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The blockage of Ras/ERK pathway augments the sensitivity of SphK1 inhibitor SKI II in human hepatoma HepG2 cells

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ABSTRACT

The treatment of hepatocellular carcinoma (HCC) remains a challenge and the future of cancer therapy will incorporate rational combinations directed to molecular targets that cooperate to drive critical pro-survival signaling. Sphingosine kinase 1 (SphK1) has been shown to regulate various processes important for cancer progression. Given the up-regulated expression of SphK1 in response to the silence of N-ras and other interactions between Ras/ERK and SphK1, it was speculated that combined inhibition of Ras/ERK and SphK1 would create enhanced antitumor effects. Experimental results showed that dual blockage of N-ras/ERK and SphK1 resulted in enhanced growth inhibitions in human hepatoma cells. Similarly, MEK1/2 Inhibitor U0126 potentiated SKI II-induced apoptosis in hepatoma HepG2 cells, consistently with the further attenuation of Akt/ERK/NF-κB signaling pathway. It was also shown that the combination of SKI II and U0126 further attenuated the migration of hepatoma HepG2 cells via FAK/MLC-2 signaling pathway. Taken together, the dual inhibition of SphK1 and Ras/ERK pathway resulted in enhanced effects, which might be an effective therapeutic approach for the treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common malignancy worldwide and considered to be the most lethal of cancer systems. The complicated molecular mechanisms of carcinogenesis and the involvement of multiple oncogenes and crucial signaling pathways in HCC reveal the difficulty of pharmacologic treatment. Ras signaling pathway plays a key role in gene transcription, cell growth, survival and angiogenesis and malignant transformation [1]. Mutated Ras is constitutively activated and highly prevalent in many human cancers [2,3]. It has emerged that N-ras, a member of the Ras family, is activated by mutation in HCC with a high frequency of 30%, such as hepatoma HepG2 cells [4].

Sphingolipid metabolites have emerged as important players in a number of fundamental biological processes. Among these metabolites, ceramide and sphingosine mediate cell cycle arrest and induce apoptosis, where as Sphingosine 1-phosphate (S1P) promotes cell growth, proliferation and survival. The balance between ceramide/sphingosine and S1P forms a so-called "sphingolipid rheostat", which decides the cell fate. Sphingosine kinase 1 (SphK1), which converts sphingosine to S1P, is a key controller of the "sphingolipid rheostat". The role of SphK1 in cancer has received considerable attention and SphK1 has been identified as an emerging target in cancer chemotherapy [5,6].

It is well established that SphK1 activity is involved in H-ras-mediated transformation [7] and the mechanism of SphK1 activation involves ERK1/2-mediated phopshorylation of SphK1 on Ser225 [8]. In addition, S1P which is produced by activated SphK1 can be exported from the cell to engage a family of five cell surface S1P receptors (S1PR1-5) in an autocrine and/or paracrine manner to activate classic GPCR signaling pathways, including PI3K/Akt pathway, ERK pathway and Rac pathway, leading to control of cell proliferation, survival, and migration [6]. Furthermore, our previous study revealed that SphK1 was up-regulated after N-ras-siRNA treatment [9]. Given the interactions between SphK1/S1P signaling pathway and Ras/ERK signaling pathway and their overlap and cooperation in proliferation and survival signaling, it was hypothesized that combined inhibition of SphK1 and N-ras/ERK would result in increased killing of cancer cells. Our results revealed that the combination of SKI II, an inhibitor with the highest selectivity towards SphK1 [10], and siN-ras/MEK1/2 inhibitor U0126 led to enhanced inhibition of cancer cells growth and survival through Akt/ERK/NF-κB signaling. In addition, the combination also resulted in an increased inhibitory effect on cell migration.

Abbreviations: HCC, hepatocellular carcinoma; SphK1, sphingosine kinase 1; S1P, sphingosine 1-phosphate; S1PR1-5, S1P receptor type1-5; CI, combination index; Fa, fraction affected; FAK, focal adhesion kinase; MLC-2, myosin light chains-2.

