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SphK1 inhibitor II (SKI-II) inhibits acute myelogenous leukemia cell growth *in vitro* and *in vivo*

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ABSTRACT

Previous studies have identified sphingosine kinase 1 (SphK1) as a potential drug target for treatment of acute myeloid leukemia (AML). In the current study, we investigated the potential anti-leukemic activity of a novel and specific SphK1 inhibitor, SKI-II. We demonstrated that SKI-II inhibited growth and survival of human AML cell lines (HL-60 and U937 cells). SKI-II was more efficient than two known SphK1 inhibitors SK1-I and FTY720 in inhibiting AML cells. Meanwhile, it induced dramatic apoptosis in above AML cells, and the cytotoxicity by SKI-II was almost reversed by the general caspase inhibitor z-VAD-fmk. SKI-II treatment inhibited SphK1 activation, and concomitantly increased level of sphingosine-1-phosphate (S1P) precursor ceramide in AML cells. Conversely, exogenously-added S1P protected against SKI-II-induced cytotoxicity, while cell permeable short-chain ceramide (C6) aggravated SKI-II's lethality against AML cells. Notably, SKI-II induced potent apoptotic death in primary human AML cells, but was generally safe to the human peripheral blood mononuclear cells (PBMCs) isolated from healthy donors. *In vivo*, SKI-II administration suppressed growth of U937 leukemic xenograft tumors in severe combined immunodeficient (SCID) mice. These results suggest that SKI-II might be further investigated as a promising anti-AML agent.

1. Introduction

Acute myeloid leukemia (AML) remains to be a deadly disease for many affected adults [1,2]. It has drawn significant attentions from both oncologists and cancer biologists in past decades [3–5]. Indeed, major improvements have been achieved in both basic research and/or clinical treatments for AML [3–5]. However, the overall survival of AML patients has not been significantly changed, especially for those with advanced diseases [1,2]. Further, the molecular heterogeneity of AML hinders the uniform application of specific molecularly targeted agents [3–5]. Therefore, the search for novel and more efficient anti-AML agents is extremely important and urgent.

Sphingolipids are ubiquitous membrane constituents of all eukaryotic cells [6–8]. Meanwhile, sphingolipids are also important signaling molecules that are involved in regulating many key cellular functions [6–8]. For instance, sphingosine-1-phosphate (S1P), produced from sphingosine by sphingosine kinases (SphKs), is a lipid signaling molecule involving several pro-cancer behaviors, including cell growth, survival and apoptosis-resistance [9]. On the other hand, accumulation of S1P precursors, including sphingosine and ceramide, will promote cell growth arrest and apoptosis [10]. As such, the balance between these sphingolipids has been viewed as a cellular rheostat determining cell fate [6–8]. This balance is tightly controlled mainly by sphingosine kinase 1 (SphK1), which catalyzes the formation of S1P through phosphorylating sphingosine [6–8]. Currently, SphK1 inhibitors are being evaluated in pre-clinical and clinical studies for their anti-tumor potentials [11–13]. Some of these SphK1 inhibitors have display promising results against AML [14,15] and other solid tumors [11–13].

In this study, we investigated the anti-leukemic activity of a novel and specific SphK1 inhibitor: SphK1 inhibitor II (SKI-II) [16–18]. Our results demonstrated that SKI-II potently induced growth inhibition and apoptosis in both primary and transformed human AML cells, and suppressed AML xenograft tumor growth in severe combined immunodeficient (SCID) mice.

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2. Material and methods

2.1. Chemicals and reagents

SKI-II, or 4-[[4-(4-Chlorophenyl)-2-thiazolyl]amino]phenol, was purchased from Tocris Bioscience (Ellisville, Mo). SK1-I, or (2R,3S,4E)-N-methyl-5-(4′-pentylphenyl)-2-aminopent-4-ene-1,3-diol, was synthesized by Suzhou Ming-de Biotech (Suzhou, China) [14]. FTY720 was purchased from Sigma Chemical Company (St. Louis, Mo.). Cell permeable short-chain C6 ceramide was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). S1P was purchased from Cayman Chemical Co. (Ann Arbor, MI). z-VAD-fmk was purchased from Calbiochem (Beijing, China).

