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SKLB70326, a novel small-molecule inhibitor of cell-cycle progression, induces G_0/G_1 phase arrest and apoptosis in human hepatic carcinoma cells

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ABSTRACT

We previously reported the potential of a novel small molecule 3-amino-6-(3-methoxyphenyl)thieno[2.3-b]pyridine-2-carboxamide (SKLB70326) as an anticancer agent. In the present study, we investigated the anticancer effects and possible mechanisms of SKLB70326 *in vitro*. We found that SKLB70326 treatment significantly inhibited human hepatic carcinoma cell proliferation *in vitro*, and the HepG2 cell line was the most sensitive to its treatment. The inhibition of cell proliferation correlated with G_0/G_1 phase arrest, which was followed by apoptotic cell death. The SKLB70326-mediated cell-cycle arrest was associated with the downregulation of cyclin-dependent kinase (CDK) 2, CDK4 and CDK6 but not cyclin D1 or cyclin E. The phosphorylation of the retinoblastoma protein (Rb) was also observed. SKLB70326 treatment induced apoptotic cell death *via* the activation of PARP, caspase-3, caspase-9 and Bax as well as the downregulation of Bcl-2. The expression levels of p53 and p21 were also induced by SKLB70326 treatment. Moreover, SKLB70326 treatment was well tolerated. In conclusion, SKLB70326, a novel cell-cycle inhibitor, notably inhibits HepG2 cell proliferation through the induction of G_0/G_1 phase arrest and subsequent apoptosis. Its potential as a candidate anticancer agent warrants further investigation.

1. Introduction

Hepatocellular carcinoma (HCC) ranks as the third-leading cause of neoplasm-related death in the world [1,2]. Chemotherapy is a common therapeutic method for the treatment of unresectable HCC; however, it has a poor efficiency and conventional cytotoxicity [3]. Currently, targeted small-molecule anti-tumor drugs is a promising strategy for a more successful treatment of HCC because of its more effective and less toxic due to their high selectivity.

The majority of human cancers, including HCC, have abnormalities in one or more components associated with cell-cycle regulation [4]. Under normal conditions, the cell cycle functions as a carefully regulated, predictable process and G_1/S checkpoint is a central regulatory step in cell-cycle progression. The cyclin-dependent kinases (CDKs), including CDKs 4/6 and CDK 2, play important roles in the G_0–G_1 and G_1–S phase transitions by associating with the cyclins [5]. In most cases of HCC (>90%), alterations in the proteins that regulate the cell cycle have been observed and associated with the development and recurrence of HCC as well as a poor patient prognosis [6,7].

Based on these observations, the modulation of CDKs and/or cyclins proteins could serve as a novel approach for the treatment of HCC. The most appropriate approaches to inhibit cancer progression would target the key CDKs, CDK2, CDK4 and/or CDK6 [8,9]. For example, BAY1000394 and PD0332991, inhibitor of CDK2, CDK4 or CDK6, which is currently in clinical trial [10].

Our research group tried to develop a small-molecule compound that potently and selectively inhibited cell-cycle progression and was suitable for HCC therapy. In a previous study that utilized computer-aided drug design that focused on the cell cycle, we discovered that 3-amino-thieno[2.3-b]pyridine derivatives could efficiently inhibit the growth of hepatocellular carcinoma cell lines [11,12]. We then optimized the thienopyridine derivatives to obtain more efficient anticancer candidates, synthesized a novel series of thienopyridine derivatives and performed a structure–activity relationship (SAR) on the compounds. Among the tested compounds, 3-amino-6-(3-methoxyphenyl)thieno[2.3-b] pyridine-2-carboxamide (SKLB70326) demonstrated a strong anti-HCC activity. However, the mechanism behind the efficacy of SKLB70326 treatment remained unclear. Therefore, investigating the molecular

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mechanisms of SKLB70326 is necessary for the development of SKLB70326 as an anticancer treatment and the further optimization of the thienopyridine derivatives.