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2. Materials and methods

2.1. Cell culture and reagents

Human hepatoma HepG2 cells were purchased from ATCC (Frederick, MD). Human hepatocellular SMMC-7721 cells were obtained from Cell Bank of Institute for Biological Sciences, China Academy of Sciences (Shanghai, China). All cell lines were routinely cultured in the MEM-EBSS medium (Hyclone, UT) with 10% FBS (Gibco, USA) and 1% penicillin–streptomycin and incubated at 37 °C with 5% CO2. SKI II (Sigma, USA) and U0126 (Cell Signaling Technology, MA, USA) were diluted in DMSO and stored at −20 °C.

2.2. SiRNA and transfection

The siRNA was synthesized by Ribobio (Guangzhou, China) with following sequences: Mock-siRNA: 5′-UUCUCCGAACGUGUCACGUdTdT-3′, N-ras-siRNA: 5′-GUGUGAUUUGCCAACAAGGdTdT-3′. Transfection of siRNA was performed with Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer′s instructions.

2.3. Cell cytocoxicity assay

For cytotoxicity assay, human hepatoma cells were counted and plated at the initial density, followed by the treatment of U0126/siN-ras and/or SKI II for 48 h as indicated. Cell cytotoxicity was determined by SRB assay.

2.4. Wound healing assay

HepG2 cells cultured in 6-well plates with 10% FBS in medium were carefully scratched with a 10 μl sterile pipette tip to draw a linear "wound" in the cell monolayer of each well when cells were grown to a nearly confluent cell monolayers. The monolayers were washed twice with PBS so as to remove debris or the detached cells and then treated with 10 μM U0126 and/or 10 μM SKI II in a medium with 2% FBS for 24 h. Images of wounds were taken at 0 h and 24 h by the Eclipse TE2000-U image analysis system. The area covered by the migrating cells was calculated using Image J 1.45 m. The experiments were performed in triplicate.

2.5. Transwell migration assay

The ability of cell to migrate was measured by the transwell filter (8 μm pores, Millipore). HepG2 cells were plated at 4000 cells per well in serum-free MEM-EBSS with the treatment of 10 μM U0126 and/or 10 μM SKI II in the upper chamber. The bottom chambers were filled with medium containing 20% FBS. Following an incubation period of 24 h at 37 °C, assays were terminated as a result of the removal of the medium from the upper wells and the filters were fixed with methanol. The cells in the upper chamber were wiped off and the cells migrating to the lower side of the upper chamber were stained with hematoxylin. Random fields were scanned (6 fields per filter) under a light microscope (magnification, ×100) for the presence of the cells at the lower membrane side only.

2.6. Western Blot

Whole cell lysates were used for immunoblotting as previously described. Cells were lysed with RIPA lysis buffer supplemented with protease and phosphatase inhibitor after twice washes of cold PBS. The cells were subject to centrifugation at 13,200*g* for 30 min at 4 °C to remove the cell debris. Protein concentrations were measured with the Bradford assay according to the standard protocol. Equal amounts of protein were separated by SDS–PAGE and then blotted onto the PVDF membranes (Millipore Corp., Bedford, MA, USA). The blots were blocked in TBST with 5% bovine serum albumin, followed by incubation with specific antibodies overnight at 4 °C. Then the membranes were blotted with an appropriate horseradish peroxidase-linked secondary antibody after thrice washes of TBST. Electochemiluminescence was performed according to the manufacturer's instructions with ChemiImager 5500 imaging system (Alpha Innotech Co., CA USA). The antibodies against phospho-ERK1/2, ERK1/2, caspase-3, caspase-7, PARP, cleacved PARP, phospho-Akt (Ser473), Akt, phospho-NF-κBp65(Ser536), NF-κBp65, phospho-FAK (Tyr-925) FAK, phospho-MLC-2 (Ser-19), MLC-2, HRP-linked secondary antibodies were obtained from Cell Signaling Technology (MA,USA). The antibody against β-actin was purchased from Sigma–Aldrich (St Louis, MO). The antibody against bcl-2 was from Santa Cruz Biotechnology (CA, USA).

2.7. Apoptosis detection with Annexin-V/propidium iodide (PI) staining

To determine the percentage of apoptotic cells, HepG2 cells were treated with U0126 and/or SKI II for indicated times and evaluated by dual staining with Annexin-V and PI, using the Annexin-V/PI apoptosis detection kit (Beyotime, China), and analyzed by FACSCalibur flow cytometry. After treatment of indicated time, cells were harvested with trypsin and washed twice with PBS. The cells were resuspended in 300 μl cold binding buffer and then stained with 8 μl FITC-labeled Annexin V for 30 min at 4 °C in the dark according to the manufacturer′s instructions. 4 μl PI was added followed by 200 μl cold binding buffer for resuspension. And then the cells were analyzed by a FACSCalibur flow cytometer.

