A Novel Anticancer Agent, SKLB70359, Inhibits Human Hepatic Carcinoma Cells proliferation via G0/G1 Cell Cycle Arrest and Apoptosis Induction

Xiao-Yun Dai¹,*; Xiu-Xiu Zeng¹,*; Feng Peng²,*; Yuan-Yuan Han¹; Hong-Jun Lin¹; You-Zhi Xu¹; Tian Zhou¹; Gang Xie¹; Yi Deng¹; Yong-Qiu Mao¹; Luo-Ting Yu¹; Li Yang¹ and Ying-Lan Zhao¹

¹State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu, ²Department of Geriatric, West China Hospital, West China Medical School, Sichuan University, Chengdu, *These authors contributed equally to this work

Key Words
SKLB70359 • G0/G1 cell cycle arrest • Apoptosis

Abstract
Hepatocellular carcinoma is one of the most common cancers in worldwide. We previously reported a novel thienopyridine derivative 3-amino-6-(3,4-dichlorophenyl) thieno[2,3-b]pyridine-2-carboxamide (SKLB70359) which possesses anticancer activity against hepatocellular carcinoma. In present study, we further investigated its anticancer activity and possible mechanism. The SKLB70359 treatment decreased the viability of a panel of hepatocellular carcinoma cell lines in a concentration- and time-dependent manner with IC₅₀ 0.4~2.5 µM. The mechanism study showed that SKLB70359 induced G0/G1 cell cycle arrest and then led to apoptotic cell death of HepG2 cell. The SKLB70359 induced G0/G1 cell cycle arrest was characterized by down-regulation of cyclin-dependent kinase 2 (CDK2), CDK4, CDK6 expression and up-regulation of p53, p21WAF1. Activating of caspase-3 and caspase-9 was also observed. Meanwhile, proliferation inhibitory effect of SKLB70359 was associated with decreased level of phosphorylated p44/42 mitogen activated protein kinase (p44/42 MAPK) and phosphorylated retinoblastoma protein (Rb). Moreover, SKLB70359 exhibit less toxicity to non-cancer cells than tumor cells. In conclusion, the findings in this study suggested that SKLB70359 have potential anticancer efficacy via G0/G1 cell cycle arrest and apoptosis induction. Its potential to be a candidate of anticancer agent is worth being further investigated.

Introduction
Hepatocellular carcinoma (HCC) is the sixth most common newly diagnosed cancer and the third most common cause of cancer mortality in worldwide. Its treatment outcome is far from satisfactory and the dismal 5-year survival rate is approximately 10% [1]. Liver transplantation is considered to be the only curative therapy at present, however, a majority (>80%) of patients with advanced and unresectable HCC are not suitable for transplantation or surgical resection [2, 3].Chemotherapy using conventional cytotoxic drugs, such
as doxorubicin, cisplatin and fluorouracil is one of the commonly treatment options, especially for patients with unresectable tumors. However, because of poor response rates, severe toxicities and high recurrence rates, the mean survival time is approximately 6 months [2, 4]. Thus, more effective agents are in urgent demand to better combat this malignancy. Recent years, the success of sorafenib, which significantly prolong the survival of patients with advanced HCC, suggested that small-molecule targeted chemotherapy is a promising strategy to combat this cancer [5].

Cell cycle regulation plays an important role in HCC proliferation and survival. The eukaryotic cell cycle is regulated by signal transduction pathways mediated by a series of cell-cycle regulators, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs). Cyclin molecules regulate the progression of each phase of cell cycle by associating with corresponding phase-specific CDKs. Cyclin D activate the CDK4 and CDK6, which help maintaining and progressing through the early G1 phase of the cell cycle. Cyclin E and CDK2 proteins play a role in the transition from the G1 to S phase of the cell cycle [6]. p21WAF1 and p27Kip1 belong to the family of CDKIs, whose expression regulates the G1 phase CDKs [7]. Increased expression of CDKs and cyclins has been observed in most cancer cells. Especially, the loss of cell cycle control in G1 has been implicated in tumor development and proliferation, including HCC [8, 9]. Therefore, induction of cell cycle arrest by chemopreventive drugs could be an effective approach to treat uncontrolled cell proliferation and survival in tumor cells.

