, our results suggest that SNX-2112 is a promising therapeutic agent for the treatment of AML.

Acknowledgements

The present study was supported by the Postdoctoral Science Foundation of China (2015M582472), the Natural Science Foundation of Guangdong Province (2014A030310434), and the National Natural Science Foundation of China (81500227)

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