AB4 inhibits Notch signaling and promotes cancer cell apoptosis in liver cancer

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Abstract. The etiology for liver cancer has been clearly defined. Unfortunately, therapeutic approaches for liver cancer are rather limited, and liver cancer is insensitive to chemotherapy and radiotherapy. Traditional Chinese medicine (TCM) has become a promising strategy for cancer treatment as TCM elicits broad spectrum anticancer activity. In the present study, we evaluated the anticancer efficacy of AB4, an extract from the medical herb *Pulsatilla chinensis* (Bunge) Regel, in liver cancer *in vitro* and *in vivo*. We found that AB4 readily dose- and time-dependently inhibited liver cancer HepG2 and Huh-7 cell proliferation and colony formation. Western blot and flow cytometry analyses suggested that AB4 treatment induced liver cancer cell apoptosis. Moreover, these findings could be readily recaptured *in vivo*, in which the AB4 regimen resulted in tumor suppression and cancer cell apoptosis in xenograft tumor-bearing nude mice. Importantly, we noted that treatment with a Notch signaling inhibitor DAPT produced very similar anticancer efficacy in both HepG2 and Huh-7 cell lines, and administration of DAPT also efficiently suppressed HepG2 xenograft outgrowth. To this end, we anticipated that AB4 and DAPT may act on the same signaling pathway, probably through inhibition of the Notch pathway. Indeed, we found decreased expression of Notch1 protein, as well as downstream targets Hes1 and Hey1, after AB4 treatment. Immunohistochemistry analysis further confirmed the suppression of Notch signaling in HepG2 xenograft-bearing mice. Taken together, our study highlighted the anticancer efficacy of AB4 in liver cancer. We also provided preliminary data showing Notch as a therapeutic target of AB4. It would be interesting to investigate the anticancer efficacy of AB4 in other types of cancer with elevated Notch activity.

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Introduction

Liver cancer is one of the leading malignancies worldwide. The etiology of liver cancer varies, which makes the diagnosis and therapeutic strategy a significant challenge. For example, hepatitis B virus infection is the prevalent cause of liver cancer in China and South Africa, whereas >70% of liver cancer cases in Japan and the US are associated with chronic infection with hepatitis C virus (1). Although numerous clinical trials and translational studies have increased the understanding of the molecular mechanisms that drive the initiation and progression of liver cancer, the prognosis of advanced liver cancer remains poor, and novel therapeutics are urgently required (2). For this purpose, traditional Chinese medicine (TCM) has attracted increasing interest as Chinese medicinal herbs can target multiple signaling pathways that are involved in cancer cell survival and proliferation (3,4). Additionally, TCM facilitates conventional chemotherapy to promote liver cancer cell apoptosis and reduce dose-related cytotoxicity of chemotherapeutics (5). Therefore, TCM deserves further investigation as an optimal strategy for advanced liver cancer.

The Chinese medicinal herb *Pulsatilla chinensis* (Bunge) Regel and its derivates exert various bioactivities, including anti-inflammatory (6), anti-oxidative (7), anti-bacterial and anti-fungal effects (8). Notably, *Pulsatilla chinensis* (Bunge) Regel extracts have been demonstrated to inhibit cancer cell proliferation *in vitro* and *in vivo*, including in A549 lung cancer cells (9), SK-MEL-2 melanoma cells (10,11) and MCF-7 breast cancer cells (12,13). These results have prompted innovative research investigating the isolation of biologically active components of this herb. Previous studies have suggested that

Pulsatilla saponin A is one of the anticancer components, which kills cancer cells through the induction of cell cycle arrest and apoptosis (14,15). However, Pulsatilla saponin A exhibits severe hemolytic toxicity, which could be a major obstacle for its clinical application as an anticancer agent (16). Anemoside B4 (AB4) is another major component of *Pulsatilla chinensis* (Bunge) Regel extracts. However, to the best of our knowledge, its biological activity is largely unknown. AB4 is well tolerated and exhibits minimal dose-related cytotoxicity. Therefore, deciphering its pharmacological potential could be a promising strategy for the treatment of patients with advanced liver cancer.

Notch signaling was initially noticed due to the appearance of a notch in the wings of fruit flies, and was subsequently found to serve a critical role in embryonic development. Activation of Notch signaling by its ligand leads to Notch intracellular domain (NICD) release and nucleus translocation, where NICD cooperates with the DNA-binding protein CSL to form a transcriptional active heterodimer, which activates Notch-targeted gene expression (17). Notably, aberrant activation of Notch has been detected in multiple malignancies, and further in-depth laboratory investigations have demonstrated that activation of Notch signaling not only directly leads to tumorigenesis, but also cross-talks with numerous other pathways implicated in tumor invasion, migration and metastasis (18). For example, transgenic mice carrying liver-specific constitutively activated Notch develop liver cancer once they reach adult age, and upregulation of Notch1 expression is an unfavorable prognostic biomarker for patients with liver cancer (18). Notch1 activation contributes to liver cancer cell growth and proliferation, whereas Notch1 downregulation inhibits invasion and migration by inactivating the cyclooxygenase-2/Snail/E-cadherin signaling pathway or via its interaction with PTEN and focal adhesion kinase (19). These data indicate that Notch is a potent therapeutic target for liver cancer. In agreement with this notion, several ongoing phase I/II trials are evaluating the safety and efficacy of Notch inhibitors in multiple solid tumors (20-22). The γ-secretase inhibitors (GSIs) have shown anticancer efficacy in multiple Notch-mutant solid tumors, while the dose-related cytotoxicity of GSIs largely limits their clinical application (23). Therefore, increasing efforts have been made to develop novel Notch inhibitors (24).

The present study reported the anticancer efficacy of AB4 in liver cancer *in vitro* and *in vivo*. AB4 inhibited liver cancer cell proliferation and induced cell apoptosis in a time- and dose-dependent manner. Further mechanical experiments demonstrated that inhibition of Notch signaling might be implicated in the anticancer efficacy of AB4. Overall, the present study highlighted the anticancer activity of the medicinal herb extract AB4, and targeting of Notch signaling by AB4 is a potential therapeutic approach for advanced liver cancer.