In the present study, we demonstrate that SKLB70326 treatment inhibits the proliferation of HCC cells by inducing G_0/G_1 cell-cycle arrest, which is due to the downregulation of CDK2, CDK4, CDK6 and p-Rb as well as the upregulation of p21 and p53. Moreover, the SKLB70326-induced G_0/G_1 arrest led to apoptotic cell death *via* the intrinsic apoptotic pathway in HepG2 cells.

2. Materials and methods

2.1. Compounds and reagents

3-Amino-6-(3-methoxyphenyl)thieno[2.3-b] pyridine-2-carboxamide (SKLB70326) was synthesized in State Key Laboratory of Biotherapy, Sichuan University (Sichuan, China), and its structural formula is shown in Fig. 1A. SKLB70326 was dissolved in dimethyl sulfoxide (DMSO) and diluted with the relevant medium for the *in vitro* experiments. The final concentration of DMSO was 0.1%.

DMSO, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), Triton X-100, rhodamine-123 (Rh-123) and deoxyribonucleotide thymine (TdR) were purchased from Sigma Chemical (St. Louis, MO, USA). The Annexin V-FITC apoptosis detection kit was purchased from Keygen (Nanjing, China). All of the primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The EdU-Apollo®567 DNA Proliferation Detection kit was purchased from RiboBio (Guangzhou, China). All of the chemicals employed in this study were culture grade and of analytic purity.

2.2. Cell lines

The human HCC SMMC-7721 and Bel-7404 cell lines were obtained from the China Center for Type Culture Collection (CTCCC, Wuhan, China). All other human cancer cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM or RPMI 1640 media containing 10% FBS and 1% antibiotics (penicillin and streptomycin) under humidified conditions with 5% CO_2 at 37 °C.

2.3. The cell viability assay

The cells (3×10^3 cells/well) were seeded in a 96-well plate. After 24-h incubation, the cells were treated with SKLB70326. Then, 20 μl of a 5 mg/ml MTT solution was added, and the plates were incubated for an additional 2 h at 37 °C. The medium was subsequently removed, and DMSO was added. The OD_{570} was measured using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, USA), and the IC_{50} values were calculated.

2.4. The colony formation assay

The cells (800 cells/well) were seeded in a 6-well plate. After 24-h incubation, the cells were treated with various concentrations of SKLB70326 and then cultured for an additional 10 days. Finally, the cells were stained with a 0.5% crystal violet solution, and the colonies (>50 cells) were counted under an inverted microscope.

2.5. The EdU incorporation assay

EdU, a thymidine analog used for the labeling of proliferating cells, can incorporate into replicating DNA during S phase [13]. HepG2 cells growing in a 96-well plate (5×10^3 cells/well) were

