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SKLB316, a novel small-molecule inhibitor of cell-cycle progression, induces G2/M phase arrest and apoptosis *in vitro* and inhibits tumor growth *in vivo*

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ABSTRACT

Benzothiazole derivatives have received considerable attentions for their potencies in cancer therapy. In the present study, we reported that SKLB316, a novel synthesized benzothiazole derivative, exhibits activities to inhibit colorectal and pancreatic cancer *in vitro* and *in vivo* by inducing G2/M cell cycle arrest and apoptosis. *In vitro*, it exhibited significant anti-proliferative activities against human cancer cells derived from different histotypes including the colorectal cancer cell line HCT116 and pancreatic cancer cell line CFPAC-1. We chose these cell lines to study the possible anti-tumor mechanism because they are sensitive to SKLB316 treatment. Flow cytometry assays showed that SKLB316 could induce G2/M cell cycle arrest. Mechanistically, SKLB316 could decrease the activities of cdc2/cyclin B1 complex, including decreasing the synthesis of cyclin B1, cdc2 and cdc25c, while accumulating the levels of phosphorylated cdc2 (Tyr15) and checkpoint kinase 2. SKLB316 could also decrease the level of cyclin E and A2. Moreover, SKLB316 could induce cancer cell apoptosis, which was associated with activation of caspase 9, downregulation of Bcl-2 and upregulation of Bax. SKLB316 could also decrease the mitochondrial membrane potential and induce the generation of reactive oxygen species in cells. The results implied that SKLB316 may induce apoptosis via the mitochondria-mediated apoptotic pathway. Moreover, SKLB316 could suppress the growth of established colorectal and pancreatic cancer tumors in nude mice without causing obvious side effects. TUNEL assays confirmed that SKLB316 could also induce tumor cell apoptosis *in vivo*. Taken together, these findings demonstrate the potential value of SKLB316 as a novel anti-tumor drug candidate.

Introduction

Colorectal cancer is the third most common cancer and the third leading cause of cancer death in the United States [1]. However, its treatment is not satisfactory because of the poor survival rates and frequent liver metastases when the patients are diagnosed [2]. Pancreatic cancer has the highest mortality rate of all the major tumor types [3], being notorious for late diagnosis, aggressive local invasion, early systemic metastasis, and resistance to chemo- and radiotherapy [4]. Presently, many obstacles exist on the road to treat this disease [5]. Therefore, it remains an urgent need to identify novel strategies to treat them.

Dysregulation of cell cycle regulation is one hallmark of cancer [6], and disruption of the cancer cell cycle can lead to tumor growth arrest and ultimately to apoptosis, contributing to cancer therapy [7]. Moreover, inducing cell cycle arrest of abnormally proliferating cancer cells could increase the efficacy of cancer treatment with other therapeutic approaches, such as traditional cytotoxic drugs [8]. Thus, induction of cell cycle arrest could be an effective way to cope with unchecked cancer cell proliferation and survival of tumor cells [9,10]. Some promising anti-cancer agents targeting the cell cycle, such as MK-7965 (Phase II) [11] and PD-0332991 (Phase III) [12], are under clinical evaluation. Many more of such kind of agents

Abbreviations: chk2, check point kinase 2; CDK1, cyclin-dependent kinase 1; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PI, propidium iodide; Rh123, rhodamine-123; FCM, flow cytometry; ΔΨm, mitochondrial membrane potential; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SD, standard deviation.

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are under preclinical evaluations in cancer therapy [13,14], implying the promising potential of this strategy in anti-cancer therapy. Evasion of apoptosis is another hallmark of most, and maybe all, types of cancer [6]. Deregulation of apoptosis leads to the uncontrolled growth of tumors and development of resistance to chemotherapy. Therefore, drugs that restore apoptosis might be effective against many types of cancers [15]. Thus, drugs that could induce cell cycle arrest and apoptosis may be effective to treat colorectal and pancreatic cancer.

Benzothiazole derivatives possess diverse biological activities, including antidiabetic and antitumor activities [16,17]. Recently, our research group has been interested in developing novel benzothiazole derivatives as potential anticancer agents. Based on the preceding effort to develop new anticancer molecules, some compounds with good inhibitory activities on cancer cell growth *in vitro* were found [18]. Among these compounds, SKLB316 showed outstanding antiproliferative activities against a panel of human cancer cells. However, its possible mechanism underlying the inhibition remains unknown.

In this study, we evaluated the activities of SKLB316 to inhibit colorectal and pancreatic cancer *in vitro* and *in vivo*. Mechanistically, we elucidated the possible mechanism through induction of G2/M cell cycle arrest by inhibiting the activities of cdc2/cyclin B1 complex and inducing apoptosis through the reactive oxygen species (ROS)–mitochondrial apoptotic pathway. Moreover, we evaluated the *in vivo* anti-tumor activities of SKLB316 in colorectal and pancreatic tumor models in nude mice. SKLB316 also showed good safety profiles in the sub-acute toxicity test. The results showed that SKLB316 may be a potential novel anti-tumor drug candidate and its further investigation is warranted.

Materials and methods

Preparation of SKLB316

2-Chloro-N-(2-(2-(5-chloropyridin-2-ylamino)-2-oxoethylthio)benzo[d]thiazol-6-yl)acetamide (SKLB316) was synthesized previously at the State Key Laboratory of Biotherapy, Sichuan University (Sichuan, China) [18], and its structural formula is shown in Fig. 1A. SKLB316 was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM and diluted with the relevant medium for the *in vitro* experiments. The final concentration of DMSO was less than 0.1%. For *in vivo* studies, SKLB316 was dissolved in ultrapure water and Cremophor EL/ethanol (50:50; Sigma Cremophor EL, 95% ethyl alcohol).

