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Silybin exerts therapeutic efficacy in non-small cell lung cancer by targeting Skp2

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ABSTRACT

Silybin (SB), a natural flavonoid isolated from *Silybum Marianum*, has been clinically used to treat hepatic fibrosis and also used as a dietary supplement owing to its hepatoprotective potentials. Numerous studies have shown that SB also exerts promising anticancer property; however, the anticancer targets of SB and the underlying mechanism remain unclear. In our study, we found that SB significantly inhibited the proliferation of non-small cell lung cancer (NSCLC), without causing cytotoxicity toward normal bronchial epithelial cells Beas-2B. Mechanistically, SB can bind to F-box protein Skp2 and disrupt Skp1-Skp2 interaction, thereby decreasing the protein level of Skp2 and inducing the accumulation of its substrates, leading to cell-cycle G1 phase arrest and cell migration suppression. In lung orthotopic xenografts, SB also significantly decreased Skp2 expression while increased p27/Kip1 (p27) protein level. SB administration inhibited tumor growth and metastasis in the lung tissue, prolonging the survival time of the mice without causing obvious toxicity. Taken together, SB can be considered a Skp2-targeting agent that warrants further clinical investigation.

Key words: silybin, NSCLC, Skp2, p27, cell-cycle arrest

1. INTRODUCTION

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Lung cancer is one of the most common malignant cancers and the leading cause of cancer deaths worldwide [1]. Non-small cell lung cancer (NSCLC) that represents 80% of lung cancers has a poor prognosis owing to tumor metastasis or drug resistance, impeding the rapid progress of targeted therapies [2]. The Identification of novel targeted drugs that attenuate aberrant proliferation and tumor metastasis are still urgently needed.

Skp1-Cullin1-F-box protein (SCF) complex, a critical E3 ligase in ubiquitin proteasome system, has emerged as a therapeutic target in various cancers [3]. Numerous anticancer drugs have been developed to target SCF complex components, especially the substrate-recognizing F-box protein Skp2. Skp2 interacts with Skp1 and forms two binding pockets around the F-box motif in Skp2, thus facilitating drug targeting [4]. Compounds such as betulinic acid and compound #25, can directly bind to Skp2 and interrupt Skp1-Skp2 interaction [5, 6]. These Skp2 inhibitors abrogate proteolytic ubiquitination of p27 and E-cadherin and also non-proteolytic ubiquitination of Akt, thereby inhibiting cell-cycle progression, tumor metastasis, glycolysis, and cancer stemness [5]. Furthermore, the high expression level of Skp2 in multiple human cancers is associated with a poor prognosis of patients [7]. Therefore, Skp2 serves as a rationale therapeutic target for anticancer drugs development.

Silybin (SB), also referred to silibinin, is the main active component of silymarin that isolated from the seeds of *Silybum Marianum*. SB and silymarin are mostly used as hepatoprotective agents in clinics to treat hepatitis, used in hepatitis therapy [8], owing to its strong antioxidant and radical-scavenging properties. SB as a natural flavonoid also possesses anti-fibrotic, anti-inflammatory, anti-cancer, anti-diabetic, and neuroprotective effects [9]. Recent studies have shown that SB exhibits interesting anticancer activities [10]. SB can modulate drug transporters, apoptotic proteins, and cell-cycle regulators, thus exhibiting both chemosensitizing and chemopreventive activities in cancer treatments [11]. SB may also regulate the activity of Stat3, Cdk4/2, MMP-2, PI3K/AKT, and ERK to attenuate cancer cell proliferation and migration [12]. Notably, evidence also demonstrates that SB can increase the protein levels of p21/p27 and induce cell-cycle G1 phase arrest [13, 14], contributing to the anticancer effects. Therefore, the therapeutic targets and underlying anticancer mechanisms of

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SB worth further investigation.

Here, we demonstrated that SB can directly bind to Skp2 and disrupt the integrity of Skp2-SCF E3 ligase in NSCLC, leading to the accumulation of p27 both *in vitro* and *in vivo*, illustrating the underlying molecular mechanisms of SB in cell-cycle arrest induction and tumor metastasis suppression.