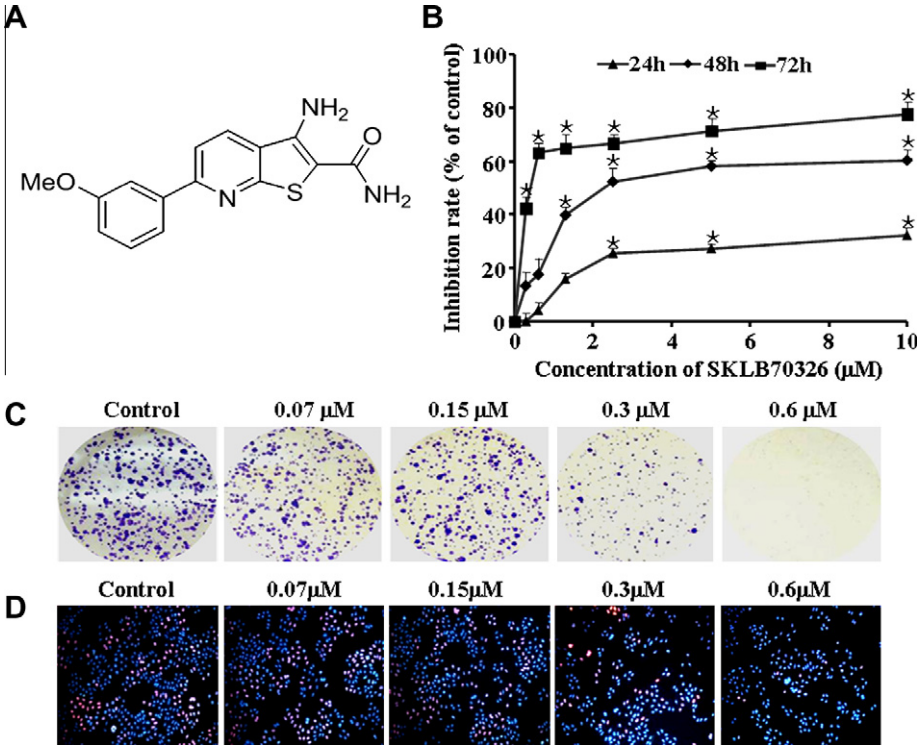

Fig. 1. The proliferation inhibition caused by SKLB70326 treatment in HepG2 cells. (A) The structural formula for SKLB70326. (B) The cells were treated with SKLB70326 for 24, 48 or 72 h. The cell viability was determined by the MTT assay (*$P < 0.05$ vs. the control group). (C) The colony clusters were detected after a 10-day SKLB70326 treatment. (D) The proliferating cells were evaluated using an EdU kit after a 24-h SKLB70326 treatment. The red nuclei represent the cells in S phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treated with SKLB70326 for 24 h. Then, the proliferation cells were assayed with an EdU-Apollo®567 DNA Proliferation Detection kit according to the manufacturer's instructions.

2.6. Cell cycle analysis

The cells were seeded in 60 mm culture dishes. After growing for 24 h, the cells were treated with SKLB70326 for 48 h. Then, the cells were incubated with 1 ml of a solution containing 50 μg/ml propidium iodide (PI) and 0.1% Triton X-100 for 30 min in the dark. The cells were then analyzed by flow cytometry (FCM) using an ESP Elite cell sorter (Beckman-Coulter, Miami, FL, USA). To further investigate the effects of SKLB70326 treatment on the cell cycle, HepG2 cells were treated with 2.5 mM deoxyribonucleotide thymine (TdR) for 24 h, then cultured with 1.25 μM SKLB70326 for various time intervals. The cells were stained with PI and subjected to FCM.

2.7. Apoptosis analysis by FCM

After a 24- or 48-h SKLB70326 treatment, the cells were harvested and washed twice with ice-cold PBS. The level of apoptosis was determined using an Annexin V-FITC apoptosis detection kit according to manufacturer's instructions.

2.8. Western blotting analysis

The cells were treated with SKLB70326 for various times and concentrations and lysed in RIPA buffer. Western blotting was performed using primary antibodies against CDK2, CDK4, CDK6, p-Rb, p53, p21, cyclin D1, cyclin E, PARP, caspase-3, caspase-9, Bcl-2 and Bax. β-Actin was used as an internal control. After incubation with the relevant secondary antibodies, the bands were visualized using the enhanced chemiluminescence method (Amersham Biosciences Corp., Piscataway, NJ).

2.9. The analysis of the mitochondrial membrane potential

The changes of mitochondrial membrane potential ($\Delta\Psi_m$) after SKLB70326 treatment were analyzed by FCM using rhodamine (Rh)-123 staining [14]. The cells that treated with SKLB70326 were stained in PBS containing 3 μg/ml Rh-123 at 37 °C in the dark for 30 min. The stained cells were then washed with ice-cold PBS, and the Rh-123 fluorescence was detected by FCM.

2.10. Acute toxicity study in rats

Seven-week-old female and male SD rats (180–200 g) (n = 10, respectively) were orally administrated 2 g/kg SKLB70326 within 24 h. We observed the rats' clinical symptoms including mortality, clinical signs, and gross findings once a day. After 14 days, the rats were sacrificed, and their major organs got histological examinations after dissection.

