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Article

RETRACTED: Schisandrin B Induces Apoptosis and Cell Cycle Arrest of Gallbladder Cancer Cells

Shan-Shan Xiang [1,2,3,†], Xu-An Wang [1,2,3,†], Huai-Feng Li [1,2,3,†], Yi-Jun Shu [1,2,3], Run-Fa Bao [1,2,3], Fei Zhang [1,2,3], Yang Cao [1,2,3], Yuan-Yuan Ye [1,2,3], Hao Weng [1,2,3], Wen-Guang Wu [1,2,3], Jia-Sheng Mu [1,2,3], Xiang-Song Wu [1,2,3], Mao-Lan Li [1,2,3], Yun-Ping Hu [1,2,3], Lin Jiang [1,2,3], Zhu-Jun Tan [1,2,3], Wei Lu [1,2,3], Feng Liu [4,*] and Ying-Bin Liu [1,2,3,*]

1 Department of General Surgery, Xinhua Hospital, Affiliated to Shanghai Jiao Tong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China

2 Laboratory of General Surgery, Xinhua Hospital, Affiliated to Shanghai Jiao Tong University School of Medicine, No. 1665 Kongjiang Road, Shanghai 200092, China

3 Institute of Biliary Tract Disease, Shanghai Jiao Tong University School of Medicine, No. 227 South Chongqing Road, Shanghai 200025, China

4 The First Affiliated Hospital Nanchang University Emergency Unit, No. 17 Yongwai Road, Nanchang 330006, China

† These authors contributed equally to this work.

* Authors to whom correspondence should be addressed; E-Mail: washar1717@163.com (F.L.); liuybphd@126.com (Y.-B.L.); Tel.: +86-21-2507-7880.

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Abstract: Gallbladder cancer, with high aggressivity and extremely poor prognosis, is the most common malignancy of the bile duct. The main objective of the paper was to investigate the effects of schisandrin B (Sch B) on gallbladder cancer cells and identify the mechanisms underlying its potential anticancer effects. We showed that Sch B inhibited the viability and proliferation of human gallbladder cancer cells in a dose-, time -dependent manner through MTT and colony formation assays, and decrease mitochondrial membrane potential (ΔΨm) at a dose-dependent manner through flow cytometry. Flow cytometry assays also revealed G0/G1 phase arrest and apoptosis in GBC-SD and NOZ cells. Western

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blot analysis of Sch B-treated cells revealed the upregulation of Bax, cleaved caspase-9, cleaved caspase-3, cleaved PARP and downregulation of Bcl-2, NF-κB, cyclin D1 and CDK-4. Moreover, this drug also inhibited the tumor growth in nude mice carrying subcutaneous NOZ tumor xenografts. These data demonstrated that Sch B induced apoptosis in gallbladder cancer cells by regulating apoptosis-related protein expression, and suggests that Sch B may be a promising drug for the treatment of gallbladder cancer.

Keywords: schisandrin B; gallbladder cancer; apoptosis; cell cycle arrest; mitochondrial pathway

1. Introduction

Gallbladder cancer, the most common malignancy of the bile duct, is an extremely aggressive, lethal neoplasm [1–6]. Due to the absence of specific symptoms and signs, most patients are detected at an advanced stage [7]. Curative resection remains the only effective treatment for gallbladder cancer, however, the majority of the patients will have frequent recurrences after surgery. For unresectable and recurrence patients, chemotherapy or radiotherapy will be the only treatment, but unfortunately, it does not have satisfactory results [8,9]. As a result, gallbladder carcinoma is associated with a very poor prognosis, and the overall survival rate for this malignancy ranges from 5.2 to 24.4 months [10–12]. Therefore, novel effective drugs are urgently needed in order to improve the outcome of patients with advanced gallbladder cancer.

Sch B (Figure 1), isolated from the fruit of *Schisandra chinensis*, is used in traditional Chinese medicine for the treatment of inflammation-related conditions [13], such as hepatitis and osteomyelitis, gynecological diseases (due to its antibacterial activity) and myocardial disorders [14]. Besides, Sch B has been shown to possess multiple functions against cancer [15–20], and previous studies have reported that Sch B can induce apoptosis in gastric cells with very low toxicity to normal cells, attenuate cancer invasion and metastasis [21,22], and also enhance doxorubicin-induced apoptosis in cancer cells through activation of the mitochondrial apoptotic pathway [23]. However, the effect of Sch B on gallbladder cancer cells and the underlying mechanisms have not been reported previously. In this study, we investigated the anti-neoplastic activity of Sch B in gallbladder cancer cell lines (including GBC-SD and NOZ cell lines) *in vitro* and *in vivo*, and explored the possible molecular mechanisms underlying this action, which could provide experimental evidence for the potential application of Sch B as a new natural anti-tumor medicine for gallbladder cancer.

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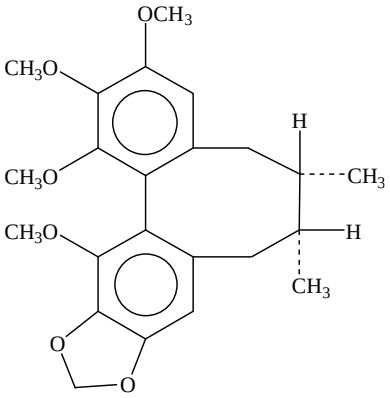
Figure 1. The chemical structure of Sch B.