Recently, many studies have shown that induction of cell cycle arrest via the inhibition of CDK activity is one of the most promising strategies for the discovery and design of novel anticancer agents. Small molecule inhibitors of cell cycle kinases, such as AT-7519 and CY-202 were demonstrated to be able to inhibit tumor growth in animal and human. Moreover, there are smaller molecule inhibitors of cell cycle kinases, such as SNS-032 and AG-24322 are currently under clinical evaluation [8, 10].

Our research group has been interested in the design, synthesis, screening and biological evaluation of novel cell cycle inhibitors as potential new anticancer agents. Based on above improved understanding of cell cycle regulation and its role in HCC, we gained into development novel cell cycle kinases inhibitors against HCC. We used computer-aided drug design, targeted cell cycle kinases and synthesized a series of compounds. In a previous cell-based screening study, we found a novel thienopyridine derivatives can efficiently inhibit the growth of HepG2 cells, and induce apoptosis in vitro [11]. Among them, 3-amino-6-(3,4-dichlorophenyl)thieno[2,3-b]pyridine-2-carboxamide (SKLB70359) has predominant anti-HCC activity. In order to provide the theoretical guide of SKLB70359 development and further optimization of the thienopyridine derivatives, it is necessary to investigate the molecular mechanism of SKLB70359.

In present study, we investigated the molecular mechanism of SKLB70359 against HCC. We demonstrated that SKLB70359 exhibited antitumor activity by inducing G0/G1 cell cycle arrest and apoptosis. This effect was characterized by down-regulation CDK2, CDK4, CDK6, p44/42 MAPK and p-Rb (Ser780) and up-regulation of p21WAF1, p53. We also found that SKLB70359 induced apoptosis through the mitochondria-dependent pathway. Its potential to be a candidate of anticancer agent is worth being further investigated.

Materials and Methods

Preparation of SKLB70359

3-amino-6-(3,4-dichlorophenyl)thieno[2,3-b]pyridine-2-carboxamide (SKLB70359) was initially synthesized in the State Key Laboratory of Biotherapy, Sichuan University (Sichuan, China). Its structural formula was shown in Fig. 1. SKLB70359 was prepared as 20 μM stock solution in DMSO and diluted in the relevant assay media.

Materials

3-(4,5)-dimethylthiazolium-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). All the chemicals employed in this study were of analytic pure and were of culture grade. The primary antibodies against β-actin, CDK2, CDK4, CDK6, p44/42 MAPK, phosphorylated p44/42 MAPK (Thr202/Tyr204), p-Rb, PCNA, Cyclin D1, p21WAF1, caspase-3 and caspase-9 were acquired from Cell Signaling Technology (Beverly, MA). The protein assay kit was purchased from Bio-Rad (Hercules, CA).

Cell culture

All the human cancer cell lines were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle medium or RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; Gibco, Auckland, N.Z.), 100 units/mL penicillin and 100 units/mL streptomycin in a 5% CO2 humidified atmosphere at 37°C.

Cell viability assay

The cell viability of SKLB70359 treated cancer cells and normal cells were determined using MTT assay. Briefly, Cells
(3×10^3~5×10^3 cells/well) were seeded in 96-well plates and cultured for 24 h, followed by various concentrations of SKLB70359 (0.3~20 µM) treatment for 24, 48, 72 h, respectively. The 20 µl of 5 mg/mL MTT was added to each well and incubated for an additional 2~4 h. Then, the medium was discarded and 150 µl DMSO was added to dissolve the formazan. Absorbance was measured at 570 nm using SpectraMax M5 (Molecular Devices). The effect of SKLB70359 on cells viability was expressed by IC50 of each cell lines.

**Clonogenic assays**

HepG2 Cells (300~400 cells/well) were seeded in 6-well plates and cultured for 24 h, followed by various concentrations of SKLB70359 treatment (0~5 µM) for two weeks. After washing by PBS, colonies were fixed with methanol and stained with methylene blue (0.04%). Colonies with > 50 cells were counted under microscope.