Materials

3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide(PI), rhodamine-123 (Rh123) and Hoechst 33258 were purchased from Sigma (St. Louis, MO).The Annexin V-FITC apoptosis detection kit was purchased from Roche (Indianapolis, IN). The primary antibody against α-tubulin and the FITC-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The other primary and second antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Cell lines and cell culture

The human hepatic carcinoma cell lines SMMC-7721 and Bel-7402 were obtained from the China Center for Type Culture Collection (CTCCC, Wuhan, China). The normal human liver cell line LO2 was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). All of the other human cancer cell lines used in this study were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM, IMDM or RPMI 1640 media containing 10% foetal bovine serum and 0.1% amikacin sulfate under humidified conditions with 5% CO_2 at 37 °C.

Cell viability assay

The cell viability assay was conducted as described previously with some modifications [19]. The cells ($2–5 \times 10^3$ cells per well in 100 μL of medium) were seeded in 96-well plates for 24 hours. Next, 100 μL of medium containing various concentrations of SKLB316 was added to each well. After the indicated time incubation, 20 μL of a 5 mg/mL MTT solution was added to each well and incubated for additional 2~4 hours at 37 °C. Next, the medium was removed, and 150 μL of DMSO was added to each well. The OD_{570} was measured using a Spectra Max M5 microplate spectrophotometer (Molecular Devices). The percentage of inhibition and IC_{50} values were then calculated. Doxorubicin served as a positive control. Each assay was replicated 3 times.

The colony formation assay

The cells (800 cells/well) were seeded in a 6-well plate and cultured for 24 h, followed by various concentrations of SKLB316 treatment for two weeks. Finally, the cells were stained with a 0.5% crystal violet solution after washing with PBS and fixing with methanol, and the colonies (>50 cells) were counted under an inverted microscope.

The EdU incorporation assay

EdU can incorporate into replicating DNA when the cells are dividing, and it is used to label proliferating cells [20]. Briefly, cells growing in 96-well plates (5000 cells/well) were treated with different concentrations of SKLB316 for 24 hours, and then were assayed using the Cell-Light™ EdU DNA Cell Proliferation Kit according to the manufacturer's instructions. Each assay was replicated 3 times.

Morphological analysis of cell nuclei

Treated cells were washed with PBS and fixed with methanol for 15 min. Next, the cells were incubated with Hoechst 33258 (10 μg/mL) containing 0.1% Triton X-100 for 30 min in the dark at room temperature and washed with PBS twice. Images were taken using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Cell cycle and apoptosis analysis by flow cytometry (FCM)

To analyze the cell cycle distribution, the cells were treated with SKLB316 for the indicated time periods. Next, the cells were incubated with 0.5 mL of a solution containing 50 μg/mL propidium iodide (PI) and 0.1% Triton X-100 for 30 min in the dark and analyzed by FCM (BD Biosciences). Data were analyzed using Modfit 2.8 software.

To further confirm the apoptosis-inducing effect of SKLB316, the Annexin V-FITC apoptosis detection kit was used as described previously [21]. Briefly, cells (1×10^5 cells/well) were seeded in a 6-well plate for 24 hours and treated with SKLB316 for the indicated time periods. The cells were harvested and washed twice with ice-cold PBS. The levels of apoptosis were examined by FCM using the apoptosis detection kit according to the manufacturer's instructions. The data were analyzed with FlowJo software. Each assay was replicated 3 times.

Mitochondrial membrane potential ($\Delta\Psi m$) assay

The mitochondrial membrane potential was determined by FCM following staining with Rh123. Cells were treated with the indicated doses of SKLB316 for 24 hours and then incubated with 5 μg/mL Rh123 for 30 min in the dark. The stained cells were then washed with cold PBS, and then the fluorescence emitted from Rh123 was detected by FCM.

Measurement of ROS levels in cells

DCFH-DA was applied to determine the ROS levels in cells. Briefly, after treatment with SKLB316 for 12 hours, HCT116 and CFPAC-1 cells were treated with PBS containing 10 μM DCFH-DA. After incubation for 30 min at 37 °C, cells were washed twice with PBS, and then the ROS levels were detected by FCM.

Western blotting analysis

1 mM phenylmethylsulfonyl fluoride (PMSF) was added to RIPA buffer (Beyotime, Shanghai, China) prior to use. After treatment with SKLB316, cells were lysed in RIPA buffer on ice. Next, cell lysates were centrifuged at 13,000 g at 4 °C for 20 min. The protein concentration in the cell lysate was measured by the Lowry method. Equal amounts of protein were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Bioscience, Piscataway, NJ). After incubation with the specific primary and secondary antibodies, the protein bands were visualized using an enhanced chemiluminescent substrate to horseradish peroxidase (Amersham, Piscataway, NJ) as described previously [22]. The quantitation of the Western blot results was based on three independent experiments using Image J.

Immunofluorescence microscopy staining of microtubules

The study was conducted as described previously with some modifications [23]. Cells (5000 cells/well) are seeded in Millicell EZ SLIDE 8 well glass slide (Millipore, Ireland) for 24 hours and then treated with different concentrations of SKLB316 (1, 2 and 4 μM) for 12 hours. Then the cells were washed with PBS twice and fixed with