2. Results and Discussion

2.1. Sch B Inhibits the Proliferation and Viability of Gallbladder Cancer Cells

To test the effect of Sch B on the proliferation of gallbladder cancer cells, GBC-SD and NOZ cells were treated with various concentrations (0, 20, 40, 60, 80 and 100 μmol/L for both cells) of Sch B for 24, 48 and 72 h, their A570 values were measured by a MTT assay. Sch B exhibited a potent cytotoxic effect on GBC-SD and NOZ cells in a dose- and time- dependent manner (Figure 2A). The IC_{50} values (the concentration of drug inhibiting 50% of the cells) of GBC-SD and NOZ cells at 48 h were around 40 μmol/L and 50 μmol/L, respectively. According to the curve, we choose 30, 60, and 90 μmol/L as the optimum concentration range for both cells in the following experiments. The ability of GBC-SD and NOZ cells to form colonies in the presence of Sch B was assessed by the flat plate colony formation assay (Figure 2B). The colony count indicated that Sch B induced a dose-dependent decrease in the colony formation ability. Moreover, statistical analysis demonstrated that the mean sizes of the control colonies were larger than those of the Sch B-treated groups (Figure 2C). The findings indicate that Sch B may exert a significant influence on GBC-SD and NOZ cell viability and proliferation. In Figure 2A, we found that the effects at 48 h were more obvious than at 24 h and more stable than the groups at 72 h, so the groups at 48 h were chosen to detect changes in molecular events during the subsequent experiments.

2.2. Sch B Induces Apoptosis in Gallbladder Cancer Cells

To further confirm whether Sch B induces apoptosis in GBC-SD and NOZ cells, we evaluated the effects of Sch B on GBC-SD and NOZ cells by using annexin V-FITC and propidium iodide double staining. In normal live cells, phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external environment. Annexin V-FITC binds to exposed PS on apoptotic and necrotic cells, and propidium iodide (PI) nucleic acid dye gains entry into late apoptotic cells and necrotic cells but not into early apoptotic cells and living cells. In the scatter plot of double variable flow cytometry, Q3 quadrant (FITC−/PI−) shows living cells; whereas Q2 quadrant (FITC+/PI+) and Q4 quadrant (FITC+/PI−) represents late and early apoptotic cells. As assessed by flow cytometry and shown in Figure 3A, Sch B reduced the number of surviving cells and increased

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the number of both early (4.8% ± 1.37%, 10.9% ± 2.13% and 20.3% ± 3.41% *vs*. 1.8% ± 0.22% in the control group in GBC-SD cells, $p < 0.05$; 5.8% ± 1.62%, 7.3% ± 1.91% and 16.5% ± 1.71% *vs*. 0.9% ± 0.21% in the control group in NOZ cells, $p < 0.05$) and late apoptotic cells in a dose-dependent manner (Figure 3B). It indicated that apoptotic pathway played an important role in the proliferation inhibition of Sch B on GBC-SD and NOZ cells.

Figure 2. Sch B inhibits the proliferation and viability of gallbladder cancer cells. (**A**) GBC-SD and NOZ cells were treated with Sch B (0, 30, 60 and 90 μmol/L) for 24, 48 and 72 h. Cell viability and IC50 were determined by MTT assay. (**B**) GBC-SD and NOZ cells were treated with various concentration of Sch B (0, 3, 6 and 9 μmol/L) and were allowed to form colonies in fresh medium for 14 days. The photomicrographic differences and (**C**) influence of colonies (mean ± SD, n = 3) on colony formation are shown. All data were from three independent experiments. ***** $P < 0.05$, ****** $P < 0.01$ *vs*. the control group.