**Cell cycle and apoptosis analysis by Flow Cytometry**

The cell cycle was analyzed as described previously with slight modifications [12]. Briefly, HepG2 cells cultured on 6-well plates were synchronized at the G1/S boundary by Thymidine (4 µg/mL, Sigma) for 24 h. To allow S-phase progression, cells were washed extensively and treated with SKLB70359. The cells were suspended in 1 mL hypotonic hypotonic solution containing 50 µg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100, and then analyzed by Flow Cytometer (ESP Elite, Beckman-Coulter, Miami, FL). The data were analyzed using Listmode software.

**Mitochondrial membrane potential (ΔΨm) assay**

The change of mitochondrial membrane potential (ΔΨm) was measured by flow cytometry as described previously [12]. After treated with various concentrations of SKLB70359 for 48 h, ΔΨm was determined by the retention of the dye rhodamine 123 (Rh123) (Sigma) in HepG2 cells. HepG2 cells were incubated with Rh123 (5 µg/ml) at 37°C for 30 min in the dark. After washing twice, the cells were incubated again in PBS and fluorescence was then measured as described above.

**Assay DNA synthesis by cell light EdU (5-ethynyl-2'-deoxyuridine)**

EdU staining was conducted using EdU-Apollo™EdU assay kit (Invitrogen, Carlsbad, CA) according to the *Cell Physiol Biochem* 2012;29:281-290

### Table 1. The proliferation inhibitory effect of SKLB70359 against cancer cell lines.

MTT assay was employed to detect the cell viability. Each cell line was treated with various concentrations of SKLB70359 for 48 h, respectively. Data are expressed as the mean from three experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>Human hepatocellular carcinoma cell line</td>
<td>0.44±0.11</td>
</tr>
<tr>
<td>SMMC-7721</td>
<td>Human hepatocellular carcinoma cell line</td>
<td>0.6±0.12</td>
</tr>
<tr>
<td>Bel7404</td>
<td>Human hepatocellular carcinoma cell line</td>
<td>3.36±0.73</td>
</tr>
<tr>
<td>A375</td>
<td>Human malignant melanoma cell line</td>
<td>5.93±0.85</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervix adenocarcinoma cell line</td>
<td>8.55±1.04</td>
</tr>
<tr>
<td>Raji</td>
<td>Human Burkitt's lymphoma cell line</td>
<td>4.36±0.83</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>Human ovary adenocarcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast adenocarcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>DU-145</td>
<td>Human prostate carcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>SW620</td>
<td>Human colorectal adenocarcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>PC-3</td>
<td>Human prostate adenocarcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>786-O</td>
<td>Human renal carcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>PANC-1</td>
<td>Human pancreas epithelioid carcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>A431NS</td>
<td>Human epidermoid carcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>SW480</td>
<td>Human colorectal adenocarcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>Human large cell lung cancer cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>OSRC2</td>
<td>Human renal carcinoma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>U251</td>
<td>Human glioma cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>HCT116</td>
<td>Human colorectal adenocarcinoma cell line</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>

**Table 2. The effect of SKLB70359 on normal cell lines viability.**

MTT assay was employed to detect the cell viability. Each cell line was treated with various concentrations of SKLB70359 for 48 h, respectively. Data are expressed as the mean from three experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>HK-2</td>
<td>Human kidney cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>HB1</td>
<td>Human bronchial epithelial cell line</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>

SKLB70359 Induces G0/G1 Cell Cycle Arrest and Apoptosis
manufacturer’s instruction. HepG2 cells (8×10⁴ cells/well) were seeded in 6-well plates and treated with different concentration of SKLB70359 for 20 h. Then, cells were fixed with formaldehyde for 15 min and washed with PBS. Cells were permeabilized with 0.2% Triton-100 for 5 min, followed by incubation with the EdU-ApolloTMEdU for 30 min in dark. After incubation, cell was washed with 0.5% Triton-100 in PBS for 10 min. For subsequent DNA staining, sections were incubated with Hoechst 33342 (5 µg/mL) for 10 min. The plates were then washed twice with PBS and observed under a fluorescent microscope.