2.8. Statistical analysis

Experiments were repeated at least 3 times in triplicate with consistent results. Data are expressed as the arithmetic mean ± SD. Statistical analysis performed using the *t* test. *P* values less than 0.05 were considered statistically significant. Combination Index (CI) was assessed by CompuSyn Software, where CI < 0.9, CI = 0.9 to 1.1, and CI > 1.1 indicated synergistic, additive, and antagonistic effects, respectively.

3. Results

3.1. U0126. and siN-ras enhanced the cytotoxicity of SKI II in hepatoma cells

To investigate whether the combination of selective MEK1/2 Inhibitor U0126 and SKI II produces enhanced effect, human hepatoma HepG2 and SMMC-7721 cells were exposed to U0126 and/or SKI II for 48 h and then cell survival was determined by SRB assay. As shown in Fig. 1, the combination of U0126 and SKI II led to increased cell cytocoxicity. The cytotoxicity of the combination was compared with that of U0126 or SKI II using the combination index (CI). The CI values of the combination assessed by CompuSyn Software indicate the synergistic, additive, and antagonistic effects, where CI < 0.9, CI = 0.9 to 1.1 and CI > 1.1, respectively [11]. The graphs of CI values versus fraction affected (Fa) revealed that most of combinations with different doses produced synergistic (CI < 0.9) or additive (CI = 0.9–1.1) killing of cancer cells in HepG2 and SMMC-7721 cells (Fig. 1). The combination of 20 μM U0126 and 10 μM SKI II in HepG2 cells and SMMC-7721 cells both exhibited strong synergism (CI < 0.3) in cell cytotoxicity. To further confirm that the blockage of Ras/ERK signaling could potentiate the cytotoxicity of SKI II, N-ras was specifically knocked down by siRNA. As shown in Fig. 1C, the combination of siN-ras and SKI II