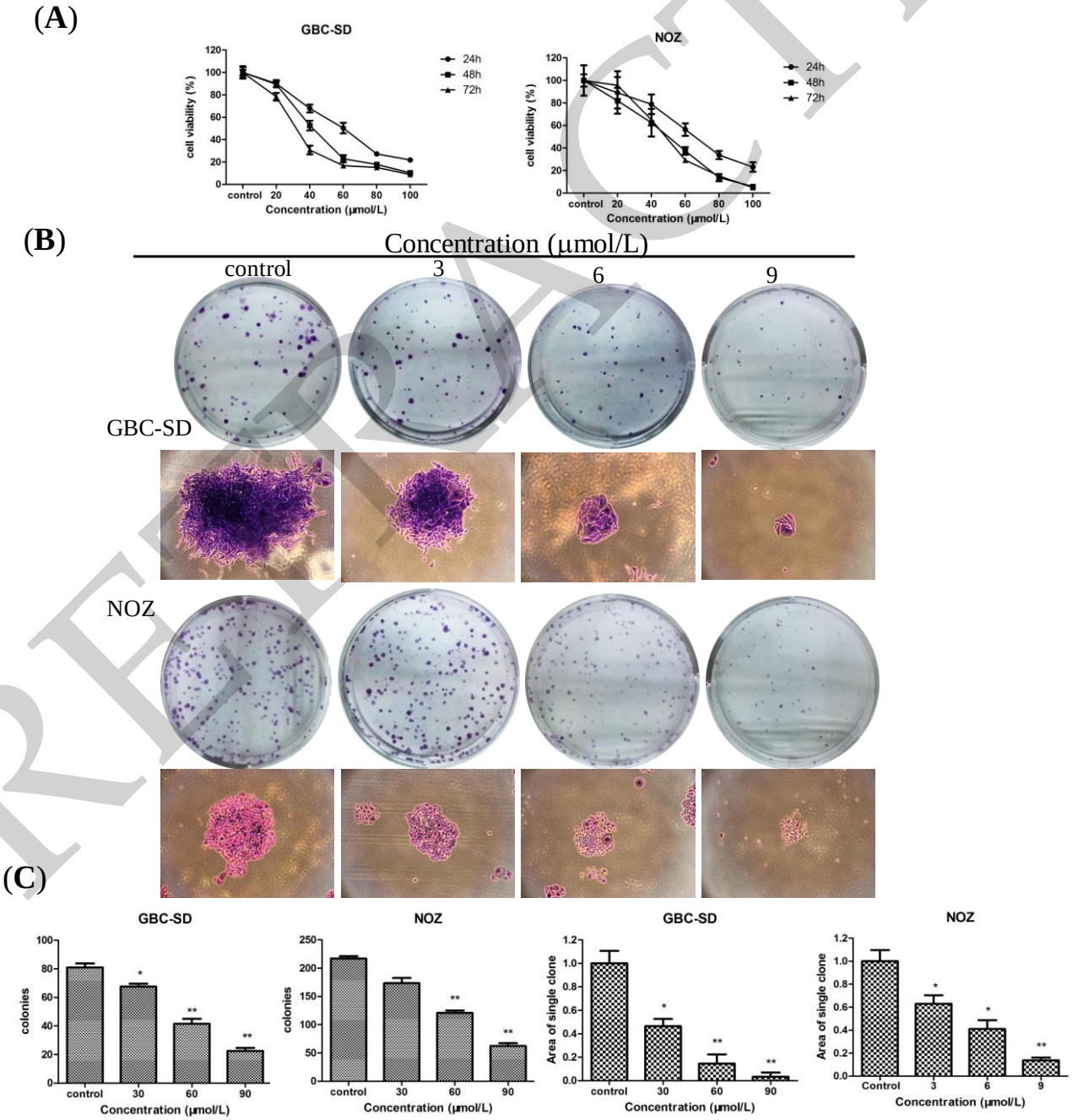

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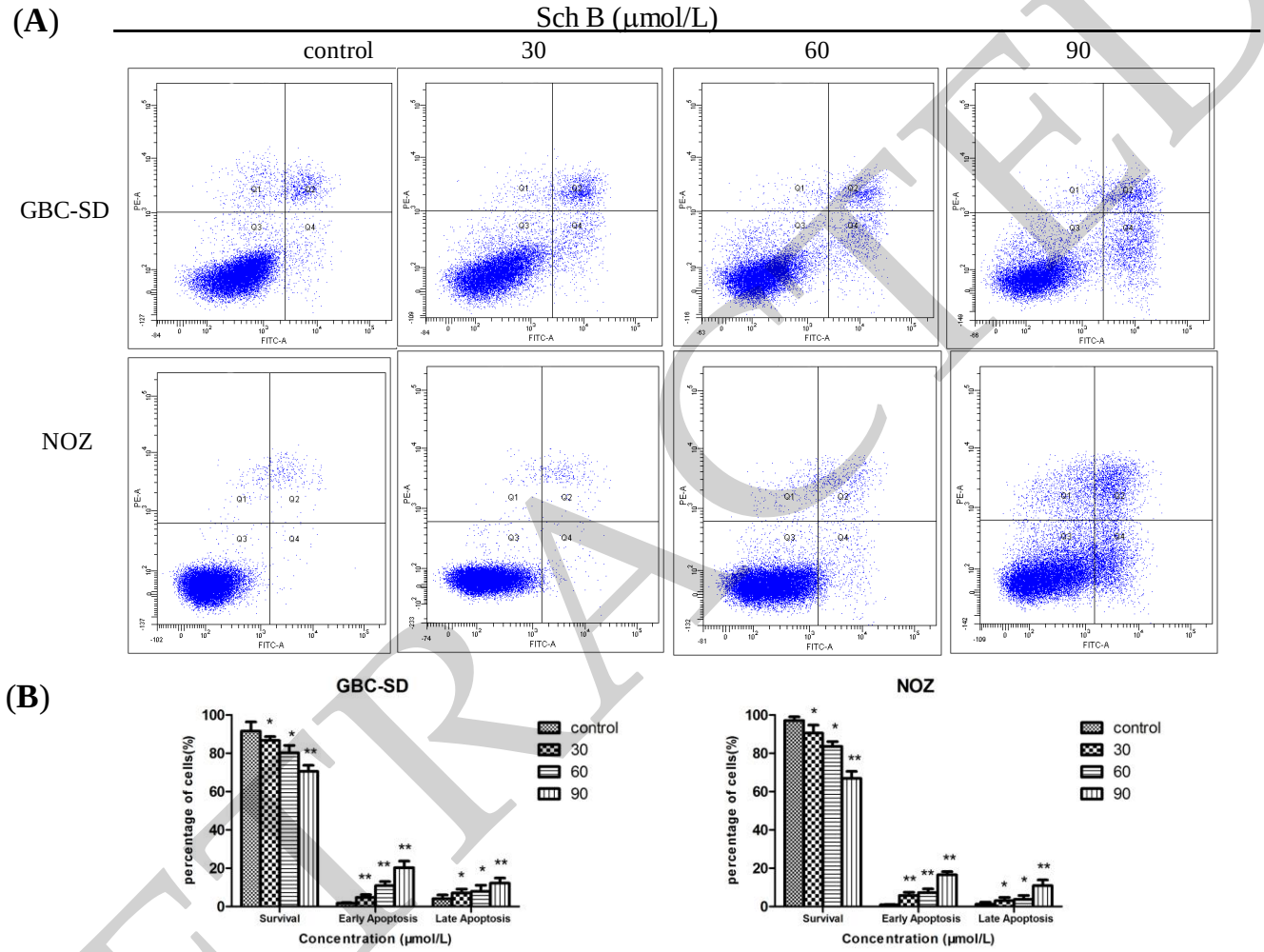
Figure 3. Sch B induces apoptosis in gallbladder cancer cells. (**A**) GBC-SD and NOZ cells were treated with Sch B (0, 30, 60, and 90 μmol/L) for 48 h. Sch B-treated GBC-SD and NOZ cells were stained with annexin V-FITC/PI and analyzed by flow cytometry. (**B**) The percentage of apoptotic cells is presented as the mean ± SD (n = 3); Results shown were representative data from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ *vs.* the control group.



2.3. Sch B Decreases Mitochondrial Membrane Potential (ΔΨm) in Gallbladder Cancer Cells

Mitochondria play an important role in the regulation of apoptosis, and apoptosis mediated by the mitochondrial pathway is often associated with the decrease of ΔΨm. After treatment of Sch B for 48 h, the ΔΨm changes of GBC-SD and NOZ cells were investigated by staining with Rhodamine 123 [24], and the staining was detected by flow cytometry. The decreased intensity of Rhodamine 123 fluorescent staining reflected the loss of the ΔΨm. As shown in Figure 4A,B, compared with the control group, Sch B treatment induced a dose-dependent reduction in ΔΨm.