Western blotting analysis

Western blot analysis was performed as described previously [13]. Cells cultured on 100 mm² diameter dishes were washed with PBS and scrapped with RIPA buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholic acid and 0.05% SDS). The protein concentration in the cell lysate was measured by the Lowry method. Equal amounts of protein were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham Bioscience, Piscataway, NJ). After incubation with the primary and secondary antibodies, the reactive band was identified using an enhanced chemiluminescent substrate to horseradish peroxidase (Amersham, Piscataway, NJ).

Statistical analysis

Data represented as means ± SD of three independent experiments. Student’s test was employed to assess the statistical significance of difference between control and SKLB70359-treated groups. A statistically significant difference was considered to be present at P < 0.05. Autoradiograms/bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, Inc. San Jose, CA).

Results

Effects of SKLB70359 on cell proliferation

In order to investigate the growth inhibitory properties of SKLB70359, we performed a chemo-sensitization assay based on the ability of viable cells to reduce MTT to formazan crystals. A comparison between the nineteen cell lines examined showed that the effect of SKLB70359 on hepatic carcinoma cells such as HepG2, Bel-7404, SMMC-7721 was more sensitive than other cancer cells (Table 1). After exposure to SKLB70359 for 48 h, the IC₅₀ of HepG2, Bel-7404, SMMC-7721 were 0.44 µM,
3.3 µM and 0.6 µM, respectively (Table 1). Exposure of cells to SKLB70359 for 24 h, 48 h and 72 h, respectively, resulted in decrease of the cell proliferation ($P < 0.05$) (Fig. 2C). These results suggested that SKLB70359 inhibited hepatic carcinoma cells proliferation in a concentration- and time-dependent manner. Moreover, no apparently toxicity on normal cells was observed (Table 2).

**Effect of SKLB70359 on clonogenicity in HepG2 Cells**

To further determine whether SKLB70359 could inhibit the proliferation of HepG2, we conducted clonogenic assay after SKLB70359 treatment. Clonogenic assay clearly showed that clone formation of HepG2 cells was reduced in a concentration-dependent manner after exposure to SKLB70359 (Figs. 2A,B). At the higher concentration of 0.63 µM, there was no colony formation. Furthermore, the size of the colonies treated with SKLB70359 was significantly smaller than the control.

**Labeling of cellular DNA with EdU**

We use EdU assay to investigate the cell proliferation after treated with different concentration of SKLB70359. When HepG2 cells were exposed to SKLB70359 for 20 h, the number of Hoechst-stained cells was almost same with control. However, the EdU staining cells was greatly reduced which indicated that DNA replication was blocked by treatment with SKLB70359 (Fig. 3).
SKLB70359 induced cell cycle arrest and apoptosis

To examine the mechanism responsible for SKLB70359-mediated cell proliferation inhibition, cell cycle distribution was evaluated using flow cytometric analysis. As shown in Fig. 4A, exposure of HepG2 cells to growth suppressive concentration of SKLB70359 (0.63 µM) resulted in significant accumulation of cells in G0/G1 phases that was accompanied by a decrease in cells with S and G2/M DNA content. For example, the percentage of cells in G0/G1 phases was increased to 77.2%, after treatment with 0.63 µM SKLB70359 for 20 h, whereas the G0/G1 phase cells in control is 33.6% (Fig. 4A).

Furthermore, 48 h after SKLB70359 exposure, the apoptosis induction effect was apparently observed (Fig. 7A). When the cells were treated with 0.31 µM SKLB70359, the percentage of apoptosis cells was 29.8%, whereas the apoptosis cells increased to 47.9% and 72.1% when cells were treated with 0.63 µM and 1.25 µM SKLB70359. The results directly suggested that SKLB70359 induced the apoptosis of HepG2 cells in a concentration-dependent manner.

**Fig. 5.** Effects of SKLB70359 on the protein expression of G1 regulators. (A) The expression of CDK2, CDK4, CDK6, Cyclin D1 and p-Rb after SKLB70359 treatment for 24 h at different concentrations (0–2.5 µM). (B) The expression of P53, P21WAF1 and PCNA after SKLB70359 treatment for 24 h at different concentrations (0–2.5 µM). β-actin in each sample was employed as a standard. Data shown are representative of at least three independent experiments.