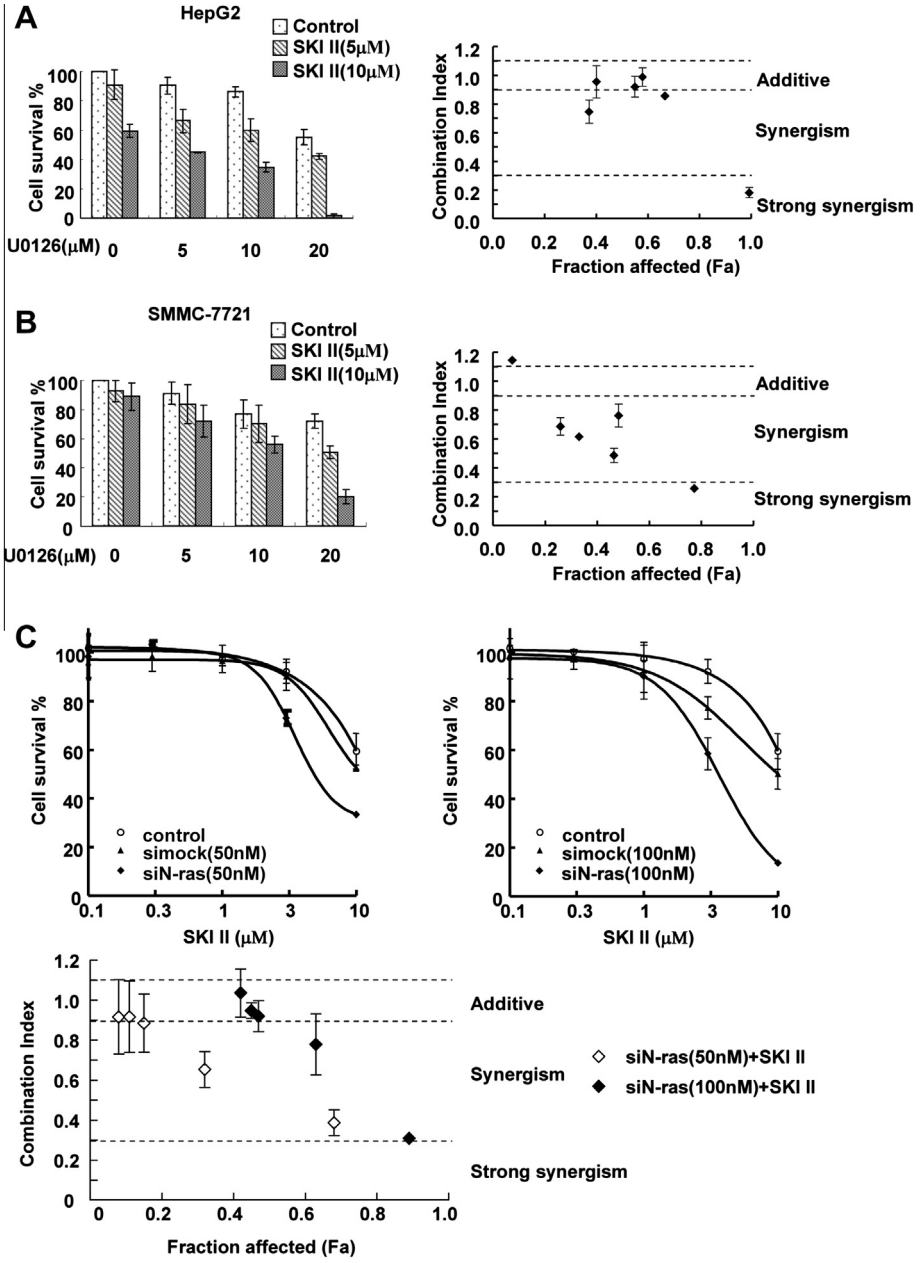

Fig. 1. U0126/siN-ras augments the cytotoxicity of SKI II in human hepatoma cells. Hepatoma HepG2 (A), SMMC-7721 (B) cells were treated with the indicated concentrations of U0126 and/or SKI II for 48 h, and then measured by SRB assay. siN-ras-transfected HepG2 cells were treated with or without 10 μM SKI II for 48 h, and then measured by SRB assay (C) . The CI-Fa plots for the combination indicated synergistic (CI < 0.9), additive (C = 0.9–1.1), and antagonistic (CI > 1.1) effects respectively.

(⩾3 μM) led to synergistic (CI < 0.9) killing of cancer cells as well. In addition, U0126/siN-ras also enhanced the growth inhibition induced by SKI II in colony formation (data not shown). These results confirmed that the blockage of Ras/ERK pathway augmented the sensitivity of human hepatoma HepG2 cells to SKI II.

3.2. Enhancement of SKI II-induced apoptosis by U0126 in HepG2 cells

It is well documented that sphK1 can induce protection against apoptosis. To investigate whether U0126 potentiates the SKI II-induced apoptosis, hepatoma HepG2 cells were exposed to U0126 and/or SKI II for the indicated times. Annexin V-FITC/PI staining revealed that U0126 could augment SKI II-induced apoptosis. Compared with the little apoptosis induced by 10 μM U0126 or 10 μM SKI II alone, the percentage of apoptosis induced by the combination was dramatically increased in a time-dependent manner (Fig. 2A). The percentage of apoptosis of the combination was 7.4% of 48 h treatment, and it was increased to 83.7% of 72 h treatment. When the dose of U0126 was increased to 20 μM (Fig. 2B), or the dose of SKI II was increased to 15 μM (Fig. 2C), the percentage of apoptosis of 48 h treatment induced by combination of U0126 and SKI II was increased to 51.4% and 37.3%, respectively.

3.3. U0126 increased SKI II-mediated inhibition of HepG2 cell migration

It has been reported that SphK1 promotes cancer cell migration and invasion [12,13]. It was interesting to investigate whether SKI II inhibits the migration of HepG2 cells and whether the combination of U0126 and SKI I II leads to further inhibition of migration. Cell migration was measured by wound-healing assay and transwell assay, where cells were exposed to 10 μM U0126 and/or