The two major pathways involved in the initiation of apoptosis include the mitochondria-mediated intrinsic pathway and the death receptor induced extrinsic pathway [25]. In the experiment, a decrease in ΔΨm was detected in both gallbladder cancer cell lines treated with Sch B, which indicates that Sch B promotes apoptosis of gallbladder cancer cells through the mitochondrial-dependent apoptotic pathway.

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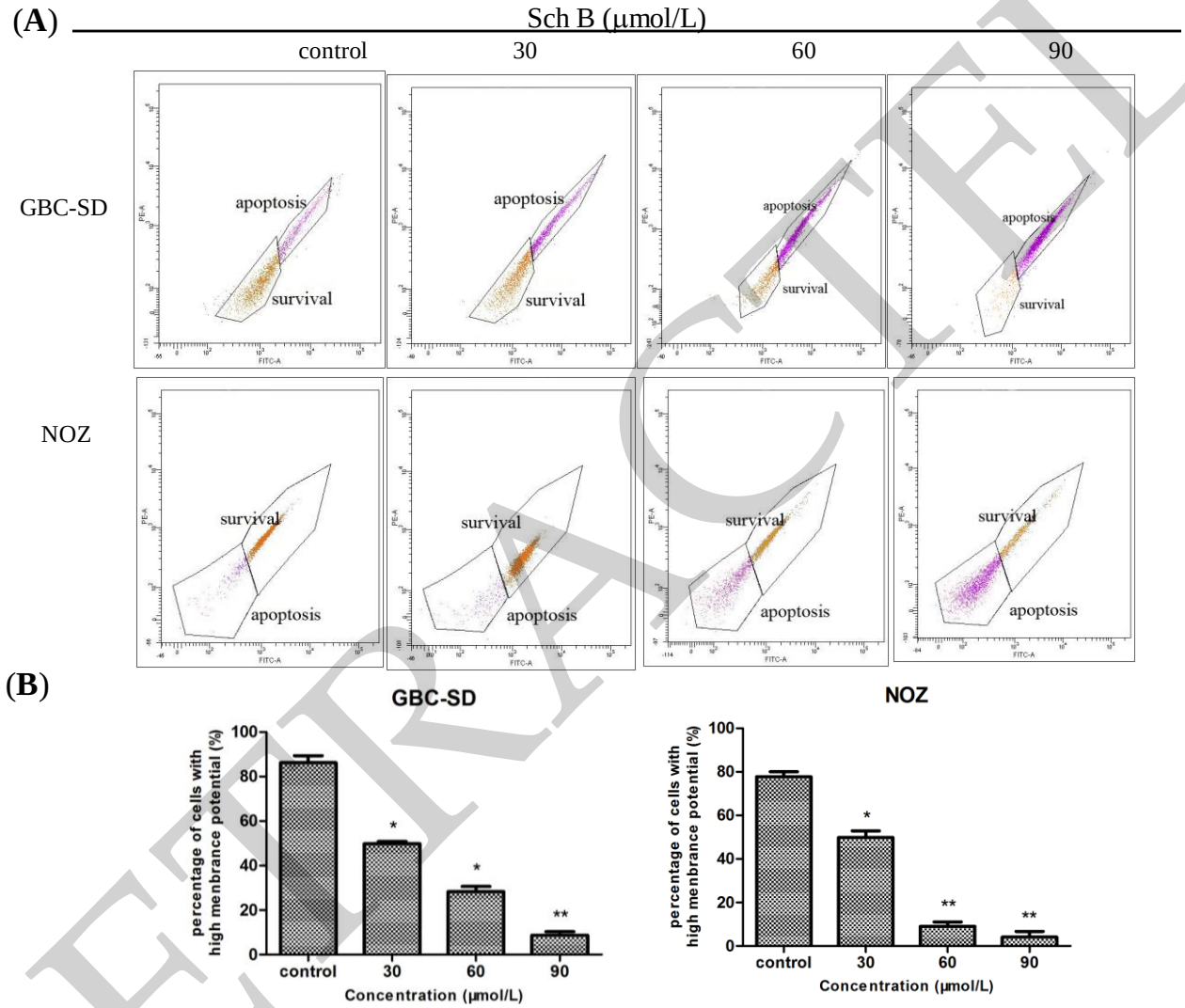
Figure 4. Sch B decreases mitochondrial membrane potential (ΔΨm) in gallbladder cancer cells. (**A**) GBC-SD and NOZ cells were treated with Sch B (0, 30, 60, and 90 μmol/L) for 48 h. Rhodamine retention was measured by flow cytometry. (**B**) The corresponding histogram shows the percentages of survival cells (mean ± SD, n = 3); The results shown were representative data from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ *vs*. the control group.



2.4. Sch B-Induced Apoptosis via Regulation of Caspase and Bcl-2 Family Members in Gallbladder Cancer Cells

It is well known that proteins in the caspase family, Bcl-2 family, NF-κB, and PARP play critical roles in the apoptotic process [26–29]. To investigate the underlying molecular mechanism of Sch B-induced apoptosis on GBC-SD and NOZ cells, the expression of apoptosis-related proteins were evaluated by western blot analysis after treatment of cells with various concentrations of Sch B for 48 h. As shown in Figure 5A, Sch B increased the amount of Bax, cleaved caspase-9, cleaved caspase-3 and cleaved PARP but decreased the amount of Bcl-2 and NF-κB in dose-dependent manner. Furthermore, we found that in Sch B-treated groups, the Bcl-2 (antiapoptoic) to Bax (proapoptotic) ratio was significantly lower than in the control group (Figure 5B).