**Fig. 6.** Effects of SKLB70359 on p44/42 MAPK expression. HepG2 cells were treated with SKLB70359 at different concentrations (0–1.25 µM) for 24 h and the phosphorylation of p44/42 MAPK in HepG2 were inhibited by SKLB70359. β-actin in each sample was employed as a standard. Data shown are representative of at least three independent experiments.

**Fig. 7.** The effect of SKLB70359 on apoptosis progression. (A) The apoptosis induction was determined by flow cytometry after PI-staining. Apoptosis rate after SKLB70359 treatment for 48 h at various concentrations (0–1.25 µM) was 1.2%, 4.3%, 10.2%, 29.8%, 47.9% and 72.1% apoptotic cells, respectively. Histograms shown are representative of triplicate experiments. (B) HepG2 cells were treated with SKLB70359 at different concentrations (0–1.25 µM) for 48 h, and the change of ΔΨm was analyzed by flow cytometry after Rh123 staining. Data shown are representative of at least three independent experiments.
SKLB70359 inhibited CDK level and phosphorylation of protein retinoblastoma in HepG2 cells

To eliminate the mechanism of the G0/G1 arrest, we investigated the change of CDK2, CDK4 and CDK6 expression which were associated with G0/G1 cell cycle regulation after SKLB70359 treatment. Our results showed that the levels of CDK2, CDK4 and CDK6 in HepG2 cells were significantly down regulated at 24 h by SKLB70359 treatment whereas cyclin D1 levels not changed (Fig. 5A). Due to the Rb plays a critical role in governing cell cycle progression, apoptosis, and differentiation, especially for the transition from the G1 to S phases [14], the phosphorylation level of Rb was detected. Our results showed that p-Rb significantly decreased in a concentration-dependent manner 24 h after SKLB70359 treatment (Fig. 5A).

SKLB 70359 induced G0/G1 arrest through a P53-depended mechanism

The previously studies show that p21 WAF1 and p53 play an important role in G0/G1 arrest. Therefore, in order to determine whether these two proteins play a role in cell cycle arrest, the HepG2 cells were exposed to SKLB70359 and analyzed for changes on protein level of p21 WAF1 and p53. The results showed that, after 24 h, the positive rate of p21 WAF1 and p53 protein expression was significantly higher in HepG2 cells than control (Fig. 5B). This change associated with down regulation of PCNA which is crucially regulated by the tumor suppressor protein p21 WAF1[15].

The effect of SKLB70359 on p44/42 MAPK expression

To further elucidate the mechanism of proliferation inhibitory effect of SKLB70359 on HepG2 cells, we examined the expression of the p44/42 MAPK in HepG2 after SKLB70359 treatment. As shown in Fig. 6, SKLB70359 significantly decreased the level of phosphorylated p44/42 MAPK (Thr202/Tyr204), whereas total p44/42 MAPK were unaffected by the SKLB70359 treatment as judged by comparisons with β-actin as a loading control. This data indicated that SKLB70359 can inhibit the phosphorylation of p44/42 MAPK.

Requirement of caspase-3 and caspase-9 activation during SKLB70359-induced apoptosis

To investigate whether SKLB70359-induced apoptosis was associated with the caspase family proteins or some other apoptotic associated proteins, the expression of caspase-3, caspase-9 was examined after SKLB70359 treatment at 24 h and 48 h. As shown in Fig. 8, the levels of caspase-3 and caspase-9 were no change at 24 h after SKLB70359 treatment, indicated that no apoptosis was induced. However, at 48 h of SKLB70359 treatment, the level of procaspase-3, procaspase-9 significantly decreased and cleaved caspase-3, caspase-9 increased in a concentration-dependent manner, indicating the activation of caspase-3 and caspase-9 (Fig. 8).

Effects of SKLB70359 on Mitochondrial membrane potential (ΔΨm)

A key step in the intrinsic apoptotic pathway is the mitochondrial membrane permeability disruption and loss of ΔΨm. ΔΨm was measured by flow cytometry using
green fluorochrome rhodamine 123 (Rh123). Disruption of ΔΨm is associated with a lack of Rh123 retention and a decrease in fluorescence [12]. As shown in Fig. 7B, a significantly decrease of Rh123 fluorescence in HepG2 cells was detected followed by SKLB70359 treatment for 48 h compared with the control, indicating the collapse of the mitochondrial membrane potential induced by SKLB70359.