2.2. Cell lines

Human AML cell lines HL-60 and U937, obtained from American Type Culture Collection (ATCC, Rockville, MD), were cultured in RPMI 1640 supplemented with 10% FBS and 1% L-glutamine at 37 °C, in a humidified atmosphere with 5% CO_2.

2.3. Primary culture of human AML cells

Leukemic blasts were obtained from two AML patients (male, 35 and 41 years old) undergoing routine diagnostic aspirations. Samples, which contained 85% blasts in each case, were separated by centrifugation over Ficoll/Hypaque (specific gravity: 1.077–1.081; Sigma–Aldrich, Shanghai, China) at 400 g at room temperature. The interface layer, containing primarily blasts, was removed using a sterile Pasteur pipette, and resuspended in medium containing 10% FBS. Cells exhibited over 95% viability by trypan blue exclusion were cultured in the presence of 50 ng/mL rhGM-CSF, 100 ng/mL rhIL-3 and 10 ng/mL rhG-CSF (all purchased from Sigma, Shanghai, China).

2.4. Primary culture of human peripheral blood mononuclear cells (PBMCs)

PBMCs of two healthy donors were isolated by centrifugation over lymphocyte separation medium (Sigma). After three washes in PBS, PBMCs were counted and cultured in DMEM supplemented with 10% FBS, 2 μg of phytohemagglutinin (PHA) per mL, 10 ng of phorbol 12-myristate-13-acetate per mL, nonessential amino acids, 5 mM β-mercaptoethanol, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate (all purchased from Sigma, Shanghai, China). The study was approved by the institutional review board of the authors' institution (Shanghai Jiao Tong University School of Medicine), and written informed consent was obtained from each participant. All investigations were conducted according to the principles expressed in the Declaration of Helsinki.

2.5. AML cell proliferation assay

AML cells were seeded at a density of 5000 cells per well in a 96-well plate. After 24 h of plating, cells were stimulated with indicated treatment for 72 h, the signal for Cell Titer Glo Assay (Promega, Shanghai, China) was determined to quantify cell proliferation, and the value was normalized to that of untreated control group.

2.6. Trypan blue staining assay

After indicated treatment, the number of dead AML cells, with trypan blue staining, was counted, and cell death percentage was calculated by the number of the trypan blue positive cells divided by the number of total cells. AML cells with trypan blue exclusion were considered viable cells.

2.7. Annexin V assay of AML cell apoptosis

Apoptosis was determined by the analysis of Annexin V and propidium iodide (PI). AML cells were cultured at a density of 5×10^6 cells/mL in RPMI with 10% FBS containing indicated treatment. After 48 h, 100 μL of the cell culture was collected and transferred to FACS tubes containing 100 μL FACS buffer with 10 μg/mL Annexin V and 10 μg/mL PI. AML cells were incubated for 30 min and analyzed within 30 min by flow cytometry through a FACS Calibur (Becton Dickinson, Shanghai, China). The apoptotic cells were determined by calculating the percentages of the Annexin V^-/PI^+ AML populations.

2.8. Histone/DNA ELISA assay of AML cell apoptosis

The cell death detection ELISA plus Kit was applied for quantitatively analyzing apoptosis in AML cells following indicated treatments, in accordance to the protocol by the manufacturer. Briefly, AML cells were lysed and the cell lysates were overlaid and incubated in microplate plate modules coated with anti-histone antibody. Samples were then incubated with anti-DNA peroxidase followed by color development with ABTS substrate. The absorbance of the samples was determined with a microplate reader at 405 nm, which was utilized as a quantitative measurement of cell apoptosis.