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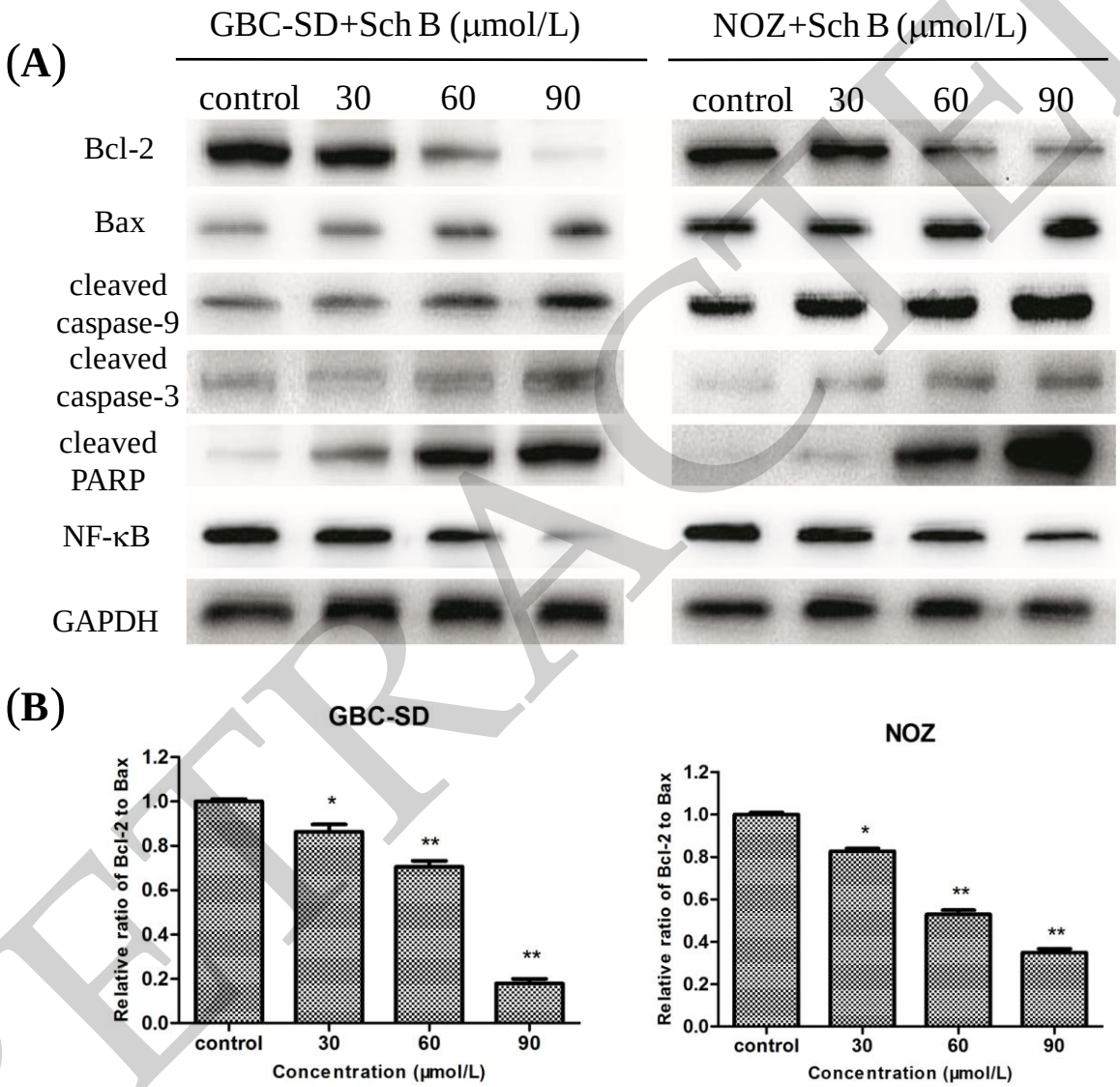
Figure 5. Sch B-induced apoptosis via regulation of Caspase and Bcl-2 family members in gallbladder cancer cells. (**A**) GBC-SD and NOZ cells were treated with Sch B (0, 30, 60, and 90 μmol/L) for 48 h. The expression levels of Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3, cleaved PARP and NF-κB were detected by western blot analysis, and GAPDH was used as a loading control; (**B**) The data of the relative ratio of Bcl-2 to Bax (mean ± SD); The results shown were representative data from 3 independent experiments. ***** $P < 0.05$, ****** $P < 0.01$ *vs.* the control group.



Apoptosis is a programmed process that is responsible for the deletion of cells in normal tissues, and decreased apoptosis is strongly associated with the beginning and progress of cancers, thus, the induction of apoptosis has been proposed as an important strategy to treat cancers [30,31].

This study evaluated potential mechanisms for Sch B induced apoptosis, expression of cell apoptosis associated proteins were measured. The caspase family consists of cysteine proteases that are indispensable in the execution process of apoptosis, caspases-3 is a key regulator and caspase-9 is activated in the mitochondria-mediated intrinsic apoptosis pathway. In this experiment, cleaved capase-3 and cleaved caspase-9 was up-regulated with the cleavage of PARP increased accordingly.

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These data showed that Sch B could activate caspase-3 and 9 in gallbladder cancer cells, then induce the inactivation of many key proteases in the cytoplasm, cell nucleus, and cytoskeleton, and finally cause the apoptosis of cancer cells.

The Bcl-2 gene family is one of the best studied anti-apoptosis genes, and according to the members' different biological effects, the apoptosis-promoting protein Bax and the anti-apoptotic protein Bcl-2 play an important role in regulating cell apoptosis [32,33]. In our experiment, increased expression of Bax, decreased expression of Bcl-2 and the decrease in the Bcl-2/Bax ratio is correlated with the apoptosis induced in human gallbladder cancer cells by Sch B.

NF-κB is a pro-survival transcription factor which controls the inflammatory and immune response as well as other genetic programs that are central to cell proliferation and cell survival, and also decrease the sensitivity of cancer cells to apoptosis. NF-κB inhibits apoptosis by inhibiting Bcl-2 members and inhibitors of apoptosis. In this study, inhibition of NF-κB nuclear translocation together with the down-regulation of its target Bcl-2 family suggested that activation of NF-κB was inhibited by Sch B during tumor progression.