Discussion

In the present study, we investigated the proliferation inhibitory effect of SKLB70359 against human hepatic carcinoma cells and the possible mechanism. To our knowledge, our study is first to demonstrated that SKLB70359 inhibited hepatic carcinoma cell HepG2 proliferation by inducing G0/G1 cell cycle arrest and apoptosis via the mitochondrial pathway.

SKLB70359 was obtained from the computer-aided drug design targeted cell cycle kinases and structural modification of 3-amino-thieno[2.3-b] pyridine derivative. It has novel chemical structure which is different from cell cycle kinase inhibitors in clinical research, and we already got the SKLB70359 patent from State Intellectual Property Office the People’s Republic of China (patent number: ZL 200810046188.8). In a previous cell-based screening of anticancer drugs, we found that SKLB70359 has antitumor activity against various kinds of cancer cell line and in particular, HepG2 Cells was most sensitive to its treatment with IC50 value of 0.44 μM. SKLB70359 proliferation inhibitory activity against HepG2 cell was further confirmed using clonogenicity assay and EdU assay. Moreover, IC50 of SKLB70359 against normal cell line was much higher than IC50 of cancer cell line, suggesting that a therapeutic window could be achieved because they might have a selective cytotoxicity effect on rapidly proliferating cell over quiescent or slowly proliferating normal cells. Though the pursuit for Cyclin-dependent kinases (CDKs) inhibitor has been last for 15 years, no CDK inhibitor has been approved for commercial use because of their toxicity in clinical use. Therefore, the property of high safety makes SKLB70359 suitable for anticancer therapies.

In mechanism study, SKLB70359 significantly induced G0/G1 arrest of HepG2 cells. The eukaryotic cell proliferation is a tightly regulated system controlled by cyclin-CDK complexes, and the transition from G1 to the S phase was the major regulatory checkpoint in this process. This transition is characterized by the phosphorylation of Rb, and the CDK2, CDK4, CDK6, cyclin D1 and cyclin E enzyme complex catalyzes the reaction [16, 18]. In our research, we found SKLB70359 potently inhibited the CDK2, CDK4, CDK6 and Rb phosphorylation without change of cyclin D1, thus results in G0/G1 cell cycle arrest and exert its antiproliferatory effect. To our knowledge, there are only few study reported the anticancer activity of 3-amino-thieno[2.3-b] pyridine derivative [11], and no study concerning their anti-HCC activity and cell cycle arrest effect was reported. Therefore, all of these results indicated that 3-amino-thieno[2.3-b] pyridine derivative optimization might be a new strategy for cell cycle inhibitor development and SKLB70359 have potential to be a candidate of CDK inhibitor.

The effect of SKLB70359 on cell cycle arrest might be through multiple direct and indirect mechanisms, and the primary target identification is very important for small molecular anticancer compound development and its further application. In above study, we demonstrated that SKLB70359 down regulated the CDK2, CDK4, CDK6 expression and we estimated whether these CDKs were the primary target of SKLB70359. However, the kinase assay in vitro showed that SKLB70359 didn’t directly inhibit CDK2/4/6 activity effectively (data not shown). We speculated that the upstream of CDKs might be the primary target of SKLB70359. In G1–S progression, p21WAF1 played an important role in this process. It could inhibit the activity of CDK-cyclin complexes and proliferating cell nuclear antigen (PCNA) to regulate cell cycle process. Our study showed p21WAF1 accumulation increased after SKLB70359 treatment. Besides p21WAF1, the tumor suppressor p53 also could induce cell cycle arrest and contributes to maintaining genome stability in response to conditions that generate DNA damage [19]. It is reported that p53-dependent G1 growth arrest is mediated by p21WAF1, and p21WAF1 is the only one directly CDK inhibitory protein transcriptional regulated by p53 [20, 21]. Our resulted demonstrated that SKLB70359 also upregulated p53 expression. Thus, in present study, p53 and p21WAF1 might perform their functions by inhibiting the kinase activities of CDK-cyclin complexes to stimulate G0/G1 arrest which was attributed to SKLB70359 effect. Although we investigated the upstream of CDKs, we still cannot identify the primary target of SKLB70359. We then performed bioinformatics predictions (data not show), and we interestingly found that Heat shock protein 90 (Hsp90) is a potential molecular target of SKLB70359. Previous research indicated that cancer therapy targeting HSP90 has shown great promise and HSP90 can affect...
CDKs including CDK4 and CDK6. This effect was simultaneously accompanied by hypophosphorylation of Rb which cause G0/G1 cell cycle arrest [22, 23]. We speculated that the SKLB70359 might inhibit the Hsp90, and then induced CDK2, CDK4, CDK6 reduction and p-Rb inactivation, which finally resulted in G0/G1 cell cycle arrest. The further study need to be carried out to confirm this bioinformatics prediction.