2.9. Sphingosine kinase activity assay

As previously reported [19], AML cell lysates (100 μg) were incubated with 25 μM D-erythrosphingosine dissolved in 0.1% Triton X-100, 2 mM ATP, and [γ-^{32}P] ATP. The reaction was stopped by adding 20 μL of HCl (1 N), followed by adding 800 μL of chloroform/methanol/HCl (100:200:1, v/v). After vigorous vortex, chloroform and KCl were added, and phases were separated by centrifugation. The organic layer was dried and resuspended in chloroform/methanol/HCl 37% (100:100:0.2, v/v). Lipids were resolved on silica TLC plates in 1-butanol/acetic acid/water (3:1:1, v/v). Labeled S1P spots were visualized by autoradiography and quantified by scraping and counting in a scintillation counter (LS-6500, Beckman) [20]. The sphingosine kinase activity was valued as pmol/hour/g protein, and was expressed as fold change of the untreated control group.

2.10. Enzymatic measurement of ceramide

The cellular ceramide level was analyzed using the same procedure as described in Ref. [21], and was valued as fmol by nmol of phospholipids (PLs). Its level in the treatment group was expressed as the fold change of the untreated control group. Each measurement was performed three times.

2.11. Mice xenograft assay

U937 cells (2×10^6, suspended in 100 μL of culture medium) were injected into the flanks of 6-week-old CB17 severe combined immunodeficient (SCID)/beige mice and allowed to grow to palpable tumors for 7 days. When tumors reached a volume around 100 mm^3, animals were randomly assigned to three groups: Vehicle, SKI-II (10 mg/kg) and SKI-II (50 mg/kg). SKI-II was dissolved in saline and administrated intraperitoneally (*i.p.*) for 7 consecutive days. Tumor measurements were performed every two days with calipers, and tumor volume was calculated using the

formula: ($\pi \times$ [length in millimeters] $\times$ [width in mm^2]/6. At the end of each mice experiment, the animals were killed, and the tumors were removed and weighted. All experiments involving animals were approved by Shanghai Jiao Tong University School of Medicine's Animal Care and Use Committee (IACUC).

2.12. Statistical analysis

Experimental results shown were repeated at least two-three times with similar results obtained. Results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way ANOVA through the SPSS 18.0 (SPSS Inc, Chicago, IL). Significance was set at $p < 0.05$.

3. Results

3.1. SKI-II inhibits proliferation and survival of both primary and transformed AML cells

As shown in Fig. 1A, SKI-II dose-dependently inhibited the growth of HL-60 AML cells cultured in the presence of 10% serum, which was evident after 72 h of culture (Fig. 1A). Its activity was significantly more potent than the same concentration of two other tested SphK1 inhibitors: SK1-I [14] and FTY720 [22]. SKI-II, as the concentrations of 5 μM and 10 μM, also inhibited growth of U937 AML cells (Fig. 1B). Similarly, it was most competent among all tested SphK1 inhibitors (Fig. 1B). Results of trypan blue staining assay demonstrated that SKI-II dose-dependent inhibited HL-60 and U937 cell survival, resulting in substantial cell death (Fig. 1C and D). The activity of SKI-II was again superior than SK1–I and FTY720 (Fig. 1C and D). The effect of SKI-II on primary human AML cells was also tested. As demonstrated, SKI-II (1 and 10 μM) dramatically inhibited primary AML cell proliferation (Fig. 1E and F). Thus, AML patients' blast cells were also sensitive to SKI-II. On the other hand, PBMCs from healthy donors showed resistance to the same SKI-II treatment (Fig. 1E and F). SKI-II is only cytotoxic to cancerous cells.

3.2. SKI-II inhibits SphK1 activity, while concomitantly increasing ceramide level in AML cells

As discussed, SphK1 is a critical regulator of the balance between pro-apoptotic ceramide and anti-apoptotic S1P, the influence of SKI-II on SphK1 activity and ceramide level in AML cells was then tested. SKI-II treatment led to a dramatic reduction of SphK1 activity in both HL-60 cells (Fig. 2A) and U937 cells (Fig. 2B), its activity was again more potent than two other SphK1 inhibitors (SK1–I and FTY720) in both cells (Fig. 2A and B). As a result, the intracellular ceramide level was increased after SKI-II treatment in above AML cells (Fig. 2C and D). Note that exogenously-adding S1P inhibited the activity of SKI-II against AML cells, while short-chain cell permeable ceramide (C6) intensified SKI-II-induced AML cell