2. MATERIALS AND METHODS

2.1 Reagents and antibodies

SB (purity: 98%) was obtained from TCI Chemical Co. (Tokyo, Japan), cycloheximide (CHX) and SZL P1-41 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Kits used for serum creatinine (SCR), blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) determination were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies against p-AKT (Ser473, #12694), Skp2 (#2652), p27 (#3688), E-cadherin (#14472), and GAPDH (#2118) were obtained from Cell Signaling Technology (Beverly, MA, USA); antibody against Flag (F1804) was purchased from Sigma; All secondary antibodies (HRP-conjugated anti-mouse IgG and anti-rabbit IgG) were purchased from Cell Signaling Technology.

2.2 Cell culture

The human lung cancer cell lines A549 and H1299, the mouse Lewis lung carcinoma cell line (LLC), and human normal bronchial epithelial cell line Beas-2B were purchased from the ATCC (VA, USA). The A549, LLC, and Beas-2B cells were cultured in DMEM medium and H1299 cells were cultured in RPMI-1640 medium (Gibco, USA), supplemented with 10%

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FBS (Gibco, USA), penicillin (100IU/mL), and streptomycin (100μg/mL) in a humidified incubator containing 5% CO_2 at 37℃.

2.3 Cell viability and colony formation assay

H1299 cells (3000 cells/well), LLC cells (4000 cells/well), Beas-2B cells (3000 cells/well) were plated in 96-well plates and treated with SB for 24h or 48h. The cells were incubated with MTT (0.5 mg/ml) for another 3 h. Then the absorbance at 490 nm was measured after the MTT fromazan crystal was dissolved. For cell colonies formation assay, H1299 and A549 cells (500 cells/well) were seeded in 6-well plates, treated with SB for 7 days, and stained with crystal violet, then the cell colonies over 50 cells was counted.

2.4 Cell cycle analysis

H1299 and A549 cells were treated with SB for 24h. Then the cells were harvested and washed twice with ice-cold PBS, fixed in 70% ethanol at -20℃ overnight. Subsequently, the cells were washed with PBS and stained with PI at room temperature according to the protocol of Cell-cycle Detection kit (Meilunbio, China). The cell cycle distribution was determined by using a flow cytometry (BD Bioscience, USA) and ModFit LT 5.0 software.

2.5 Cellular thermal shift assay (CETSA)

CETSA was performed to examine whether SB directly binds to the target protein of interest in cells [15]. Briefly, H1299 cells were treated with DMSO or 100 μM SB for 3h, and the cells were harvested and resuspended in PBS. Then, the cell suspension was aliquoted into seven PCR tubes and heated to indicated temperatures (42, 46, 50, 54, 58 and 62 ◦C) for 3 min using a PCR instrument. Subsequently, the cells were frozen 3 times in liquid nitrogen

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and centrifuged at 10000rpm for 10 minutes; the supernatants were collected for Western blotting.

2.6 Western blotting

NSCLC cells were treated with SB for 12 h or 24 h, the whole cell proteins were harvested using the RIPA lysis buffer (Beyotime). Then, the lysates were subjected to SDS-PAGE and total proteins were transferred to PVDF membranes. The membranes were incubated with indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The signal of indicated protein bands was detected by ChemiDoc XRS system with Quantity One software (Bio-Rad, CA, USA); the densitometry of protein bands was measured by using ImageJ software (NIH, USA). Each experiment was performed in triplicate.

2.7 Molecular docking

Molecular docking simulation was performed as we previously described [6]. In brief, the 3-dimensional structure of SB was downloaded from Pubchem; The crystal structure of Skp2-Skp1 complex (Protein Data Bank ID: 2AST) and SB were prepared using AutoDock Tools and OpenBabel, respectively. Molecular docking was performed using Autodock Vina.