Materials and methods

Reagents. AB4 was purchased from Absin Biotechnology Co., Ltd. FBS, DMEM, trypsin and penicillin-streptomycin solution were purchased from HyClone; Cytiva. Primary antibodies against cleaved caspase-3 (#9661), cleaved poly(ADP-ribose) polymerase (PARP) (#5625), cytochrome *c* (#4280), Notch1 (#4380), Jagged1 (#2620), NICD1 (#4380), hes family bHLH transcription factor 1 (Hes1) (#11988), , Ki67 (#9027) and β-actin (#4967), and HRP-labeled secondary anti-rabbit IgG (#7074) were obtained from Cell Signaling Technology, Inc. The hes related family bHLH transcription factor with YRPW motif 1 (Hey1) antibody (#ab11723) was from Abcam. The Annexin V-FITC Apoptosis Detection kit was obtained from EMD Millipore. DAPT, a γ-secretase inhibitor (GSI) that inhibits the Notch pathway, MTT and DMSO were purchased from Sigma-Aldrich; Merck KGaA. The 3,3'-diaminobenzidine reagent was purchased from Dako; Agilent Technologies, Inc.

Cell culture. The hepatoblastoma HepG2 cell line and hepatocellular carcinoma Huh-7 cell line were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cell lines were authenticated using short-tandem repeat profiling, tested for mycoplasma contamination and used at passage numbers of <10. Cells were maintained in DMEM supplemented with 10% FBS and 100 U/ml penicillin-streptomycin solution. Cells were cultured in a humidified incubator with 5% CO_2 at 37˚C.

MTT assay. Cells were seeded into 96-well plates at a density of $5x10^3$ cells per well. The cells were cultured overnight to allow attachment, and then the cells were treated with increasing concentrations of AB4 or DAPT (50 μmol/l) for different time periods. Cells were subsequently incubated with 0.5 mg/ml MTT for another 4 h. The formazan was dissolved in 100 μl DMSO and the optical density at 570 nm was determined using a micro-array reader (Bio-Rad Laboratories, Inc.). Each experiment was performed in triplicate and the data are presented as the mean ± SD.

Colony formation assay. Cells were seeded into 6-well plates at a density of $1x10^3$ cells per well. Following overnight incubation to allow cell attachment, the cells were grown in medium containing AB4 (5 and 10 μmol/l) or DAPT (5 μmol/l) for ~14 days. The medium was refreshed every 3 days. At the end of the experiment, the cell culture medium was discarded and the cells were washed with PBS three times. After being fixed with 4% paraformaldehyde for 20 min, the colonies were visualized using crystal violet staining under a microscope.

Apoptosis assay. Apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit (EMD Millipore) according to the manufacturer's protocols. Briefly, cells were seeded into 6-well plates at a density of $2.5x10^5$ cells per well, and then treated with AB4 (50 μmol/l) and DAPT (50 μmol/l) for 24 h. Cells were resuspended in 95 μl Annexin V-FITC binding buffer mixed with 5 μl Annexin V-FITC, followed by incubation with 10 μl PI. Flow cytometry was performed using a FACS Calibur flow cytometer.

Western blot analysis. After the indicated treatment, cells were harvested and lysed in lysis buffer. Equal amounts of extracted protein samples (10-30 μg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% BSA in TBS with 1% Tween-20 for 1 h, and then incubated with the corresponding primary antibodies (dilution, 1:1,000) overnight at 4˚C. Protein bands were detected using HRP-conjugated secondary antibodies (dilution, 1:5,000) and visualized using enhanced chemiluminescence (Millipore).