Mitogen-activated protein kinases (MAPKs) are essential components of the intracellular signal transduction pathways that regulate cell proliferation and apoptosis. One subgroup of MAPKs, p44/42 MAPK is an important target in the diagnosis and treatment of cancer and has been reported to be required for up-regulation of p21WAF1 that results in cell cycle arrest [24-26]. In present study, MAPK signaling pathway regulation after SKLB70359 treatments were investigated and our study found that SKLB70359 treatment down-regulated the phosphorylation of p44/42 MAPK. Therefore, p44/42 MAPK also play a role in SKLB70359 induced G0/G1 arrest.

Previously evidences have shown that the outcome of G0/G1 arrest could be lead to cell death by apoptosis [27, 28]. To demonstrate the rapid response of the HepG2 cells, we evaluated the SKLB70359 induced apoptosis. The prominent loss of mitochondrial membrane potential (ΔΨm) was observed after 48 hours which is one of the hallmarks of apoptosis. In mitochondrial-mediated apoptotic pathway, caspase family protein plays a central role and is responsible for the breakdown of several cellular components involved in DNA repair and regulation [29]. We analyzed the proteins involved in apoptosis, and found that caspase-3 and caspase-9, the key molecular in apoptosis, was activated. Thus, apoptotic changes we observed in the cells might be attributed to the fate of cell cycle arrest.

As not all the compounds which exhibit antitumor activity in vitro show anticancer activity in vivo, we also investigated the in vivo antitumor effect of SKLB70359 using HepG2 tumor model established in nude BALB/C mice. The resulted showed that SKLB70359 inhibited the tumor growth by 50% at the dose of 150 mg/kg orally (data not show). By investigating the pharmacokinetics parameter, we doubt that the modest inhibitory rate in vivo might be due to its pool solubility and bioavailability. Therefore, at further optimization should be needed to improve its drugibility.

In conclusion, our observations indicated that SKLB70359 inhibited cell cycle progression and induced apoptotic cell death which contributed to its anti-proliferation effects against hepatocellular carcinoma HepG2 cells. The most likely mechanism underlying the SKLB70359-induced growth arrest involves an initial inhibition of p44/42 phosphorylation and increase of p53 and p21WAF1 expression, which led to the reduction of the G1-related CDKs (CDK6, CDK4, and CDK2) protein and p-Rb, then ultimately arrested HepG2 cells in the G1 phase and followed apoptotic cell death. Its potential to be a candidate of anticancer agent is worth being further investigated. Our work provided strong evidence that inhibition of the cyclin kinases is a promising approach to cancer therapy, holding the potential to induce regression of a diverse range of tumor types.

**Abbreviations**

DMSO (Dimethyl Sulfoxide); PI (Propidium Iodide); EdU (5-Ethynyl-2’-Deoxyuridine); MAPK (Mitogen-Activated Protein Kinase); CDK (Cyclin-Dependent Kinase); Rb (retinoblastoma Protein); HCC (Hepatocellular Carcinoma); MTT (3-(4,5)-Dimethylthiahiazo(-z-y1)-3,5-Di-phenytetrazoliwmromide).

**Acknowledgements**

This work was supported by National S&T Major project (2011ZX09102-001-013 and 2012ZX09501001-003) and National Key Basic Research Program of China (2010CB529900).

**References**


SKLB70359 Induces G0/G1 Cell Cycle Arrest and Apoptosis

Cell Physiol Biochem 2012;29:281-290