2.5. Sch B Induces G0/G1 Phase Arrest and Regulates the Expression of Cell Cycle-Related Proteins of Gallbladder Cancer Cells

To investigate whether Sch B affects cell cycle progression, cell cycle distribution was analyzed by flow cytometry. The results showed that Sch B significantly arrested cell cycle progression in GBC-SD and NOZ cells (Figure 6A) by increasing the percentage of cells in the G0/G1 phase (50.65% ± 5.84%, 57.90% ± 5.57% and 73.16% ± 5.28% *vs*. 46.72% ± 4.23% in the control group in GBC-SD cells, $p < 0.05$; 49.80% ± 5.22%, 52.53% ± 5.28% and 60.48 ± 3.89% *vs*. 42.13% ± 2.43% in the control group in NOZ cells, $p < 0.05$, Figure 6B). In addition, the results for the sub-G1 group (blue color) indicated that Sch B induced apoptosis of GBC-SD and NOZ cells. We also evaluated the levels of cycle-related proteins cyclin D1 and cyclin-dependent kinase 4 (CDK4) by western blot analysis. Cyclin D1 is one of the important factors in cell cycle regulation [34,35], which send chromatin replication signal after binding with high leading levels of cyclin-dependent kinase, then leading G phase cells into S phase. A dose-dependent decrease of both proteins was observed in GBC-SD and NOZ cells after treatment with Sch B for 48 h (Figure 6C), indicating that Sch B induces G0/G1-phase arrest in these cells. Thus, we speculated the inhibition of cell growth with Sch B treatment might be not only due to apoptosis but also to cell cycle arrest.

2.6. Sch B Potentiates the Antitumor Effect in Vivo

To investigate the effect of Sch B on tumor growth *in vivo*, vehicle (10% DMSO and 90% PBS) or Sch B (30 mg/kg and 100 mg/kg) were injected into nude mice carrying subcutaneous NOZ tumor xenografts every 2 days for up to 15 days. The tumors removed from these animals are shown in Figure 7A,B, and their mean weights were provided in Figure 7C. The results showed an obvious inhibition of tumor growth in mice treated with Sch B in a dose-dependent manner compared with control group.

In previous research, shengmai injection, which contains Sch B as an active ingredient, intravenous administrated in rat, Sch B was found to distribute quickly from blood into most tissues, prone to fat

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and liver specifically, and had accumulation in these tissues[36],which can be explained by its high liposolubility because of structure characteristics. Sch B also has the effect of raising blood concentrations of co-administering immunosuppressive drug and decreasing the total expense of drug, so Sch B should be used carefully under guidance [37]. In summary, our study showed that Sch B has potential as a novel anti-tumor therapeutic strategy for the treatment of gallbladder cancer.

Figure 6. Sch B induces G0/G1 phase arrest and regulates the expression of cell cycle-related proteins of gallbladder cancer cells. (**A**) GBC-SD and NOZ cells were treated with Sch B (0, 30, 60 and 90 μmol/L) for 48 h. The cell cycle phases of the treated cells were evaluated by flow cytometry; (**B**) Data were expressed as mean ± SD (n = 3); (**C**) The expression levels of cyclin D1, CDK4 were measured by western blot analysis, and GAPDH was used as a loading control; Results were representative of 3 independent experiments. ***** $P < 0.05$, ****** $P < 0.01$ *vs.* the control group.