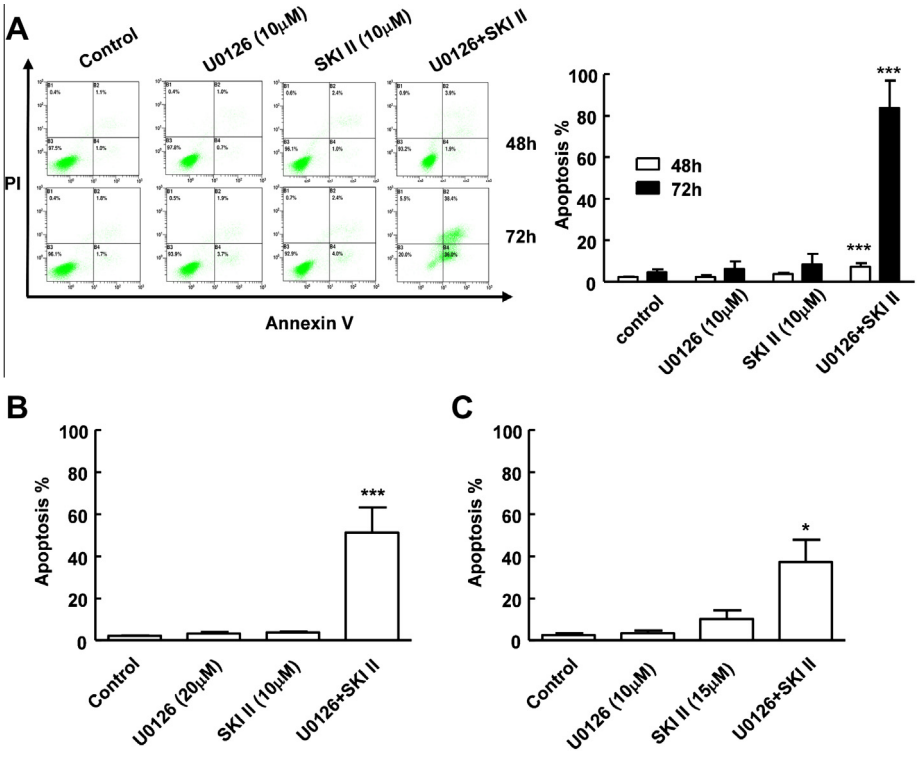

Fig. 2. U0126 enhances the apoptosis induced by SKI II. (A) HepG2 cells were treated with 10 μM U0126 and/or 10 μM SKI II for 48 h and 72 h, and the percentages of apoptosis were detected with Annexin-V/propidium iodide (PI) staining. (B) Cells were treated with 20 μM U0126 and/or 10 μM SKI II for 48 h and then analyzed by flow cytometry. (C) Cells were treated with 10 μM U0126 and/or 15 μM SKI II for 48 h and then analyzed by flow cytometry.

10 μM SKI II for 24 h. In wound-healing assay, the covered area of the wound by the migrating cells was measured using Image J and then normalized to that of control cells. The results showed that SKI II led to a decrease of 30% of the cell migration, which was augmented to 80% by combining with U0126 (Fig. 3A). In transwell assay, SKI II consistently resulted in a decrease of migrated cells to 53.3% compared with control and the combination of SKI II and U0126 further decreased the number of migrating cells to 31.7% (Fig. 3B). Taken together, U0126 could augment SKI II-mediated inhibition of HepG2 cell migration.

3.4. U0126 potentiated SKI II-induced apoptosis through Akt/NF-κB signaling pathway

In agreement with the enhanced apoptosis by the combination of U0126 and SKI II, Western Blot results confirmed that the combination significantly induced the activation of caspase-3 and -7, enhanced the cleavage of PARP, and increased the down-regulation of Bcl-2 protein (Fig 4A).

Previous studies have shown that inhibition of SphK1 contributes to apoptosis, which correlates with the Akt/NF-κB signaling [14–16]. It was of interest to investigate the effects of the combination of U0126 and SKI II on these signaling pathways. As shown in Fig. 4B, SKI II markedly suppressed the phosphorylations of Akt and NF-κB, while there was no significant reduction in the levels of Akt and NF-κB proteins. And SKI II-induced dephosphorylations of Akt and NF-κB were augmented by U0126. Therefore, U0126 augmented SKI II-induced apoptosis by further attenuating Akt/NF-κB signaling pathway. Meanwhile, SKI II enhanced U0126-induced inactivation of ERK, which contributed to the enhancement of growth inhibition. Taken together, the combination of U0126 and SKI II further inhibited cell growth and induced apoptosis via the Akt/ERK/NF-κB signaling pathway in hepatoma HepG2 cells.