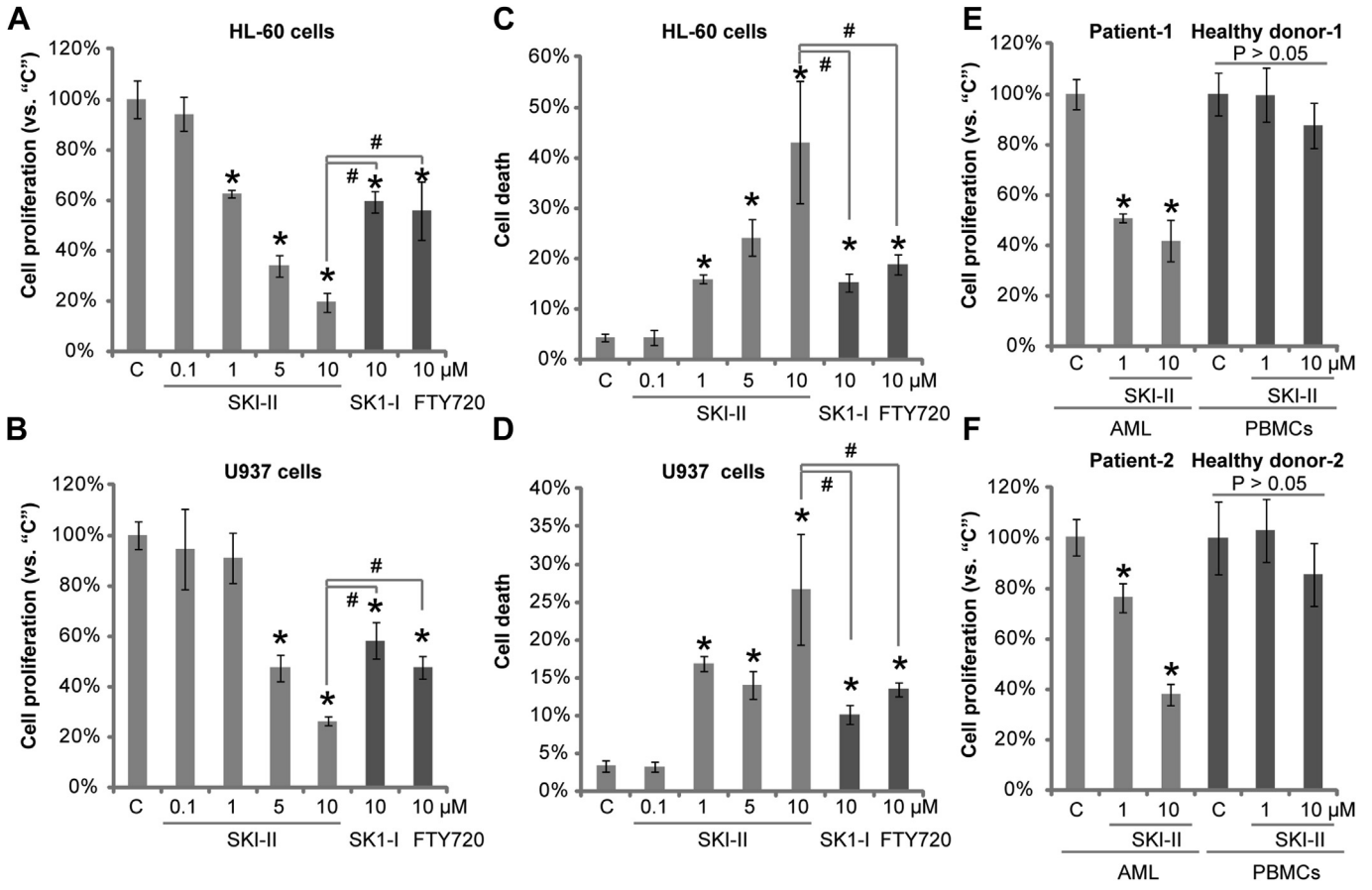

Fig. 1. SKI-II inhibits proliferation and survival of AML cells. The proliferation of HL-60 or U937 AML cells with or without indicated treatment for 72 h was analyzed by the Cell Titer Glo Assay (A–B), cell death was analyzed by the trypan blue staining assay (C and D). Primary human AML cells or the PBMCs from healthy donors were cultured and treated with or without indicated concentration of SKI-II for 72 h, cell proliferation was analyzed (E and F). Experiments in this figure were repeated 5 times, with similar results obtained. For each assay, n = 5. "C" stands for untreated control group (same for all figures). *$p < 0.05$ vs. "C" group. #$p < 0.05$ (A–D).