2.8 Animal experiments

C57/BL6 mice (5-6 weeks) were purchased from the Laboratory Animal services Center, Guangzhou University of Chinese Medicine. All experiments in this study were approved by and in accordance with the guidelines of the Animal Ethics Committee of Guangzhou University of Chinese Medicine. For orthotopic xenografts of lung cancer cells, the mice were injected with LLC cells (1.5×10^6) via the tail vein. After 7 days of cell injection, the mice

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were randomly divided into 4 groups with 10 mice each: vehicle solvent (10% DMSO, 70% cremophor EL/ethanol (3:1), and 20% saline, i.p.), SB 200 mg/kg (daily, i.p.), SB 400 mg/kg (daily, i.p.), cisplatin 2 mg/kg (once every other day, i.p.). SB was dissolved in vehicle solvent and cisplatin was dissolved in saline. As the majority of mice will die within 45 days since the day of tumor xenograft, the survival time of the mice was tested for 45 days. The mice were considered dead when they appeared moribund and then were euthanized. At the end of the experiment, all mice survived were sacrificed using CO_2 and dissected to count the number of lung tumor nodules. Differences in the distribution of survival percentages per treatment group were analyzed using the Kaplan–Meier method with the log-rank test.

2.9 Drug side effects, animal tissue preparation, hematoxylin-eosin (H&E) staining, and Immunohistochemistry (IHC)

For the drug side effects examination, the body weight of each mouse was recorded from day 8 to day 29 since no mice died before day 29. Then, the serum was harvested from these mice to measure SCR, BUN, AST, and ALT levels by using colorimetric assay according to the manufacturer's instructions. For Western blotting, the tumor nodules in the lung were dissected from 6 mice of each group and homogenized to obtain total protein extract using RIPA lysis buffer. For H&E staining, the lung tissues were fixed in 4% paraformaldehyde, dehydrated in a graded ethanol series (70%, 85%, 95%, and 100%), incubated in xylene, and embedded in paraffin. The tissues were sectioned at 4 μm thickness and stained with H&E for histological examination. IHC experiments were carried out using DAB IHC kit (Solarbio, China), followed by deparaffinization, antigen retrieval, and Skp2 antibody incubation.

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2.10 Statistical analysis

Data processing and plots were performed using GraphPad Prism 7.0 and SPSS software 17.0. All Data are presented as mean ± standard error of mean (SEM) from at least three independent experiments. Unpaired Student's t tests and Two-way analysis of variance (ANOVA) were used to determine significant differences. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

3. RESULTS

3.1 SB inhibits the proliferation of NSCLC cells by inducing cell-cycle arrest

We first examined the cytotoxicity of SB against NSCLC and Beas-2B cells. The results showed that SB dose-dependently inhibited the proliferation of A549, H1299, and LLC cells (**Figure 1A-C**); however, the drug did not show any cytotoxicity against Beas-2B cells (**Figure 1D**), which is consistent with previous evidence that SB is a safe drug without any toxicity [16]. SB also significantly inhibited colonies formation of H1299 and A549 cells (**Figure 1E, F**). As SB at appropriate concentrations (≦150 μmol/L) did not induce obvious cell death in NSCLC cells within 48 h, we examined whether the drug exhibits anti-NSCLC effects by inducing cell-cycle arrest. Similar to previous studies, we found that SB markedly induces cell-cycle G1 phase arrest in NSCLC cells (**Figure 1G, H**). As expected, SB cannot induce obvious cell-cycle arrest in Beas-2B cells (**Figure 1I**). Furthermore, we also tested the inhibitory effects of SB on cell migration of NSCLC. Interestingly, SB was also able to suppress the migration and invasion of NSCLC cells **(Figure 2A-C)**. These results revealed that SB exhibits anticancer activities by inducing G1 phase arrest and suppressing cell migration.