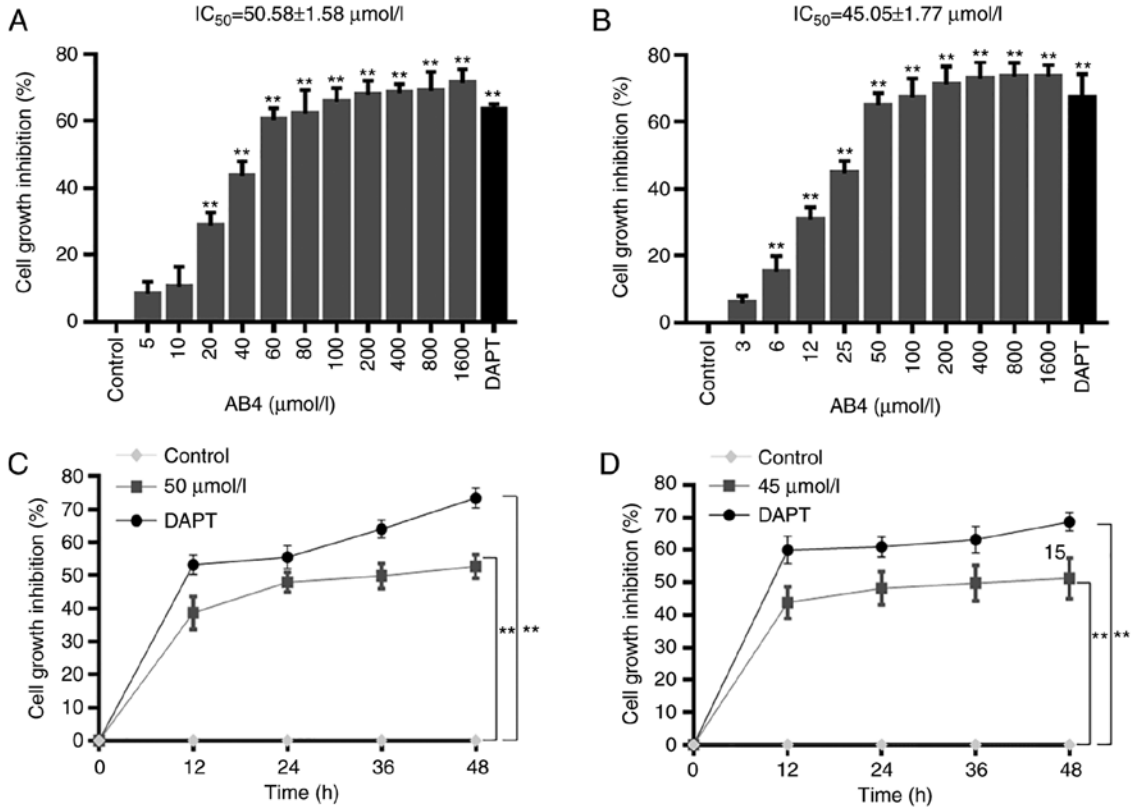

Figure 1. AB4 treatment dose- and time-dependently inhibits liver cancer cell proliferation. (A) HepG2 and (B) Huh-7 cells were treated with the indicated increasing concentrations of AB4 for 24 h. The inhibitory effect and IC_{50} of AB4 on liver cancer cells were measured by MTT assay. (C) HepG2 and (D) Huh-7 cells were treated with AB4 at a final concentration corresponding to the relevant IC_{50} value for different time intervals (0-48 h). The effect of AB4 on liver cancer cell proliferation was determined by MTT assay. Each experiment was performed in triplicate and DAPT was used as a positive control. Data are presented as mean ± SD. $^{**}P<0.01$ vs. vehicle control. AB4, an extract from the medical herb *Pulsatilla chinensis* (Bunge) Regel; IC_{50}, half maximal inhibitory concentration; DAPT, a γ-secretase inhibitor (GSI) that inhibits the Notch pathway.

Xenograft tumor model. A total number of 20 specific pathogen-free male nude mice (4 weeks old; 12±2 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were housed in temperature (25˚C)- and humidity (50%)-controlled feeding rooms under a 12 h light/dark cycle and provided with unrestricted amounts of rodent chow and drinkable water. All experiments were approved by the Ethic Committee of The Hospital 971 of The Navy of Chinese People's Liberation Army (Qingdao, China) and were performed in accordance with Animal Ethics Guidelines (#401LL-2017010). HepG2 cells (~$2x10^6$) were resuspended in 0.2 ml DMEM and subcutaneously injected into the right flank of the nude mice. When the xenograft tumor volume reached 150 mm^3, the tumor-bearing mice were randomly divided into four groups (n=5 in each group), which received vehicle as a control, low dose AB4 (60 mg/kg), high dose AB4 (120 mg/kg) or DAPT (100 mg/kg) for 19 days. Xenograft tumor growth was monitored and recorded every 2 days. At the end of the experiment, the mice were humanely sacrificed by intraperitoneal injection of pentobarbital (100 mg/kg), and tumors were carefully isolated and processed for histological examination.

Immunohistochemistry (IHC). Paraformaldehyde-fixed and paraffin-embedded tissue blocks were cut into 4-μm sections. The sections were dewaxed and dehydrated, followed by antigen retrieval and endogenous peroxidase blocking. IHC was performed with anti-Notch1, anti-Hes1 and anti-Ki67 antibodies (dilution, 1:100) at 4˚C overnight. Subsequently, the slides were washed with PBS and incubated with HRP-conjugated secondary antibodies (dilution, 1:200) for 1 h at room temperature. Indicated proteins were visualized using a DakoEnVision Detection Kit (Dako; Agilent Technologies, Inc.). Finally, all sections were rinsed with running water, counterstained with hematoxylin and dehydrated in graded ethanol.

Statistical analysis. All analyses were performed using SPSS v20.0 software (IBM Corp.) and the results are presented as the mean ± standard deviation. Statistically significant differences among groups were determined using one-way ANOVA and Dunnet's least significant difference post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Anticancer effect of AB4 in liver cancer in vitro. To determine the potential anticancer effects of AB4 in liver cancer, two liver cancer cell lines, namely the HepG2 (hepatoblastoma) and Huh-7 (hepatocellular carcinoma) cell lines, were treated with increasing concentrations of AB4. The toxic effect of AB4 was evaluated using an MTT assay. As shown in Fig. 1A, 24 h of AB4 treatment dose-dependently inhibited HepG2 cell proliferation. AB4 concentrations ranging between 20 and 1,600 μmol/l readily elicited a growth inhibitory effect, and the estimated half maximal inhibitory concentration (IC_{50})