3.5. U0126 enhanced SKI II-mediated the dephosphorylations of FAK and MLC-2

The effects of SphK/S1P on cancer cell motility and migration are perhaps the most complicated, because it's related to the S1P receptors and crosstalks with other signaling pathways [17]. It has been known that Focal Adhesion Kinase (FAK) [18] and Myosin Light Chains-2 (MLC-2) [19] are crucial regulators of cell motility. In this study, the FAK and MCL-2 proteins were focused on to explore the molecular mechanism of the enhanced inhibitory migration induced by SKI II and U0126.

As illustrated in Fig.4C, the phosphorylated activation of FAK at Tyr-925 was mildly inhibited by U0126 or SKI II, but it was significantly repressed by the combination of SKI II and U0126. In addition, the combination of U0126 and SKI II dramatically enhanced the dephosphorylations of MLC-2. These results indicated that the combination of U0126 and SKI II repressed cell migration through FAK/MLC-2 pathway.

4. Discussion

Hepatocellular carcinoma is the third most frequent cause of cancer-related death worldwide; and its incidence rate is increasing. Hepatocarcinogenesis is a stepwise process during which multiple genes are altered [2,3]. The existence of multiple oncogenes and redundant signaling pathways in cancer reveal the difficulty of pharmacologic treatment [20]. Accumulated understanding of the molecular mechanisms regulating cancer progression has led to novel development of molecularly targeted therapies [21]. The future of cancer therapy will incorporate rational rather than empirical drug combinations directed to molecular targets that cooperate to drive critical pro-survival signaling, and the appropriate drug combination will create a pharmacologic synthetic lethal

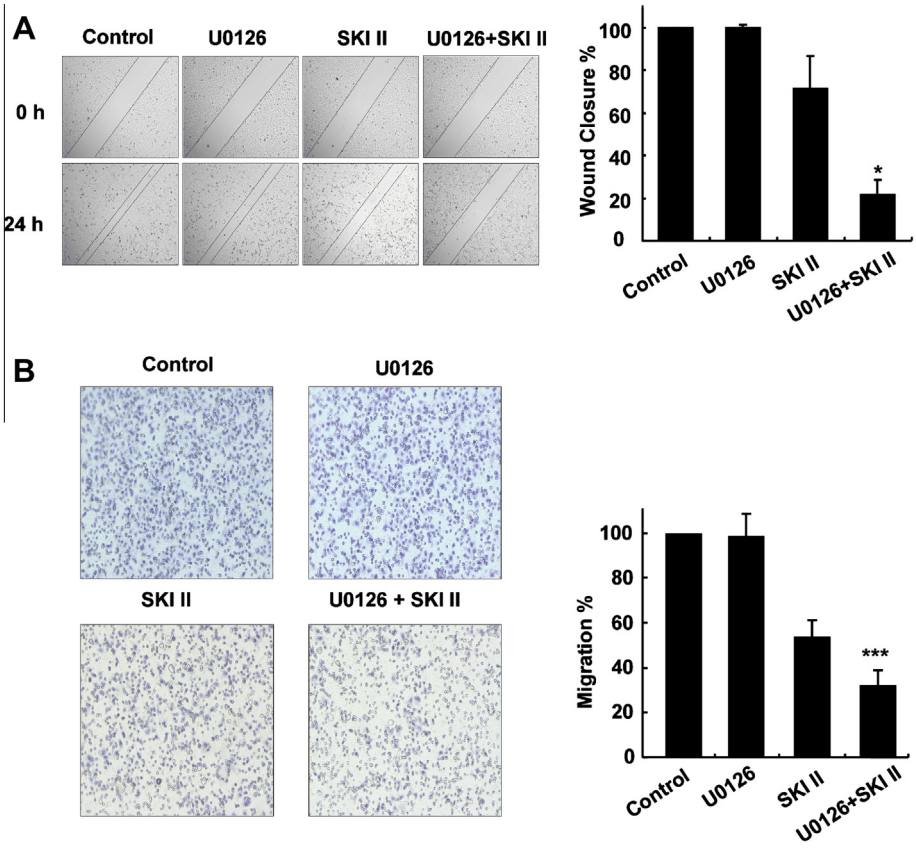

Fig. 3. Combination of U0126 and SKI II augments the inhibitory migration of HepG2 cells. (A) Cells subjected to wound healing assay were treated with 10 μM U0126 and/or 10 μM SKI II for 24 h and the covered area of the wound by the cells after incubation for 24 h was measured using Image J and normalized to control cells. (B) Transwell migration assays were performed on HepG2 cells treated with 10 μM U0126 and/or 10 μM SKI II for 24 h.

event to selectively kill the cancer cells with the sensitive context of vulnerability [22,23].