2.11. Statistical analysis

The data were expressed as the mean ± SD. The statistical comparisons were made by an ANOVA test. All of the data were analyzed using SPSS 13.0 software (Chicago, IL, USA). A P value (P) less than 0.05 was considered to be statistically significant.

3. Results

3.1. The anti-proliferation effects of SKLB70326 against cancer cells

We investigated the proliferation inhibition caused by SKLB70326 treatment on a panel of human cancer cells.

Table 1
The proliferation inhibition caused by SKLB70326 treatment in human tumor cell lines.

Cell line	Cell type	IC_{50} (μM)
HepG2	Hepatocellular carcinoma	2.5 ± 0.2
SMMC-7721	Hepatocellular carcinoma	3.7 ± 0.3
Bel-7404	Hepatocellular carcinoma	7.4 ± 0.6
H460	Lung carcinoma	>40
A549	Lung carcinoma	>40
HCT116	Colon adenocarcinoma	>40
SW480	Colon adenocarcinoma	>40
DU145	Prostate carcinoma	>40
PC-3	Prostate carcinoma	>40
MCF-7	Breast carcinoma	>40
Bcap-37	Breast carcinoma	>40
HeLa	Cervical carcinoma	>40
SKOV3	Ovarian carcinoma	>40
7860	Renal carcinoma	>40
PANC-1	Pancreatic carcinoma	>40
A375	Melanoma	>40
A431	Epithelial carcinoma	>40
OSRC	Osteogenic sarcoma	>40
U251	Glioblastoma	>40

Each cell line was treated with varied concentrations of SKLB70326 (0–40 μM) for 48 h. The cell viability was measured with the MTT assay. The data are expressed as the mean ± SD from three experiments.

Interestingly, we found that SKLB70326 treatment greatly inhibited the proliferation of human HCC cell lines (Table 1). The IC_{50} values for SKLB70326 in HCC cell lines were much lower than the IC_{50} values for SKLB70326 in normal cell lines (Table 2). Furthermore, when HepG2 cells were treated with SKLB70326 for 24 h, the IC_{50} value was greater than 10.0 μM, whereas the IC_{50} decreased to 2.5 or 0.4 μM after SKLB70326 treatment for 48 or 72 h, respectively (Fig. 1B). Therefore, these results demonstrated that SKLB70326 treatment inhibited the proliferation of HepG2 cells in a time- and concentration-dependent manner but not the proliferation of normal cells.

Moreover, SKLB70326 treatment decreased the colony size and number of colonies significantly compared to the control (Fig. 1C). The results from the EdU incorporation assay also demonstrated that the number of proliferating cells (red nuclei) significantly decreased with increasing concentrations of SKLB70326 (Fig. 1D).

3.2. SKLB70326 treatment induced G_0/G_1 phase arrest and apoptosis in HepG2 cells

Following 48-h SKLB70326 treatment, the percentage of cells in G_0/G_1 phase increased from 35.5% in the untreated cells to 49.7%, 63.1%, 68.2% or 80.0% in the cells treated with increasing concentrations of SKLB70326 (Fig. 2A). The number of cells in S and G_2/M phase correspondingly decreased. Moreover, we found that the cell-cycle distribution of the SKLB70326-treated groups was similar to the control group for less than 12 h. However, after 18 h of SKLB70326 treatment, the percentage of cells in G_0/G_1 phase increased to 73.8%, which was significantly higher than the control cells (28.5%) (Fig. 2B). These results indicate that SKLB70326

Table 2
The proliferation inhibition caused by SKLB70326 treatment in normal human cell lines.

Cell line	Cell type	IC_{50} (μM)
HEK293	Embryonic kidney	>40
HBE	Bronchial epithelial	>40
HK-2	Proximal tubule epithelial	>40

Each cell line was treated with varied concentrations of SKLB70326 (0–40 μM) for 48 h. The cell viability was measured with the MTT assay.