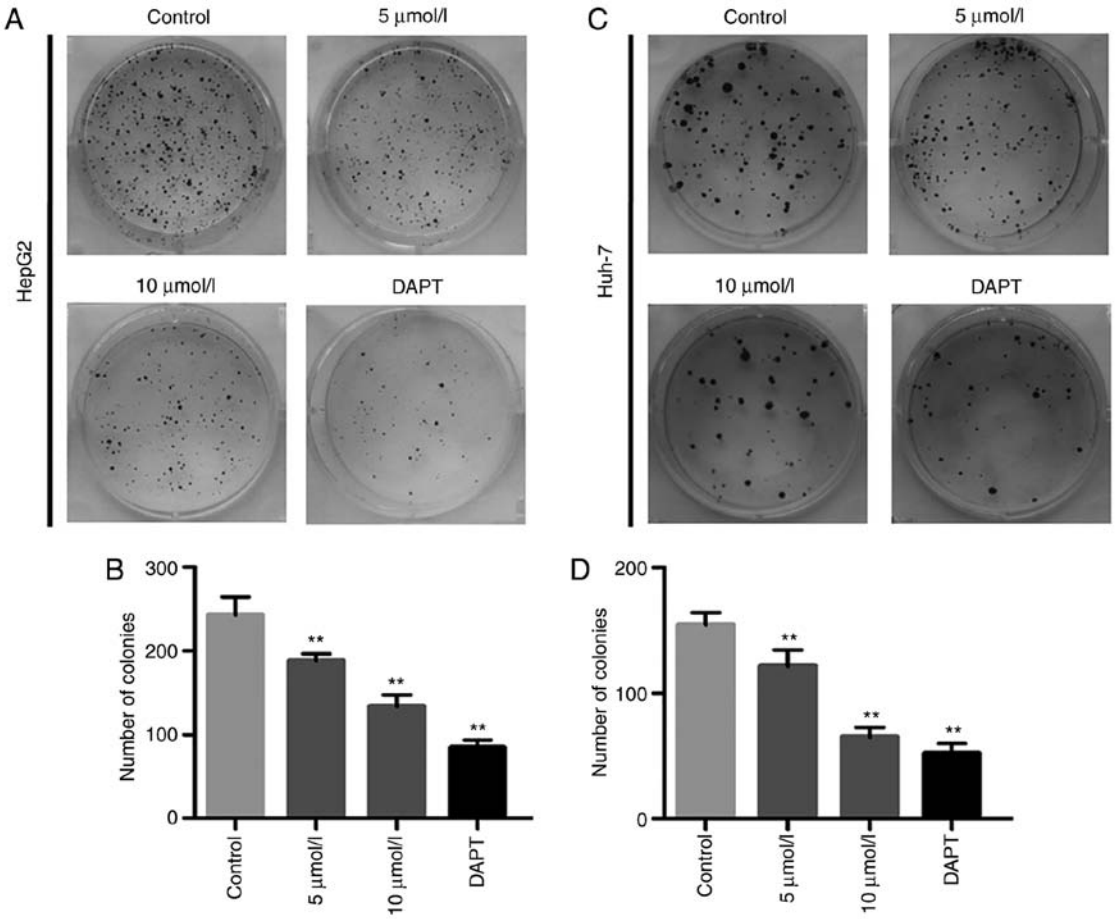

Figure 2. AB4 treatment inhibits liver cancer cell colony formation. (A and B) HepG2 and (C and D) Huh-7 cells were seeded in 6-cm culture plate and treated with the indicated low concentrations of AB4. As a positive control, cells were treated with DAPT. Cell colonies were stained and visualized with crystal violet. $^{**}P<0.01$ vs. vehicle control. AB4, an extract from the medical herb *Pulsatilla chinensis* (Bunge) Regel; DAPT, a γ-secretase inhibitor (GSI) that inhibits the Notch pathway.

at 24 h was ~50 μmol/l. As a positive control, treatment with 50 μmol/l DAPT, a GSI, led to >60% cell growth inhibition. In agreement with this, AB4 also dose-dependently inhibited Huh-7 cell proliferation. The estimated IC_{50} of AB4 at 24 h was ~45 μmol/l in Huh-7 cells (Fig. 1B).

The present study evaluated the time-dependent cytotoxicity of AB4 in both cell lines. HepG2 and Huh-7 cells were treated with AB4 at a final concentration of 50 and 45 μmol/l, respectively. Treatment with 50 μmol/l AB4 resulted in HepG2 cell growth inhibition to a comparable magnitude of that in cells treated with 50 μmol/l DAPT. This effect peaked at 24 h and was maintained at a similar level at 36 and 48 h (Fig. 1C). Furthermore, all these aforementioned findings could also be observed in Huh-7 cells, in which treatment with 45 μmol/l AB4 time-dependently suppressed Huh-7 cell proliferation (Fig. 1D). These results revealed the anticancer effects of AB4 in liver cancer cells. Therefore, AB4 at a final concentration of 50 or 45 μmol/l was used in further experiments.

AB4 inhibits liver cancer cell colony formation. Having established that AB4 elicited cytotoxicity in liver cancer cell lines after 24 h of treatment, the present study next investigated whether AB4 also efficiently killed HepG2 and Huh-7 cells in experiments with relatively longer durations. Cells were seeded into 6-cm culture plates and cell colonies were readily visualized after 2 weeks. Chronic exposure to low concentrations of AB4 (5 and 10 μmol/l) markedly inhibited colony formation in both cell lines. As shown in Fig. 2, chronic exposure to AB4 also dose-dependently decreased the numbers of HepG2 and Huh-7 cell colonies. Notably, the absolute colony number in the 10 μmol/l AB4 group was approximately half of that in the vehicle control group. Additionally, DAPT at a final concentration of 5 μmol/l efficiently inhibited HepG2 and Huh-7 cell colony formation. These findings demonstrated the cytotoxicity of AB4 in liver cancer cell lines and prompted the investigation of the underlying mechanism responsible for its anticancer efficacy.

AB4 induces liver cancer cell apoptosis. Extensive studies have indicated that liver cancer cells undergo apoptosis in response to various chemotherapeutic agents. Therefore, the present study investigated whether the anticancer effects of AB4 in liver cancer were dependent on the induction of cancer cell apoptosis. Following the indicated treatment, the cell apoptosis status was determined by flow cytometry. Fig. 3A shows that 24 h of 50 μmol/l AB4 treatment led to >50% of HepG2 cells undergoing apoptosis. Importantly, most of the apoptotic cells were at the early apoptotic stage. This was consistent with the notion that cytotoxic drugs primarily induce cancer cell apoptosis, rather than inducing necrosis. In agreement with this, treatment with AB4 at the same concentration and for the same treatment duration induced nearly 50% of Huh-7