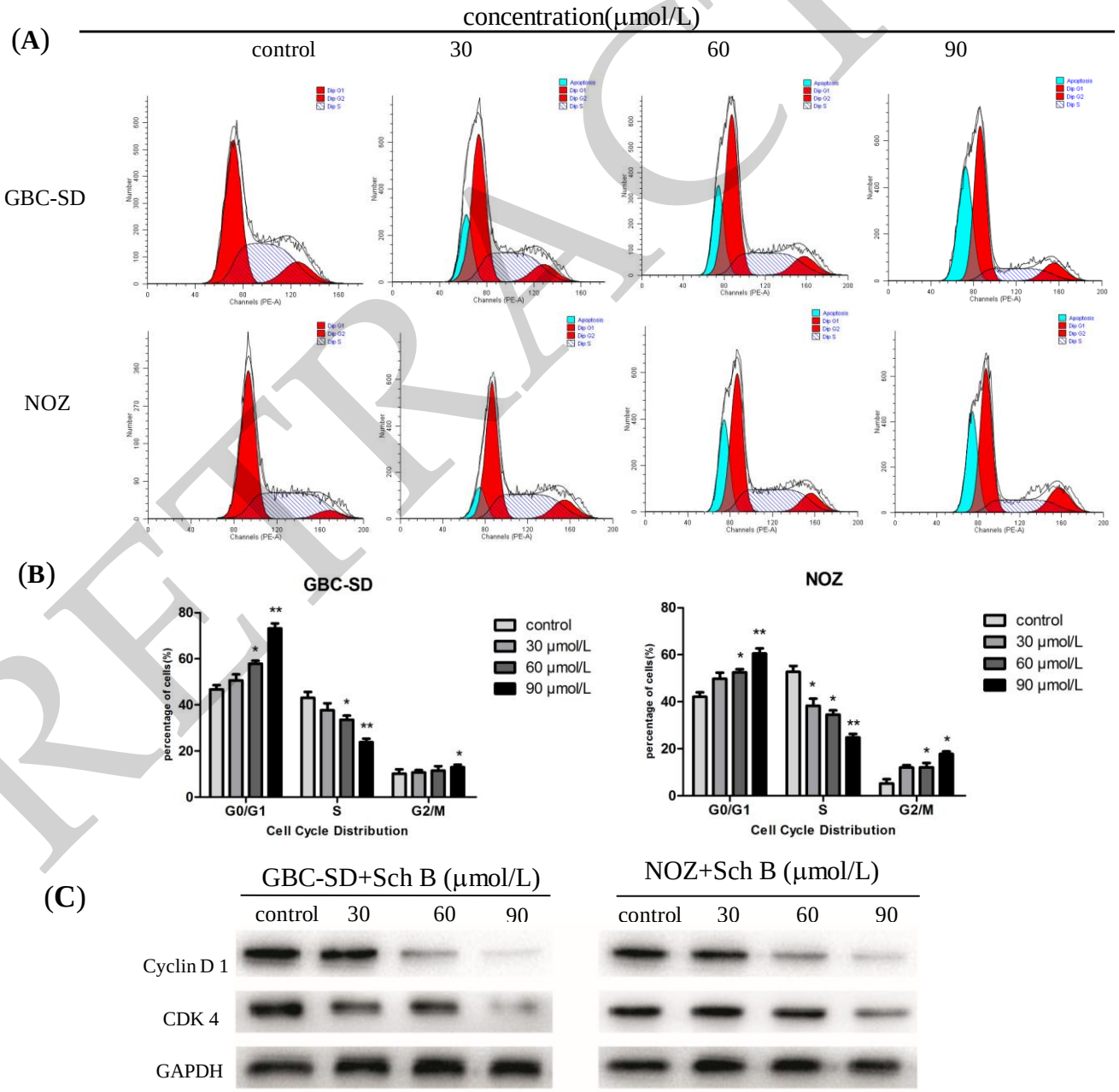

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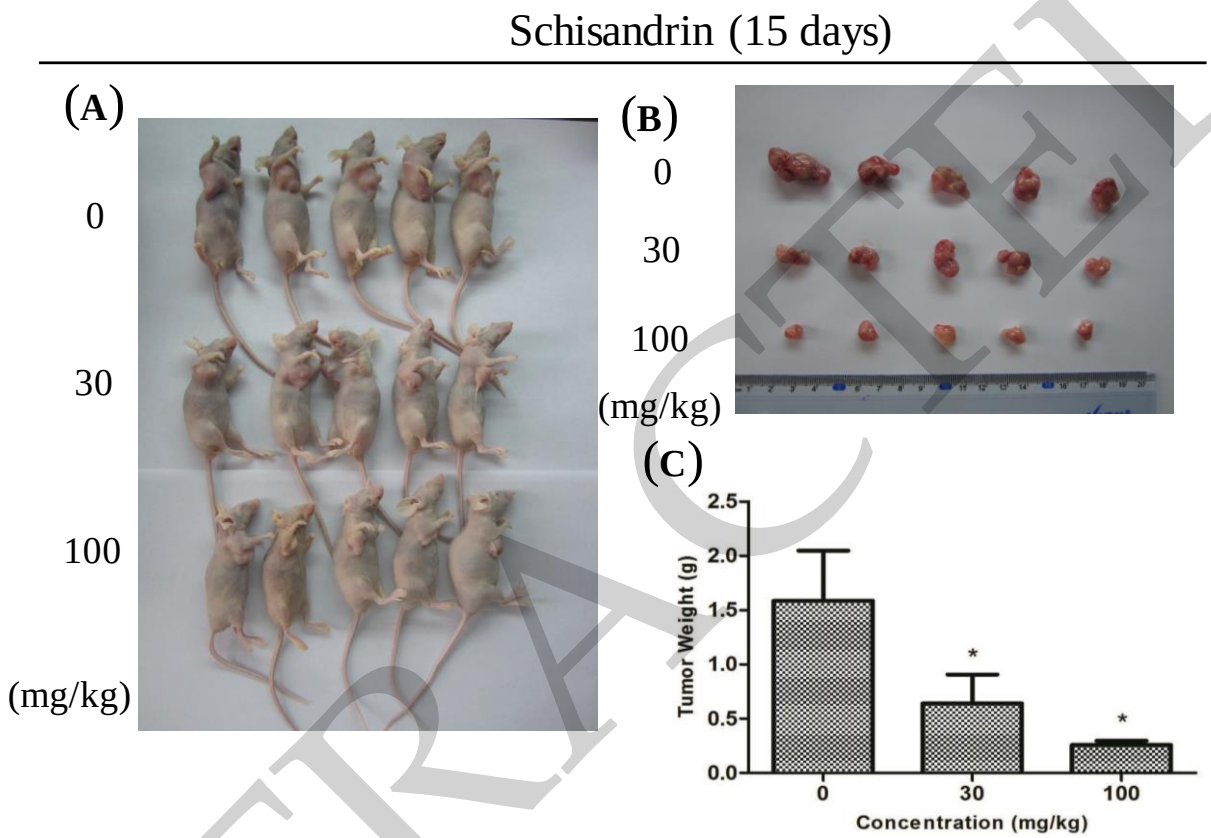
Figure 7. Sch B potentiates the *in vivo* antitumor effect. (**A)** NOZ cells were subcutaneously injected into the right flank of the nude mice; The mice were then administered 0.2 mL of vehicle (10% DMSO and 90% PBS) or Sch B (30 mg/kg and 100 mg/kg) intraperitoneally every 2 days for up to 15 days. Photos of 5 representative mice (n = 10) from each group were presented to show the sizes of the resulting tumors; (**B**,**C**) Tumors were excised from the animals and weighed. ***** $P < 0.05$ *vs.* the control group.



3. Experimental

3.1. Drugs and Antibodies

Sch B was purchased from Sigma-Aldrich (St. Louis, MO, USA). For *in vitro* studies, Sch B was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution (0.1 mol/L), which was stored at −20 °C. To prepare working solutions, the stock solution was further diluted with culture media to yield the desired Sch B concentration. Control cells were treated with an equal volume of vehicle. The DMSO concentration was kept below 0.1% in cell culture and did not have any detectable effect on cell growth or cell death. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), annexin V-FITC, propidium iodide (PI), and Rhodamine 123 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies against Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3, cleaved PARP, NF-κB, cyclin D1, CDK4 , GAPDH and secondary antibodies (goat-anti-rabbit) were purchased from Cell Signaling Technology (Danvers, MA, USA).