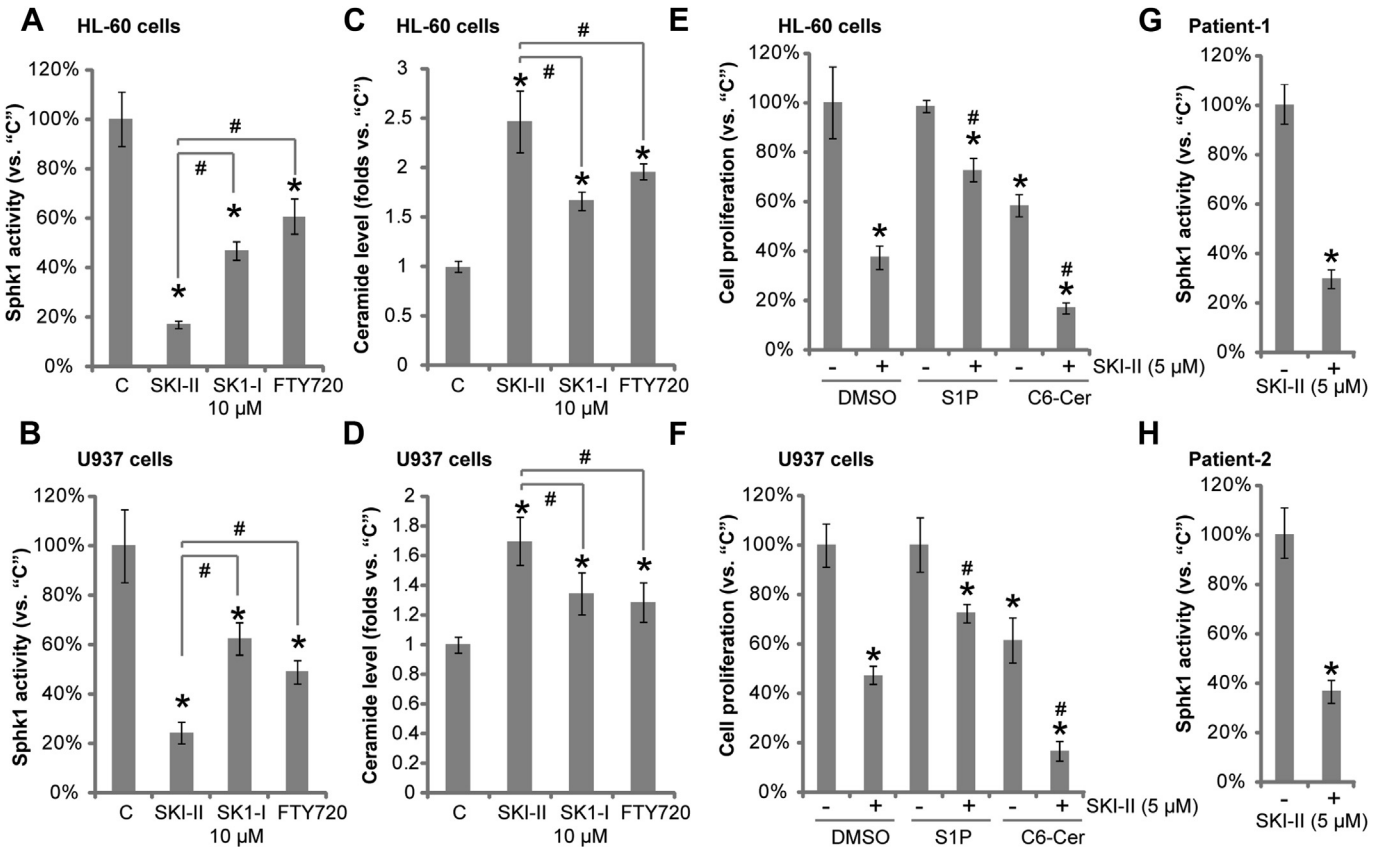

Fig. 2. SKI-II inhibits SphK1 activity, and induces ceramide production in AML cells. The SphK1 activity (A and B) and intracellular ceramide level (C and D) of HL-60 and U937 AML cells with or without indicated treatment for 12 h was shown (A and B). HL-60 and U937 AML cells, pretreated with S1P (5 μM), C6 ceramide (C6-Cer, 5 μM) or DMSO (0.1%) for 1 h, were stimulated with or without SKI-II (5 μM), cell proliferation was analyzed after 72 h (E and F). The SphK1 activity of primary human AML cells with or without SKI-II (5 μM) treatment (12 h) was shown (G and H). Experiments in this figure were repeated 3 times, with similar results obtained. For each assay, n = 5. *p < 0.05 vs. "C" group. #p < 0.05 (A–D).

lethality (Fig. 2E and F). In primary human AML cells, the SphK1 activity was also dramatically inhibited by SKI-II treatment (Fig. 2G and H).

3.3. SKI-II induces apoptotic death of AML cells

The effect of SKI-II on AML cell apoptosis was also tested. Two independent apoptosis assays including the histone-DNA ELISA assay and Annexin V FACS assay were applied. Results from both assays showed that SKI-II at the concentrations of 1 μM and 10 μM induced significant apoptosis in both HL-60 cells (Fig. 3A and B) and U937 cells (Fig. 3C and D). SKI-II at 10 μM was more potent than at 1 μM in inducing AML cell apoptosis (Fig. 3A–D). z-VAD-fmk, a general caspase inhibitor, largely inhibited SKI-II-induced HL-60 and U937 cell death (Fig. 3E and F), indicating that