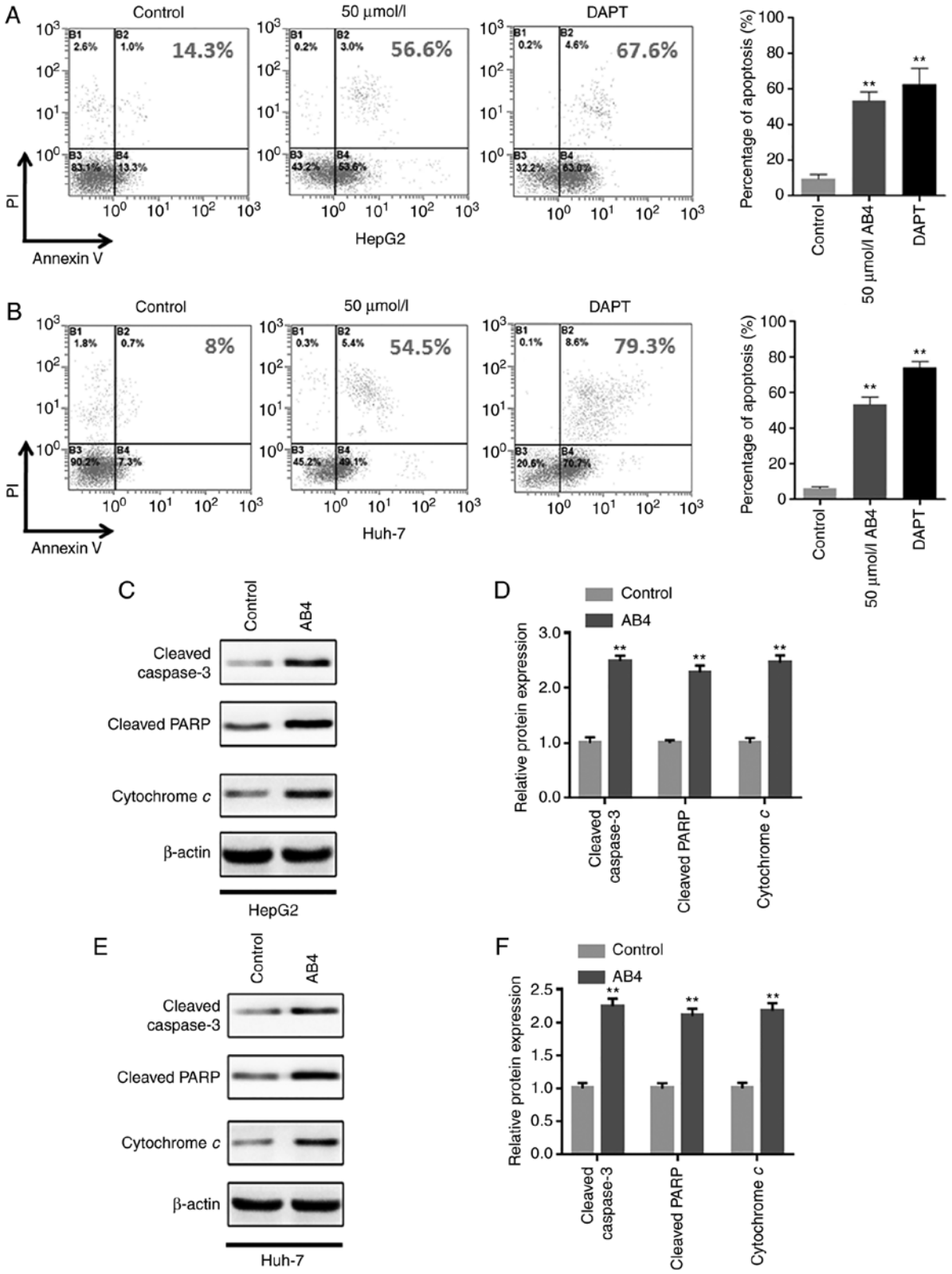

Figure 3. AB4 treatment leads to liver cancer cell apoptosis. (A) HepG2 and (B) Huh-7 cells were treated with 50 μmol/l AB4 or DAPT for 24 h and evaluated for cell apoptosis by flow cytometry. (C-F) After the indicated treatment, (C and D) HepG2 and (E and F) Huh-7 cells were analyzed for apoptosis by western blot analysis. Expression of apoptosis-related proteins, including cleaved caspase-3, PARP fragmentation and cytochrome *c* release, were determined. **P<0.01 vs. vehicle control. AB4, an extract from the medical herb *Pulsatilla chinensis* (Bunge) Regel; DAPT, a γ-secretase inhibitor (GSI) that inhibits the Notch pathway.

cells to undergo early-stage apoptosis (Fig. 3B). Furthermore, treatment with the GSI DAPT also led to robust early-stage apoptosis in both cell lines. These compelling results indicated that apoptosis induction is a crucial mechanism of liver cancer cell death following AB4 treatment. In addition, the similar findings obtained for AB4 and DAPT indicated that both drugs may act on the same signaling pathway to induce liver cancer cell apoptosis.