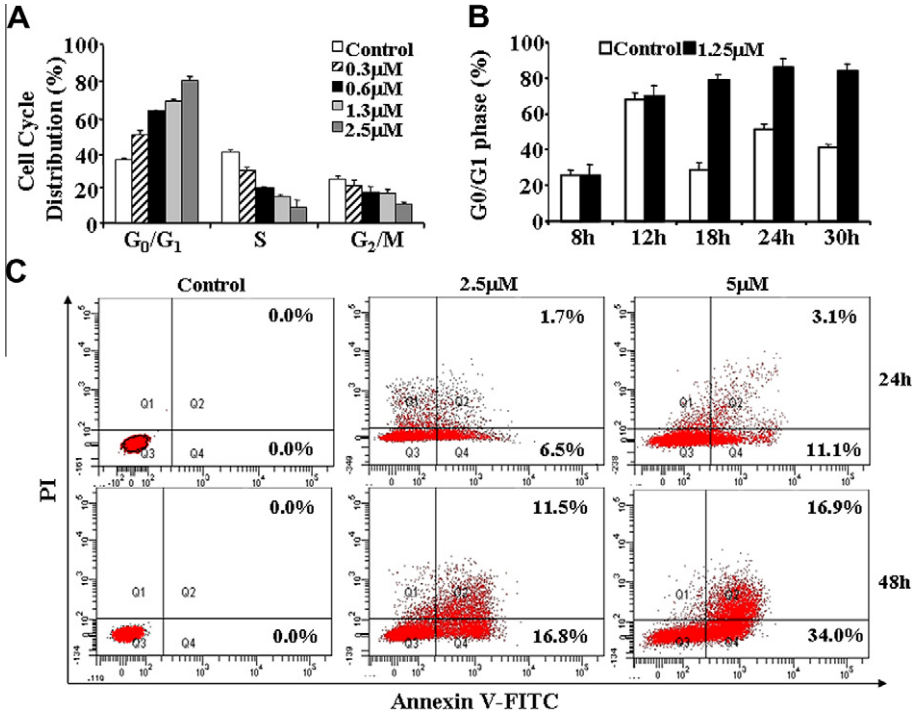

Fig. 2. SKLB70326 treatment induced a G_0/G_1 phase arrest and apoptosis in HepG2 cells. HepG2 cells were treated with (A) various concentrations of SKLB70326 for 48 h or (B) 0 or 1.25 μM SKLB70326 for different times after synchronization at G_1/S phase with TdR. The cell cycle distribution was measured by FCM. The percentages of the cell-cycle stages were presented. (C) HepG2 cells were treated with SKLB70326 and the level of apoptosis was assessed using the AnnexinV-FITC/PI dual-labeling technique. The early apoptotic cells were counted from the lower right quadrant (Q_4), and the late apoptotic cells were counted from the upper right quadrant (Q_2).

treatment induces a G_0/G_1 cell-cycle arrest in a concentration- and time-dependent manner.

Next, we explored whether SKLB70326 treatment induced apoptosis in HepG2 cells. We found that after treatment with 2.5 μM or 5 μM SKLB70326, the percentages of early apoptotic cells (in Q_4) were 6.5% or 11.1%, respectively, for 24-h SKLB70326 treatment and 16.8% or 34.0%, respectively, for 48-h SKLB70326 treatment. No cells undergoing apoptosis were detected in the control group (Fig. 2C). Therefore, these data suggest that SKLB70326 inhibits the proliferation of HepG2 cells by inducing apoptosis in a concentration- and time-dependent manner.

3.3. SKLB70326 treatment regulated the expression of proteins associated with G_0/G_1 phase arrest

Because SKLB70326 treatment induced a significant G_0/G_1 cell-cycle arrest in HepG2 cells, we further investigated the activities of CDK2, CDK4 and CDK6 after SKLB70326 treatment. We found that SKLB70326 treatment decreased the levels of CDK2, CDK4 and CDK6 in a time- and concentration-dependent manner (Fig. 3A and B).

As shown in Fig. 3C and D, SKLB70326 treatment also inhibited the phosphorylation of Rb but caused no obvious effects on the levels of cyclin D1 or cyclin E. Furthermore, SKLB70326 treatment induced the expression of p21 and upregulated the level of p53.