3.2 SB potentially inhibits Skp2/P27 pathway in NSCLC

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Considerable evidence has shown that Skp2-SCF complex plays an important role in cell cycle progression and migration by regulating the expression of its substrates such as p27 and E-cadherin. Notably, our results showed that SB markedly increases the protein level of p27 in a dose-dependent manner, accompanied by the reduction of Skp2 protein expression in NSCLC cells **(Figure 3A-C)**. Moreover, SB also obviously induced the accumulation of E-cadherin, another substrate of Skp2 **(Figure 3D)**. To examine whether SB serves as a potential Skp2 inhibitor, we further employed a potent specific Skp2 inhibitor SZL P1-41 as a reference compound [5]. Our results showed that both SB and SZL P1-41 can markedly induce the accumulation of p27, and SB exerted a more profound inhibitory effect on Skp2 expression than SZL P1-41 in NSCLC cells, demonstrating that SB may target Skp2 to inhibit proliferation and migration of NSCLC cells.

3.3 SB acts as a Skp2 inhibitor

Accumulating evidence demonstrates that Skp2 is a valuable therapeutic target in various cancers [17, 18]. We also evaluated the clinical significance of Skp2 in NSCLC by using online databases. The GEPIA database displayed that the expression of Skp2 in lung squamous cell carcinoma (LUSC) tumor tissues is significantly higher than that in normal tissues (**Figure 4A and Figure S1**). Skp2 also had a higher expression level in NSCLC cells than that in normal bronchial epithelial cells (**Figure S2**) Moreover, Skp2 expression was negatively associated with NSCLC patients' prognosis by using Kaplan-Meier plotter database (kmplot, Affymetrix ID: 203625_s_at) (**Figure 4B**). Therefore, Skp2 may serve as a rationale target for lung cancer treatment.

As Skp2 inhibitors can reduce Skp2 protein stability by disrupting the integrity of SCF complex, we examined whether SB serves as a Skp2 inhibitor. We first performed co-IP

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experiment and found that SB could obviously prevent Skp1-Skp2 protein interaction (**Figure 4C**). As expected, SB significantly decreased protein stability of Skp2 upon CHX co-treatment (**Figure 4D**). More importantly, CETSA showed that SB significantly increased the thermal stability of Skp2 protein at indicated temperatures, suggesting that SB may have a direct binding to Skp2 protein (**Figure 4E**). To predict the binding sites of Silybin in Skp2, we further adopted molecular docking based on the crystal structure of Skp2-SCF complex. SB was predicted to form four H-bonds with Pro115, Tyr128, Ser132, and Lys145 in proximity to the C terminus and the first leucine-rich repeat of Skp2, with a binding affinity energy value of -6.8 kcal/mol (**Figure 4F**), further proving the binding potential of SB with Skp2. Since Skp2 is required for epidermal growth factor (EGF)-induced Akt phosphorylation [5], we examined the effects of SB on the signaling pathway downstream of Skp2. EGF was able to stimulate Akt phosphorylation with 30 min in A549 cells, whereas SB markedly attenuated the process (**Figure 4G**). Therefore, these results demonstrated that SB may act as a potent Skp2 inhibitor.

3.4 SB has potent antitumor activity without toxicity

To assess the therapeutic potential of SB in NSCLC, we utilized high metastatic LLC cells to establish orthotopic xenografts. The mouse model can quickly develop visible tumors in the lung tissue, which lead to the mortality of the mice probably within 40 days. Our results showed that LLC cells can form multiple large tumor nodules across the entire lung tissue; however, SB and cisplatin administration significantly decreased the number and size of tumor nodules in the lung **(Figure 5A-C)**. Consistently, nine out of ten mice in the control group died within 40 days, whereas 60% of mice in the high dose SB group survived after 45 days **(Figure 5D)**. Cisplatin treatment also significantly prolonged the survival of the mice **(Figure 5D)**. As expected, SB markedly attenuated Skp2 expression while increased P27 expression in the tumor tissues **(Figure 5E)**. IHC analysis also showed that Skp2 expression in tumor tissue was markedly reduced upon SB administration **(Figure 5F)**, suggesting that SB may also exert anticancer activity via targeting Skp2 *in vivo*.