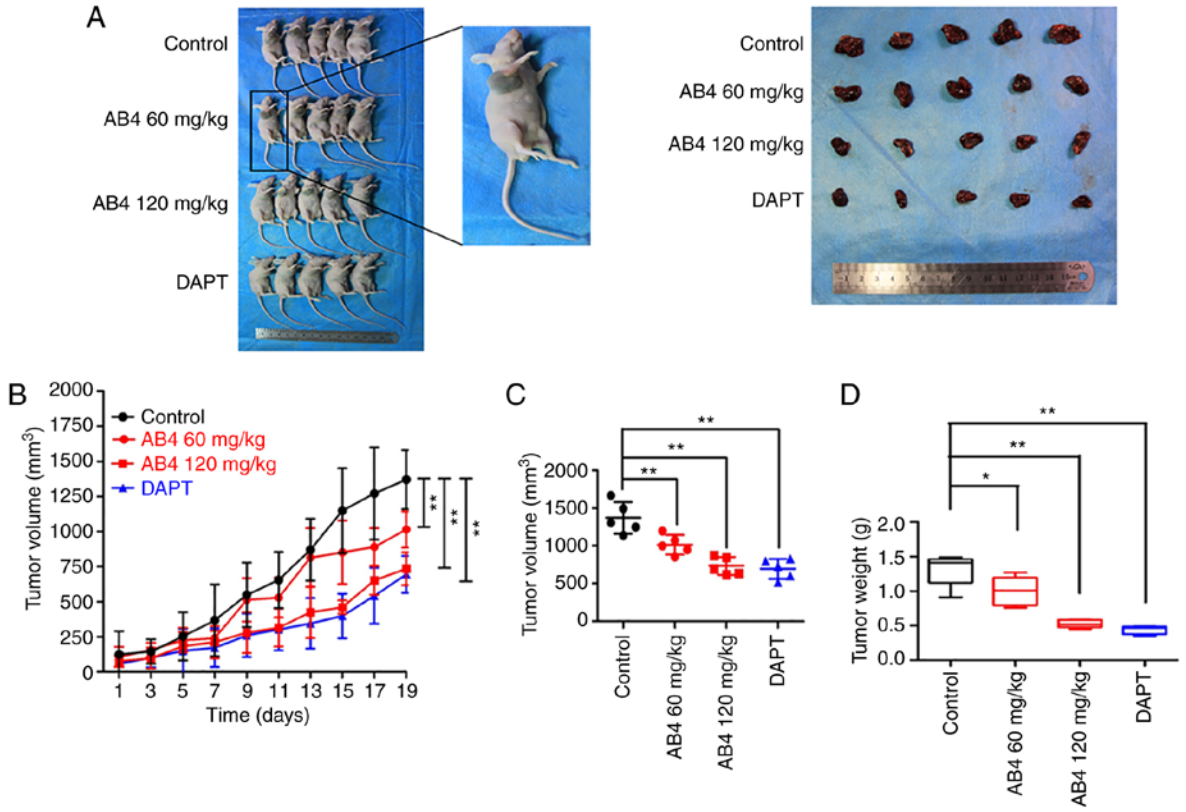

Figure 4. AB4 treatment suppresses liver cancer cell growth *in vivo*. (A) HepG2 cells were subcutaneously injected into null mice. The mice were treated with AB4 (60 and 120 mg/kg) or DAPT or vehicle as a control. At the end of the experiment, the mice were humanely sacrificed and the xenograft tumors were carefully isolated. Representative images of xenograft tumors were recorded. (B) The dynamic changes in xenograft tumor volume after the indicated treatment. The black line indicates mice receiving vehicle, the red lines indicate mice receiving AB4 (circles: 60 mg/kg and squares: 120 mg/kg, and the blue line indicated mice receiving DAPT. (C and D) At the end of the experiment, xenograft tumor (C) volume and (D) weight were measured. *P<0.05 and **P<0.01 vs. vehicle control. AB4, an extract from the medical herb *Pulsatilla chinensis* (Bunge) Regel; DAPT, a γ-secretase inhibitor (GSI) that inhibits the Notch pathway.

To validate these findings, western blot analysis was performed to evaluate the expression levels of apoptosis-related proteins following the indicated treatment. It was revealed that AB4 treatment resulted in cytochrome *c* release, caspase-3 activation and PARP cleavage (Fig. 3C and D). Notably, the changes of the aforementioned apoptosis-related proteins were consistent in both cell lines (Fig. 3E and F). Overall, these findings indicated that AB4 may be a potential anticancer candidate drug for liver cancer, and acts primarily through the induction of cancer cell apoptosis.

Anticancer effect of AB4 in liver cancer in vivo. Since AB4 exhibited anticancer activity in liver cancer cell lines, the present study next determined whether this anticancer effect could also be observed *in vivo*. Using a xenograft model, HepG2 cells were injected into the right flank of 4-week-old male null mice. In this case, only HepG2 cells were used because this cell line rapidly grows and readily forms subcutaneous tumors in null mice. Treatment was initiated when the volume of the subcutaneous tumors reached 100 mm^3. Fig. 4A shows the gross observation of subcutaneous tumors after 19 days of the indicated treatment. The dynamic change of tumor size was measured every 2 days. In Fig. 4B, it is shown that the xenograft tumors in the vehicle group markedly grew in null mice. At the end of the experiment, the average tumor volume exceeded 1,200 mm^3 (maximum tumor size, 1,600 mm^3; Fig. 4C). By contrast, the tumor volume gradually decreased in the AB4 group, and this effect was positively associated with the dose of AB4. To be specific, the subcutaneous tumor began to shrink on day 15 in the AB4 low dose group (60 mg/kg), whereas tumor growth began to shrink on day 9 in mice fed with a high dose of AB4 (120 mg/kg; Fig. 4C). After 19 days of the indicated treatment, the subcutaneous tumor size in the low and high dose groups was ~83 and 50% of that in the vehicle control group, respectively (Fig. 4B).

The anticancer effects of the GSI DAPT were also evaluated *in vivo*. Interestingly, this drug elicited profound tumor-suppressive effects in HepG2 cells in null mice (Fig. 4A-C). Notably, the 100 mg/kg DAPT regimen induced more pronounced anticancer effects. At the end of the experiment, the tumor weight in the DAPT group was ~33% of that in the vehicle control group (vs. 40% in the 120 mg/kg AB4 group; Fig. 4D). Both the *in vitro* and *in vivo* experiments highlighted that AB4 and DAPT exhibited similar anticancer efficacy in hepatocellular carcinoma (HCC), suggesting the possibility that both drugs may manipulate the same signaling pathway.

AB4 promotes liver cancer cell apoptosis in vivo. In order to investigate whether AB4 promotes apoptosis *in vivo*, the present study evaluated the histological alterations of xenograft tumors after the indicated treatment. Fig. 5A shows representative H&E staining images of xenograft tumors from formalin-fixed paraffin-embedded tissue blocks.