Ras/MEK/ERK signaling is involved in the pathogenesis of HCC [1,2]. SphK1 has been shown to regulate various processes important for cancer progression. As we mentioned above, SphK1 involves in H-ras mediated transformation and ERK/12 mediates the phosphorylation of SphK1 on Ser225. Given the interactions between Ras/ERK and SphK1 and their overlap and cooperation in proliferation and survival signaling, we evaluated the cytotoxicity of combination of siN-ras/U0126 and SKI II. The experimental results confirmed that combination of U0126 and SKI II produced enhanced cytotoxicity in human hepatoma HepG2 and SMMC-7721 cells. And the cytotoxicity of SKI II on human hepatoma HepG2 cells can be potentiated by siN-ras as well. Consistently, the combination of SKI II and U0126/siN-ras markedly suppressed long-term survival of HepG2 cells in colony formation assays (data not shown). The enhanced attenuation of cell survival could be attributed to several mechanisms. First, dual blockage of SphK1 and N-ras/ERK resulted in a more effective inhibition of ERK1/2 signaling pathway but without affecting p38 mitogen-activated protein kinase activation (data not shown), which led to enhanced suppress of the cell growth. Interestingly, SKI II had little effect on ERK activity, but it could augment the inhibition of ERK activity induced by U0126, which was different from another study reported that inhibition of SphK1 suppressed proliferation of glioma cells under hypoxia by attenuating activity of the ERK1/2 [24]. Second, U0126 enhanced SKI II-induced apoptosis. Abrogation of pro-apoptotic pathways has been demonstrated to be one of the key events to tumor development and progression. As we know, sphingosine induces apoptosis, whereas S1P promotes cell survival. SphK1 is a key controller of the "sphingolipid rheostat". SKI II as the SphK1 inhibitor,not only inhibits S1P production, but also increases sphingosine, pushing more toward cell death. Previous reports have demonstrated that SphK1 contributes to the apoptosis resistance via regulating the Akt [15,25] /NF-κB signaling [14]. Consistently, we demonstrated that U0126 enhanced SKI II-induced dephosphorylations of Akt/NF-κB, leading to the increased apoptosis.

Cell migration, as a critical step in tumor metastasis, is a major hallmark of an aggressive solitary HCC. Understanding the mechanisms underlying this process would allow for the development of effective approaches to reduce HCC mortality. It was demonstrated that SKI II could significantly inhibit cell migration. And U0126, with little effect on cell migration, could potentiate the SKI II-induced inhibition of migration significantly. FAK is a crucial signaling component that is activated by numerous stimuli and functions as a biosensor or integrator to control cell motility [18,26]. Through multifaceted and diverse molecular connections, FAK can influence the cytoskeleton, structures of cell adhesion sites and membrane protrusions to regulate cell movement [26]. At present, there is little literature about the effect of FAK in SphK1-mediated cell migration. In our study, it was demonstrated that phosphorylations of FAK at Tyr-925 was reduced by SKI II. It has been known that phosphorylation of MLC-2 at serine-19 controls the activity of myosin II [27], which has multiple functions in cells, including stimulation of cell motility as a key component of focal adhesion formation and stress fiber formation [28,29]. It has been reported that SphK1/S1P induces MLCK-mediated MLC-2 phosphorylation [30]. In our study, the phosphorylations of FAK at Tyr-925 and MLC-2 at serine-19 were significantly decreased with the combination of