We further evaluated the safety of SB for in *vivo* treatment. SB administration did not affect

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body weight of the mice **(Figure 6A)**. Furthermore, SB had no obvious side effects toward liver and kidney, evidenced by normal histological features of the liver and kidney tissues by H&E staining and the normal serum levels of SCR, BUN, AST, and ALT **(Figure 6B-E)**. However, CDDP moderately induced body weight loss **(Figure 6A)**, caused renal tubular dilatation and hepatocellular enlargement **(Figure 6B, D)**, and increased serum levels of SCR, BUN, and AST **(Figure 6C, E)**. These results demonstrated that SB exerts promising anti-NSCLC activity without inducing obvious side effects.

4. DISSCUSION

Oncogenic E3 ligase, especially Skp2-SCF complex, has been widely accepted as promising therapeutic target in various cancer types [18]. Here, we demonstrated that SB may directly target Skp2 and inhibit Skp2-SCF E3 ligase in NSCLC cells, leading to p27 protein upregulation and cell-cycle arrest, thereby providing novel mechanisms of action of SB in cancer treatment.

Skp2-SCF E3 ligase mediates the proteolytic ubiquitination of a variety of substrates such as p27, p21, E-cadherin, and FOXO1, and also the non-proteolytic ubiquitination of AKT, LKB1, and Twist [19]. The oncogenic function of Skp2-SCF E3 ligase has attracted great attention to explore novel Skp2 inhibitors. Skp2 interacts with Skp1 by means of the F-box motif and also binds its substrates via the variable protein-protein interaction domain [4]. Therefore, two types of Skp2 inhibitors have been identified based on the structure of Skp2-SCF E3 ligase: Skp2 inhibitors such as compound C1/2 can specifically attenuate the interaction between p27 and Skp2/Cks1 complex [20]; the second type of Skp2 inhibitors such as compound #25 can directly bind to residues around the F-box motif and inhibit Skp1 and Skp2 interaction [5]. Therefore, the second type of Skp2 inhibitors that disrupt SCF

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complex integrity can completely inhibit SCF E3 ligase, thus exerting profound anticancer activities by inhibiting cell proliferation, migration, glycolysis, and even stemness, presenting a promising anticancer strategy. Herein, we found that SB can bind to Skp2 and disrupt Skp1-Skp2 interaction, leading to the accumulation of p27 and E-cadherin proteins, suggesting that SB may bind to the F-box motif of Skp2. However, the potential binding residues of BA in Skp2 needs further investigation. Further, our results showed that SB significantly decreases the protein level of Skp2 in LLC xenografts, inhibiting the growth and metastasis of NSCLC *in vivo*. These results demonstrate that SB displays a favorable antiproliferative and antimetastatic potential via targeting the Skp2-SCF E3 ligase. However, SB may target several proteins besides Skp2 as we found that Skp2 expression has a weak association with SB sensitivity in NSCLC cells (R^2 = 0.33, Figure S3). Whether SB suppresses cancer cell proliferation by targeting other proteins worth further investigation.

As a relative safe drug in humans, silymarin is well tolerated at a therapeutic dose of 700 mg, three times a day for 24 weeks [16]. Therefore, silymarin has been used as hepatoprotective medicine and food supplement [21]. SB, a major constituent of silymarin, is also non-cytotoxic and non-genotoxic at concentration of 100 μM [16]. In our study, we also demonstrated that SB at concentrations of 25-200 μM did not cause cytotoxicity toward bronchial epithelial cell Beas-2B. In contrast, SB increased cell viability of Beas-2B at concentrations around 100 μM. Moreover, SB did not show obvious side effects when the mice were treated with SB at a high dose of 400 mg/kg for one month. To date, multiple clinical studies have been performed to test the effects of SB on liver and other diseases [22,

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23], novel therapeutic strategies that enhance the water solubility and bioavailability of SB can promote its clinical application [21].

In conclusion, we demonstrated that pharmacological Skp2 inhibition by SB is able to stabilize p27 and E-cadherin expression and inhibit EGF-mediated Akt activation, thereby inhibiting cell growth and metastasis of NSCLC. Our study uncovered the novel anticancer mechanisms of SB, further supporting its safety and therapeutic potential in human cancers.

DECLARATION OF COMPETING INTEREST

The authors declare no conflict of interest.