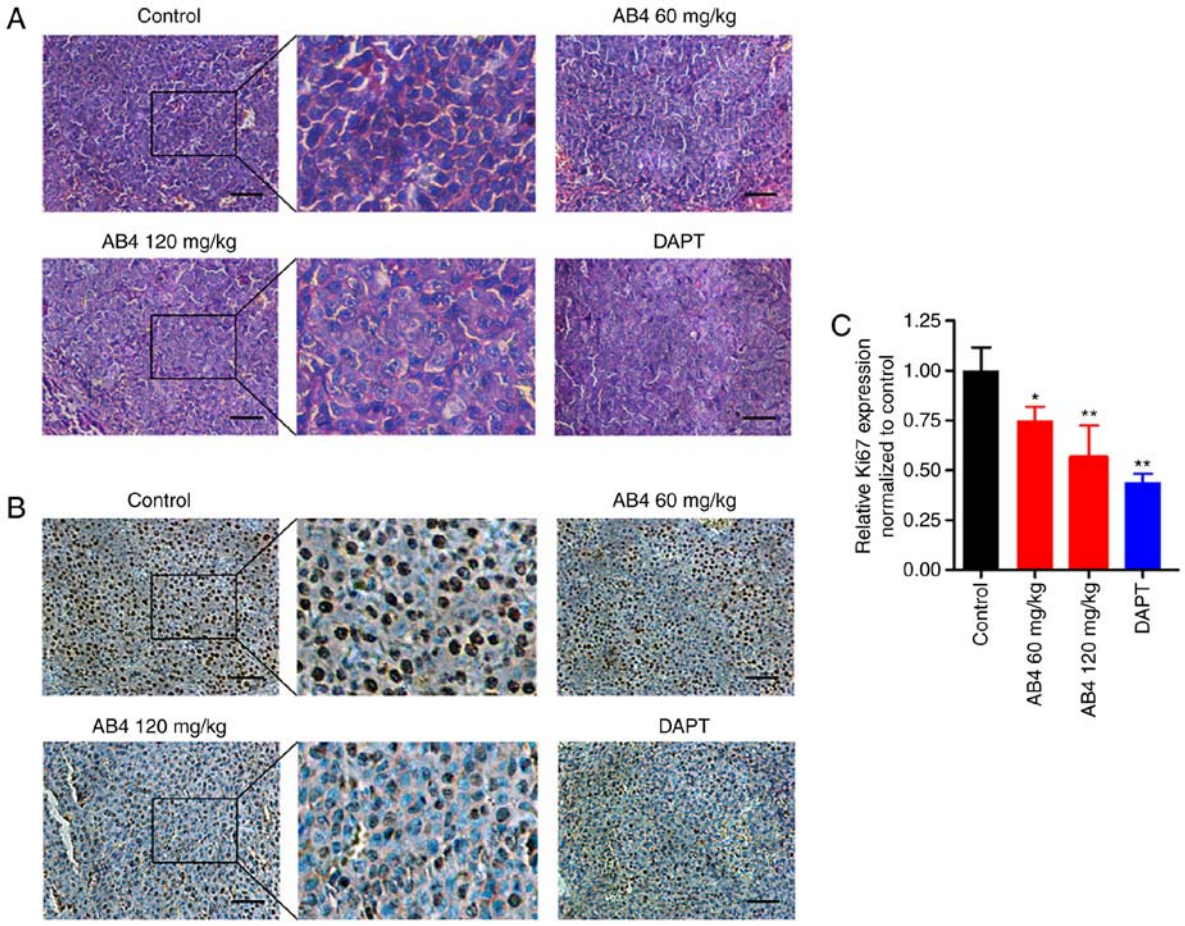

Figure 5. AB4 treatment promotes liver cancer cell apoptosis *in vivo*. (A) Representative histology images of the xenograft tumors. Scale bar, 50 μm. (B and C) Immunohistochemistry staining of Ki67 of the xenograft tumors. To visualize the cell nucleus, the slides were counterstained with hematoxylin. Scale bar, 50 μm. *P<0.05 vs. vehicle control; *P<0.05 and **P<0.01 vs. vehicle control. AB4, an extract from the medical herb *Pulsatilla chinensis* (Bunge) Regel; DAPT, a γ-secretase inhibitor (GSI) that inhibits the Notch pathway.

It was evident that AB4 treatment led to nucleus fragmentation, a morphological hallmark of cells undergoing apoptosis. In agreement with this, AB4 dose-dependently inhibited HCC cell proliferation, as examined by Ki67 staining, in xenograft tumors (Fig. 5B). Specifically, qualitative analysis of the Ki67 score indicated that AB4 at a dose of 60 mg/kg reduced the IHC score to ~75% of that in the vehicle group, while this effect was more pronounced in the 120 mg/kg AB4 group. Again, DAPT produced similar anticancer effects in xenograft tumors (Fig. 5C). These data strongly suggested that AB4 is a potential anticancer drug in liver cancer *in vitro* and *in vivo*, and mainly acts through the induction of apoptosis, and AB4 and DAPT may act on the same signaling pathway.

Notch signaling as a therapeutic target of AB4. The present experiments strongly indicated the anticancer effects of AB4 in liver cancer *in vitro* and *in vivo*. Additionally, the present study highlighted that inhibition of Notch signaling by the GSI DAPT exerted anticancer effects in a similar manner to AB4. These findings strongly suggested the possibility that AB4 and DAPT may act on the same signaling pathway and prompted the investigation of whether AB4 and liver cancer converged at the Notch signaling pathway.

To address these questions, the present study evaluated the effect of AB4 treatment on the expression levels of key components of the Notch signaling pathway, including Notch1, Jagged1, NICD1, Hes1 and Hey1. As illustrated in Fig. 6A, AB4 dose-dependently suppressed Notch signaling in HepG2 and Huh-7 cells, and this effect was more evident for higher doses of AB4 (50 and 100 μmol/l). Treatment with AB4 at final concentrations of 50 and 100 μmol/l primarily inhibited Notch1 expression and, to a lesser extent, inhibited Jagged1 and NICD1 expression, suggesting that Notch1 could be the major molecular target of AB4. Therefore, the expression levels of downstream targeted proteins, such as Hes1 and Hey1, were suppressed. Consistent with these findings, the Notch1 protein was primarily expressed in the cytoplasm in xenograft tumors, whereas its expression was readily suppressed following treatment with AB4 (Fig. 6B). Furthermore, AB4 treatment inhibited Hes1 expression *in vivo*. Fig. 6C shows representative IHC images of Hes1 expression after the indicated treatment. In contrast to the Notch1 protein, the Hes1 protein was primarily expressed in the nucleus in xenograft tumors. However, AB4 treatment markedly reduced its expression. As a positive control, DAPT readily suppressed the protein expression of Notch1 and Hes1 in xenograft tumors. Overall, Notch signaling may be a pharmacological target of AB4. Inhibition of Notch signaling by AB4 treatment suppressed liver cancer cell proliferation and induced cancer cell apoptosis.