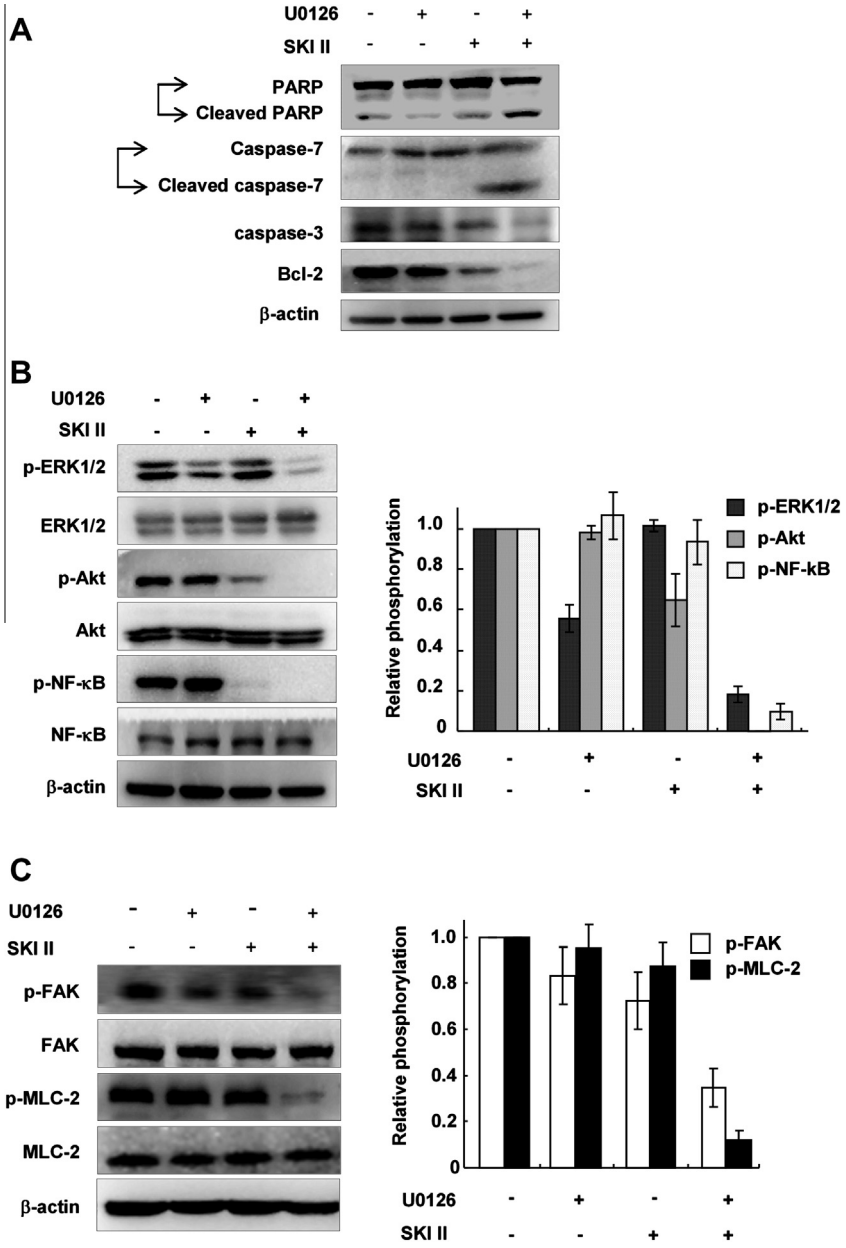

Fig. 4. The changes of signaling molecules after treatment of U0126 and/or SKI II. HepG2 cells were treated with 10 μM U0126 and/or 10 μM SKI II for 72 h (A), 48 h (B) and 24 h (C), respectively to detect the apoptosis-related, proliferation and survival-related and migration-related proteins by Western Blot.

U0126 and SKI II, compared with the barely decreases with the treatment of U0126 or SKI II alone.

The mechanisms of the enhancement induced by inhibition of Ras/ERK in SKI II-induced cell growth inhibition, apoptosis, and inhibition of migration can be speculated as follows. On the one hand, there was overlap and cooperation in proliferation and survival signaling between Ras/ERK signaling and SphK1/S1P signaling. One the other hand, SphK1 activation involves ERK1/2-mediated phosphorylation of SphK1 on Ser225. U0126, as MEK1/2 inhibitor, further inhibited the SphK1 activation indirectly via inhibition of ERK1/2 activation. However, more researches are needed to prove the speculation.

In summary, the results confirmed that combined blockage of Ras/ERK and SphK1 resulted in augmented effects, including enhanced growth inhibitions, increased apoptosis and attenuated migration of hepatoma HepG2 cells via enhanced inhibitions of Akt /ERK/NF-κB signaling pathway and FAK/MLC-2 signaling pathway. These data not only position SphK1 as a valid pharmacologic target for HCC, but also support further evaluation of combined inhibition of SphK1 signaling pathway and Ras/ERK signaling pathway.

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