caspase-dependent apoptosis mediates SKI-II-induced cytotoxicity in AML cells. In primary human AML cells, z-VAD-fmk also dramatically reduced cell death caused by SKI-II treatment (Fig. 3G and H). Together, these results indicated that SKI-II induced apoptotic AML cell death.

3.4. SKI-II inhibits U937 xenograft growth in SCID mice

We next evaluated the *in vivo* anti-leukemic activity of SKI-II in SCID mice. U937 cells were subcutaneously injected into the flanks of SCID/beige mice. When U937-xenografted tumors reached a volume around 100 mm^3, mice were injected intraperitoneally (*i.p.*) with saline ("Vehicle") or SKI-II (10–50 mg/kg) daily for a total of 7 days, the SKI-II regimen was based on other studies [16,23]. As demonstrated in Fig. 4A, SKI-II significantly decreased tumor growth, and the *in vivo* anti-leukemic activity of SKI-II was again dose-dependent (Fig. 4A). After 10 days of initial SKI-II administration, the mean volume of the U937 tumors in mice treated with SKI-II (10 mg/kg) was about 50% smaller than the tumors of the vehicle-treated mice, and tumors of 50 mg/kg of SKI-II-treated mice were more than 75% smaller than that of the vehicle-treated mice (Fig. 4A). Tumor weights at autopsy of SKI-II-treated mice were also significantly lower than vehicle group (Fig. 4B). Mice treated with SKI-II did not show signs of wasting, and the body weights were not significantly different from controls (Fig. 4C). Together, these results showed that SKI-II inhibited U937 xenograft growth in mice.