3.4. SKLB70326 treatment induced apoptosis via the intrinsic pathway

To further confirm the induction of apoptosis with SKLB70326 treatment, we analyzed the levels of PARP, caspase-3 and caspase-9. We found that SKLB70326 treatment induced increase in cleavage of PARP (89 kDa), caspase-3 and caspase-9 (Fig. 4A). Moreover, SKLB70326 treatment led to a decreased level of Bcl-2 and an increased level of Bax in HepG2 cells (Fig. 4B).

In $\Delta\Psi_m$ assay, we found that 1.25 ~ 5 μM SKLB70326 treatment led to a loss of the $\Delta\Psi_m$, with a 17.6% ~ 47.6% compared to a $\Delta\Psi_m$ loss of only 5.98% in the control group (Fig. 4C). Moreover, a time-dependent decrease in the $\Delta\Psi_m$, from 9.4% to 29.7%, was observed after treatment with 2.5 μM SKLB70326 (Fig. 4D).

3.5. SKLB70326 was well tolerated in acute toxicity evaluation

During a 14-day treatment period, SKLB70326 was well tolerated, no adverse clinical effects were observed and the no observed adverse effect level (NOAEL) value for SKLB70326 treatment was greater than 2 g/kg.

4. Discussion

SKLB70326 was obtained using a computer-aided drug design that targeted the cell cycle and the structural modification of the 3-amino-thieno[2.3-b] pyridine derivative. SKLB70326 has a novel chemical structure that is different from the cell-cycle inhibitors currently in clinical research. To our knowledge, this study is the first to demonstrate that the 3-amino-thieno[2.3-b] pyridine derivatives can significantly inhibit the proliferation of human hepatic carcinoma cells and induce a G_0/G_1 cell-cycle arrest *via* the down-regulation of the CDKs.

In G_0/G_1 phase progression, cyclin D1 binds to CDK4/CDK6, which results in the formation of the cyclin E/CDK2 complex, eventually driving the cell from G_1 to S phase [15]. We found that SKLB70326 treatment significantly decreased the expression of CDK2, CDK4 and CDK6 but not cyclin D1 or cyclin E. These results are consistent with our initial hypothesis that SKLB70326 inhibits the CDKs but not the cyclins. Moreover, this character of SKLB70326 is similar to most cell-cycle inhibitors that target the CDK-cyclin complexes by regulating the CDKs rather than the