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3.2. Cell Lines and Culture

The human gallbladder cancer cell lines GBC-SD and NOZ were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (CAS, Shanghai, China). GBC-SD cells were cultured in high-glucose DMEM (Gibco, Grand Island, NY, USA). NOZ cells were cultured in William's medium (Gibco). The media for both cell lines were supplemented with 10% fetal bovine serum (Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin (Hyclone, Logan, UT, USA) and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

3.3. Cell Viability Assay

The viability of cells treated with Sch B was measured by the MTT assay. GBC-SD and NOZ cells (5×10^3) were seeded into 96-well plates, incubated overnight, and treated with Sch B at final concentrations of 0, 20, 40, 60, 80 and 100 μmol/L for 24, 48 and 72 h. After treatment, 20 μL of MTT solution (5 mg/mL) was added to each well and the cells were then incubated at 37 °C for 4 h, then replaced the culture medium with 100 μL of DMSO. The absorbance of the solution at 490 nm was measured with a microplate reader (Quant Bio Tek Instruments, Winooski, VT, USA). The results represent the average of five parallel samples.

3.4. Colony Formation Assay

Cells in the logarithmic growth phase were aliquoted as single cell suspensions and 500 cells were placed into each well of 6-well plates (Corning, Corning, NY, USA). After adherence, cells were treated with Sch B (0, 3, 6 and 9 μmol/L for GBC-SD and NOZ) for 48 h. Then the Sch B-containing medium was removed and the cells allowed to form colonies in complete medium for 14 days. Next, the cells were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet (Sigma-Aldrich) for 30 min. After washing, the plates were air-dried, and stained colonies were photographed using a microscope (Leica, Wetzlar, Germany). The total number of colonies (>50 cells/colony) was counted manually.

3.5. Cell Apoptosis Assay

The cells were seeded in 6-well plates and treated with Sch B (0, 30, 60 and 90 μmol/L) for 48 h. cells were then harvested by trypsinization, After washing twice with cold PBS, the cells were resuspended at a density of 1×10^6 cells/mL. Then, 100 μL of binding buffer containing 5 μL of annexin V-FITC and 5 μL of propidium iodide (PI) working solution (100 μg/mL) was added to these cells and incubated in the dark, after 30 min, 400 μL of the binding buffer was added to suspension. The samples were then immediately analyzed by flow cytometry (BD Biosciences, San Diego, CA, USA).

3.6. Mitochondrial Membrane Potential (ΔΨm) Assay

Rhodamine 123 (Rho123) was used to determine mitochondrial membrane potential (ΔΨm). After treatment with different concentrations of Sch B for 48 h, GBC-SD and NOZ cells were harvested and washed twice with cold PBS. The cells were then incubated with rhodamine 123 (Sigma-Aldrich) for

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30 min at 5% CO_2 and 37 °C in the dark. Subsequently, then washed the cells twice with cold PBS and analyzed by flow cytometry (BD Biosciences).

3.7. Western Blot Analysis

The cells were treated with Sch B (0, 30, 60 and 90 μmol/L) for 48 h, and then the adherent and floating cells were harvested, washed twice with cold PBS, and lysed in RIPA buffer (Beyotime Institute of Biotechnology, Beijing, China) and protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) at 4 °C for 5 min. After centrifugation at 14,000× *g* for 5 min, the protein concentration of the cell extracts was determined by the bicinchoninic acid (BCA) assay kit (Beyotime) according to the manufacturer's instructions. Eaqual amounts of protein lysates (40 μg/lane) from each sample were separated by 10% SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). Each membrane was blocked with 5% skim milk, and then incubated with the indicated primary antibodies against Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3, cleaved PARP, NF-κB, cyclin D1, CDK4, GAPDH (1:1000) at 4 °C overnight. After washing with TBST buffer, the membrane was incubated with the secondary antibodies (HRP-conjugated goat anti-rabbit IgG, 1:5000; Abcam, Cambridge, UK) for 1 h at room temperature and the bands were visualized using a Gel Doc 2000 (Bio Rad, Hercules, CA, USA).

3.8. Cell Cycle Analysis

GBC-SD and NOZ cells were treated with different concentrations of Sch B for 48 h. Cells were then harvested by trypsinization, washed twice in cold PBS, and fixed in 70% ethanol at 4 °C overnight. After fixation, the cells were washed and resuspended in cold PBS and incubated in a solution of 10 mg/mL RNase and 1 mg/mL propidium iodide (Sigma-Aldrich) at 37 °C in the dark for 30 min. Finally, the samples were analyzed by flow cytometry (BD Biosciences). The percentage of cells in the G0/G1, S, and G2/M phases was determined using Cell Quest acquisition software (BD Biosciences).

3.9. Experimental Animals

Female athymic nude mice (4–6 weeks old with an initial body weight of 20 g ± 2 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animals were housed at a temperature of 25 °C ± 2 °C and a relative humidity of 70% ± 5% under natural light/dark conditions for 1 week and allowed free access to food and water. The animal experiments were performed in strict accordance with international ethical guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University.

3.10. Statistical Analysis

All values are expressed as the mean ± SD unless otherwise stated. Student's *t*-test was used to compare the difference between treated groups and their controls. A *P*-value of less than 0.05 was considered to be statistically significant.

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4. Conclusions

In conclusion, this study suggests that Sch B inhibits the proliferation of GBC-SD and NOZ cells and arrests the cell cycle in the G0/G1 phase. The alteration of Bax, Bcl-2, caspase-9, caspase-3, PARP and Bcl-2 protein expression revealed that Sch B induce apoptosis via the activation of the mitochondrial-mediated intrinsic caspase pathway. Nonetheless, other apoptotic mechanisms remain to be researched further.

Acknowledgments

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Author Contributions

Shan-Shan Xiang, Xu-An Wang, Huai-Feng Li, Yi-Jun Shu, Run-Fa Bao, Fei Zhang, Yang Cao, Yuan-Yuan Ye, Hao Weng, Wen-Guang Wu, Jia-Sheng Mu, Xiang-Song Wu, Mao-Lan Li, Yun-Ping Hu, Lin Jiang, Zhu-Jun Tan, Wei Lu designed and conducted the study, analyzed the data. Shan-Shan Xiang, Xu-An Wang, Huai-Feng Li wrote the manuscript. Feng Liu and Ying-Bin Liu are the principal investigators, and revised and edited the manuscript. All authors approved the final manuscript.

Conflicts of Interest

The authors declare no competing interests.

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Sample Availability: Samples of the compounds are not available from the authors.

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