4. Discussion

Numerous studies have reported that perturbations in the S1P/ceramide rheostat are involved in the regulation of apoptosis of neoplastic cells [24–28], including those of hematopoietic origin [29–31]. Further, although a possible anti-cancer activity of SKI-II, alone or in combination with other anti-cancer agents, has been proposed for many other solid tumors [32–34], its therapeutic potential in hematologic malignancies, including AML, and the underlying molecular mechanisms have been only minimally investigated. In the current study, our results showed that SKI-II dramatically inhibited the SphK1 activity in both primary and

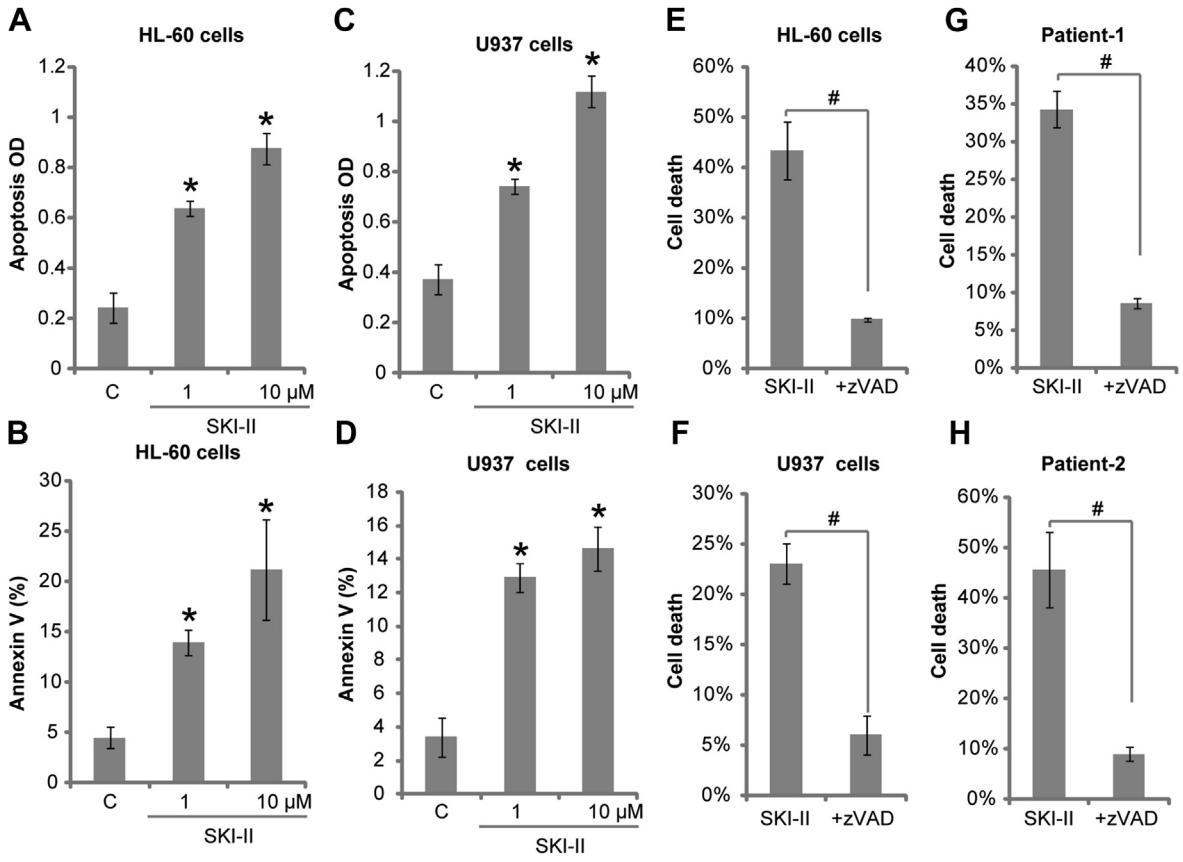

Fig. 3. SKI-II induces AML cell apoptotic cell death. The apoptosis of HL-60 and U937 AML cells with or without indicated treatment for 48 h was analyzed by the histone-DNA apoptosis ELISA assay (A and C) or Annexin V FACS assay (B and D). SKI-II (10 μM, 72 h)-induced death of HL-60 (E), U937 (F) or primary AML (G and H) cells, with or without z-VAD-fmk (zVAD, 50 μM) co-administration, was shown. Experiments in this figure were repeated 5 times, with similar results obtained. For each assay, n = 5. *p < 0.05 vs. "C" group. #p < 0.05 (E–H).