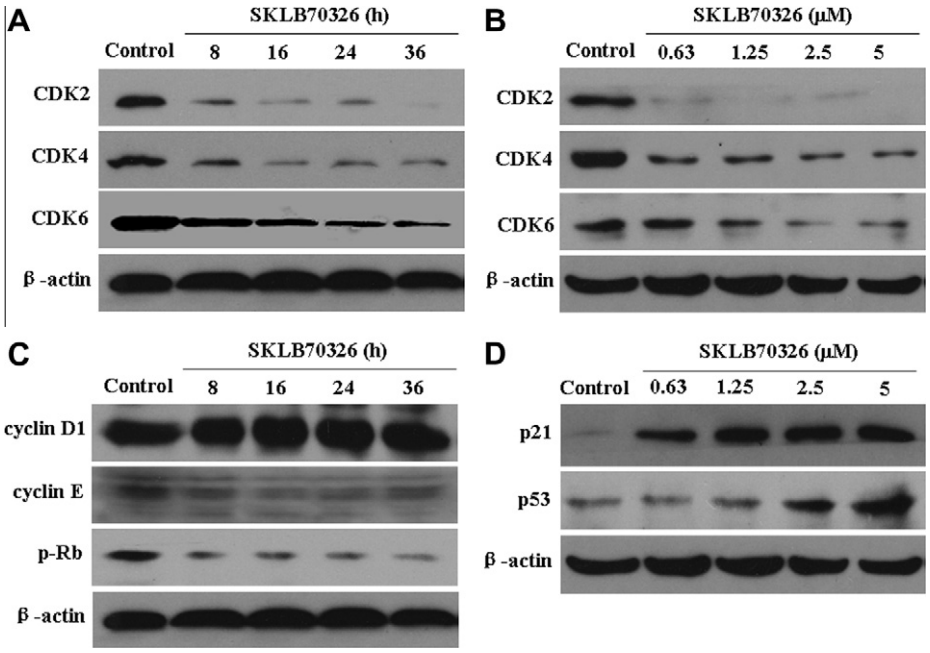

Fig. 3. SKLB70326 treatment modified the expression of proteins associated with G_0/G_1 phase. HepG2 cells were treated with (A) 2.5 μM SKLB70326 for various times or (B) different concentrations of SKLB70326 for 48 h. The levels of CDK2, CDK4 and CDK6 were detected by Western blotting. (C) HepG2 cells were treated with 2.5 μM SKLB70326 for various times, and the expression levels of cyclin D1, cyclin E and p-Rb were analyzed. (D) The expression levels of p53 and p21 were analyzed in cells after treatment with various concentrations of SKLB70326 for 48 h.

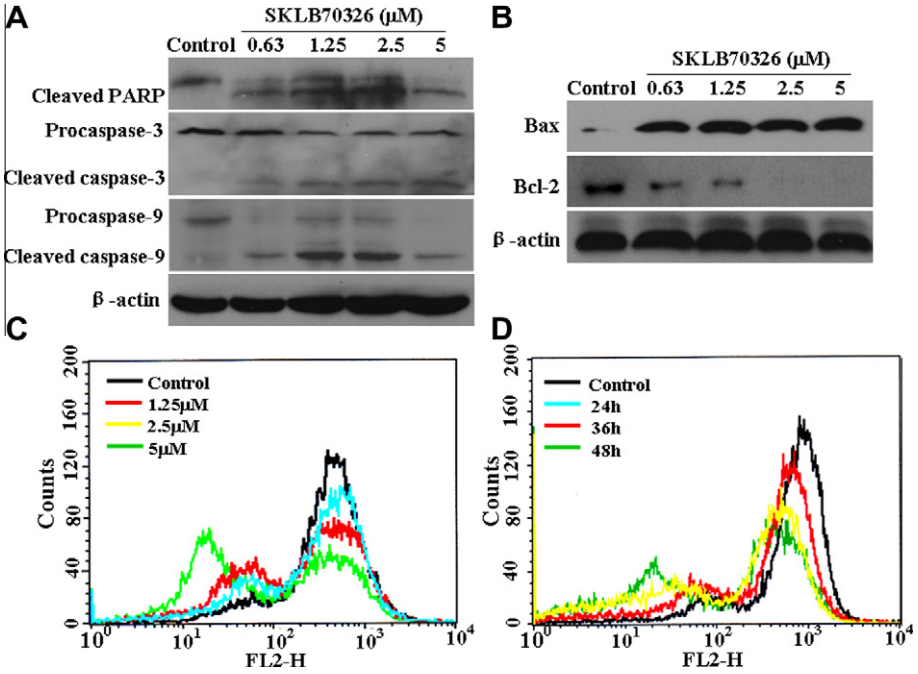

Fig. 4. SKLB70326 treatment induced apoptosis in HepG2 cells *via* the intrinsic pathway. (A) The levels of PARP, caspase-3 and caspase-9 were measured in HepG2 cells; (B) the expression of Bax and Bcl-2 were determined. After an incubation with (C) 0–5 μM SKLB70326 for 48 h or (D) 2.5 μM SKLB70326 for 24, 36 or 48 h, the change in the mitochondrial membrane potential was determined by FCM.

cyclins [16]. The retinoblastoma protein (Rb), which serves as a tumor suppressor, also plays an important role in the regulation of G_1/S phase transition. The CDK-cyclin complex promotes the phosphorylation of Rb, which then causes the release of the E2F transcription factors that facilitate cells enter into S phase [17]. We found that SKLB70326 treatment inhibited the expression of CDK2, CDK4 and CDK6, decreased the phosphorylation of Rb, which inhibited the release of E2F, and finally arrested the cell cycle in G_0/G_1 phase.