ACKNOWLEGEDMENT

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Author Contributions:

SBZ, MH, YY, and YQL designed the research, SBZ, XYS, DH, HDH, and YFC performed the experiments, SBZ, MH, XYS, and DH analyzed the data, SBZ and YQL wrote the manuscript, MH and YY reviewed the manuscript. All authors have read and approved the final submitted manuscript.

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FIGURE LEGENDS

Figure 1. SB inhibits proliferation and causes cycle arrest in NSCLC cells. (A-D) MTT assay was conducted in A549, H1299, LLC, and Beas-2B cells after the treatment of SB for 24 h and 48 h. (E-F) H1299 and A549 cells were treated with SB for seven days and cell colonies were counted. (G-I) H1299, A549, and Beas-2B cells were treated with SB for 24 h and cell-cycle distribution was determined by flow cytometry. The results are expressed as

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the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control.

Figure 2. SB inhibits migration of NSCLC cells. (A, B) Wound healing assay was conducted in H1299, A549, and LLC cells after the treatment of SB for 24 h, cells were photographed by light microscopy and relative migration rate was quantified by Image J software. (C) Transwell assay of NSCLC cells treated with SB; cells were counted to calculate relative invasion rate. All data are expressed as the means ± SEM from three independent experiments; *P < 0.05, **P < 0.01, and ***P < 0.001 versus control.

Figure 3. SB potentially targets Skp2/P27 pathway in NSCLC. (A-C) The protein levels of Skp2 and p27 were detected by Western blotting in H1299 (A), A549 (B), and LLC (C) cells after the treatment of SB for 12h and 24h. (D) The protein levels of E-cadherin were detected by Western blotting in A549 cells after the treatment of SB for 12h and 24h. (E, F) The protein levels of Skp2 and p27 were detected in A549 and H1299 cells by Western blotting after the treatment of SB and SZL (SZL P1-41) for 24h. The relative protein levels were determined by densitometry analysis from three independent experiments. The results are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control group.

Figure 4. Skp2 is a potential target of SB. (A) The gene expression of *Skp2* in LUSC or LUAD and adjacent normal tissues from GEPIA database is shown. (B) The association between Skp2 expression level and the prognosis of NSCLC patients is determined by kmplot database. (C) A549 was transiently transfected with Flag-Skp1 and Co-IP experiment was

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performed to examine the effect of SB on Skp2-Skp1 interaction. (D) H1299 cells were treated with CHX in the presence or absence of SB for indicated time points and Skp2 protein level was detected by Western blotting. (E) Cellular thermal shift assay of Skp2 was conducted by Western blotting. (F) Molecular docking was carried out to predict the binding site of SB in Skp2 protein, Skp2 and SB were shown as cartoon and sticks. (G) A549 cells were serum-starved in the absence or presence of SB for 24 h, stimulated with or without EGF, and harvested for Western blotting. LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; T, tumor tissue; N, normal tissue. All data are expressed as the means ± SEM from three independent experiments; *$P < 0.05$ versus control.

Figure 5. SB suppresses tumor growth and metastasis *in vivo*. (A-D) LLC cells were intravenously injected into the mice. At the end of the experiments, representative pictures of lung metastases were shown (A); the number of metastasis nodules in the lung tissue was counted (B); H&E staining of lung tissue sections were photographed and shown (C); overall survival analysis of the mice were shown (D). (E) The expression of Skp2 and p27 in mice lung tumor tissues were detected by Western blotting. (F) The expression of Skp2 in lung tumor tissues were detected by IHC assay. Black arrows: metastasis nodules in the lung tissue. CP, cisplatin. Bar, 50 μm. The results are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control group.

Figure 6. SB has a good safety profile *in vivo*. (A) Body weight of the mice was recorded every three days after the treatment of vehicle or SB. (B-E) At the end of the experiments, H&E staining of the kidney (B) or liver (D) tissue were performed and representative pictures

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were shown; the serum levels of BUN, SCR, ALT, and AST were measured (C, E). CP, cisplatin. The results are expressed as the mean ± SEM. ***$P < 0.001$ versus control group.