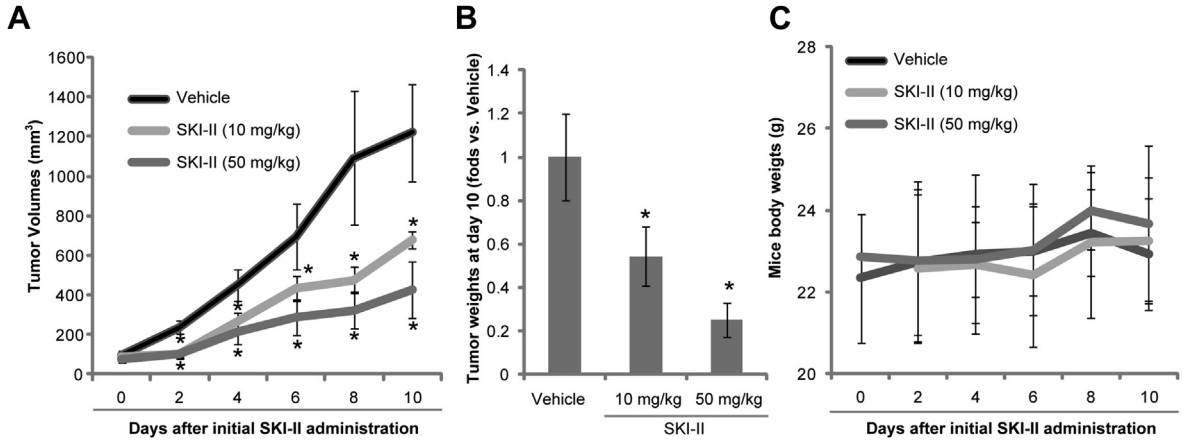

Fig. 4. SKI-II inhibits U937 xenograft growth in SCID mice. SCID/beige mice (10 mice per group) with palpable U937 cell tumors were injected intraperitoneally with saline ("Vehicle") or SKI-II (10 mg/kg or 50 mg/kg) for 7 days. Tumor volumes (A) and mice body weights (C) were recorded every 2 days for a total of 10 days. Animals were killed at day 10, and tumors were excised and weighed (B). Experiments in this figure were repeated twice, with similar results obtained. For each assay, n = 10. *p < 0.05 vs. "Vehicle" group.

transformed human AML cells, resulting in a significant increase of intracellular ceramide level, which might be responsible for AML cell apoptosis. The conclusion was supported by the fact that adding S1P inhibited SKI-II-induced apoptosis and lethality against AML cells, while the short-chain cell permeably C6 ceramide facilitated apoptosis by SKI-II in AML cells.

Indeed, recent studies have evaluated the potential activity of other SphK1 inhibitors in pre-clinical leukemic models. For example, Paolo Neviani et al., demonstrated that the SphK1 inhibitor FTY720 showed promising results against Philadelphia chromosome-positive AML cells, an activity that was associated with phosphotase 2A (PP2A) activation [15]. Interestingly, the

authors showed that PP2A activation by FTY720 was a direct effect, and was somehow independent of stimulation of the ceramide pathway [15]. Paugh et al., showed that SphK1 specific inhibitor SK1-I ((2R,3S,4E)-N-methyl-5-(4′-pentylphenyl)-2-aminopent-4-ene-1,3-diol) dramatically inhibited AML (both primary and transformed) cell proliferation and survival [14]. In this study, we showed that SKI-II was most efficient in inhibiting SphK1 activity and increasing ceramide production in all tested SphK1 inhibitors. As a result, SKI-II was more potent in inducing AML cell growth inhibition and apoptosis than those two tested inhibitors. Importantly, SKI-II induced potent apoptosis and growth inhibition in primary human AML cells, but was generally safe to PBMCs of healthy donors. *In vivo*, SKI-II dramatically inhibited U937 xenograft growth in SCID mice, without inducing apparent toxicities. These observations together warrant the future evaluations of SKI-II as clinical candidates for therapy in AML.

Conflict of interests

The authors declare no conflict of interests.

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