Because of the important roles of the CDKs in cell-cycle progression, many studies have examined the possibility of developing small-molecule inhibitors of the CDKs to block tumor growth. However, until now, no CDK inhibitors have been approved for commercial use due to the toxicity of these molecules in clinical

trials [16]. SKLB70326 treatment inhibited the expression of CDK2, CDK4 and CDK6. Moreover, our results suggest that SKLB70326 treatment is not highly toxic in normal cells and no observed adverse effect level (NOAEL) value for SKLB70326 treatment was greater than 2 g/kg in rats. Therefore, these data indicate that the *in vivo* use of SKLB70326 is very safe, which would make SKLB70326 suitable for anticancer therapies. In addition to a high level of safety, the selective inhibition of proliferation in HCC cells with SKLB70326 treatment compared to other human cancer cells makes SKLB70326 a potentially very interesting and useful drug. Moreover, SKLB70326 treatment at 150 mg/kg resulted in a 50% growth inhibition of HepG2 xenograft tumors in nude mice (data not shown). Our results suggest that SKLB70326 may be a potential anti-HCC agent, and the optimization of the 3-amino-thieno[2.3-b] pyridine derivatives could serve as a promising strategy for the development of anti-HCC drugs.

In G_0/G_1 phase regulation, p21 plays a key role in inhibiting the activity of the cyclin-CDK2 or cyclin-CDK4/6 complexes [18]. p53, a main protein upstream of p21, inhibits cell proliferation by inducing a cell-cycle arrest in cancer cells [19]. Our results demonstrated that SKLB70326 treatment caused the upregulation of p21 and p53 expression in HepG2 cells, which have been shown to express wide-type p53. These results suggest that SKLB70326 treatment may induce a G_0/G_1 cell-cycle arrest through a p53-p21-CDK-mediated pathway. Although we investigated the potential targets of SKLB70326 upstream of the CDKs, the inhibition of the CDKs does not explain the HCC-specific activity of SKLB70326. As another attempt to identify the targets of SKLB70326, we performed a bioinformatics prediction assay, which identified I-kappa-B kinase 2 (IKK-beta) and Heat Shock Protein 90 (Hsp90) as additional potential targets of SKLB70326 (data not shown). These proteins are both known to have roles in cell-cycle regulation [20,21]. The detailed mechanism that results in the HCC-specific sensitivity to SKLB70326 treatment will require further investigation.

In cancer therapy, inhibiting the cell cycle would subsequently induce apoptosis [22]. In the present study, we observed that the percentage of HepG2 cells in G_0/G_1 phase significantly increased to 80% after SKLB70326 treatment for 24 h. However, the percentage of early apoptotic cells was only 11.1% after a 24-h SKLB70326 treatment and 34% after a 48-h SKLB70326 treatment. These results suggest that apoptosis could be a result of the cell-cycle arrest, which supports the role of SKLB70326 as a cell-cycle inhibitor. Apoptosis plays an important role in growth inhibition, and the agents that induce apoptotic death in cancer cells could be useful for the treatment of malignancy [23]. In the present study, SKLB70326 treatment induced the cellular apoptosis through the intrinsic pathway. Furthermore, our results also showed SKLB70326 induced the expression of p53, which can regulate cell-cycle arrest and induce apoptosis [24]. These results suggest that SKLB70326 treatment induces apoptotic death in HepG2 cells through the intrinsic apoptotic pathway, which is mediated by p53, Bcl-2 and Bax.

Taken together, we conclude that SKLB70326 treatment significantly inhibited HCC cell proliferation by causing a G_0/G_1 arrest *via* the p53-p21-CDK pathway and inducing apoptosis through the intrinsic pathway. While the mechanisms underlying the activities of SKLB70326 remain to be understood, the potential of SKLB70326 as a candidate anti-HCC agent warrants further investigation.

Acknowledgments

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