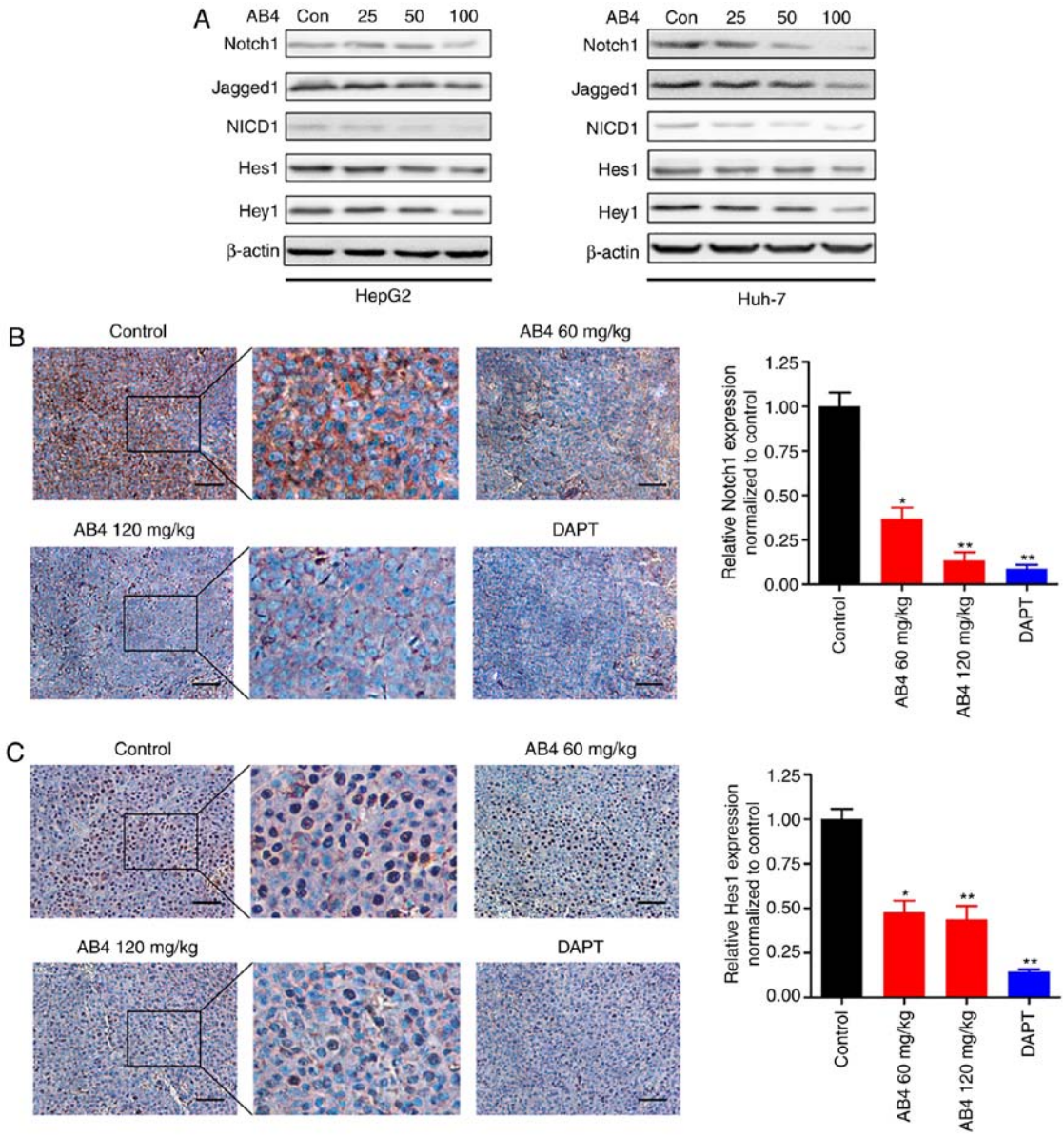

Figure 6. AB4 treatment inhibits Notch signaling. (A) Expression of Notch1 and its downstream targets in HepG2 (left panel) and Huh-7 (right panel) cells after increasing concentrations of AB4 treatment was determined by Western blot analysis. (B and C) Representative immunohistochemistry analysis of Notch1 and Hes1 expression. Scale bar, 50 μm. *P<0.05 and **P<0.01 vs. vehicle control. AB4, an extract from the medical herb *Pulsatilla chinensis* (Bunge) Regel; DAPT, a γ-secretase inhibitor (GSI) that inhibits the Notch pathway; NICD1, Notch1 intracellular domain; Hes1, hes family bHLH transcription factor 1; Hey1, hes related family bHLH transcription factor with YRPW motif 1.

Discussion

The present study revealed the anticancer efficacy of a natural medicinal herb extract, AB4, an extract from the medical herb *Pulsatilla chinensis* (Bunge) Regel, in liver cancer *in vitro* and *in vivo*. It was revealed that AB4 readily inhibited HepG2 and Huh-7 cell proliferation and induced cancer cell apoptosis. Notably, the present study provided preliminary evidence showing that direct inhibition of Notch signaling by GSI DAPT killed both cell lines in a similar manner compared with AB4. Furthermore, it was reported that AB4 treatment blocked the Notch signaling pathway *in vitro* and *in vivo*. It was concluded that AB4 is a novel therapeutic agent for liver cancer, and it was speculated that targeting of Notch signaling underlies its anticancer activity.

Natural occurring phytochemicals have attracted increasing interest, since they are well tolerated and have broad spectrum biological activities (25). Indeed, several commercially available anticancer agents, such as taxol (26) and vincristine (27), are derived from medicinal herbs. *Pulsatilla chinensis* (Bunge) Regel is another paradigm of natural occurring medicinal herbs, and the bioactivities of its components have been widely investigated. Pulsatilla saponin A is the most well documented anticancer bioactive component of *Pulsatilla chinensis* (Bunge) Regel (14). However, this agent elicits severe cytotoxicity in untransformed cells as well. Therefore, exploring other well tolerated anticancer components is urgently necessary (16). In contrast to Pulsatilla saponin A, AB4, is well tolerable and has minimal cytotoxicity in untransformed cells. It has been widely used as a 'blood-cooling' and anti-infectious regimen in TCM (28). However, most studies have focused on the isolation, purification, pharmacokinetics and distribution

pattern of AB4, and its pharmacological effect on cancer cells is largely unknown. Therefore, given the compelling clinical need for liver cancer treatment, this preliminary study was conducted to examine the anticancer efficacy of AB4.

The present study highlighted the anticancer efficacy of AB4 in the HepG2 and Huh-7 cell lines. The present study particularly focused on liver cancer because liver cancer cells are not sensitive to most cytotoxic agents. Consistently, chemotherapy fails to elicit marked and durable antitumor activity in clinical settings, and the 5-year survival of patients with advanced liver cancer is rather limited. Despite numerous successes having been achieved in the era of immunotherapy, the efficacy of immunotherapy in patients with liver cancer is far from satisfactory, and the response rate of anti-programmed cell death protein 1 (PD-1)/programmed death-ligand 1 inhibitors, including nivolumab and pembrolizumab, is <20% (29,30). Given the pressing clinical needs, the present study reported that AB4 treatment readily suppressed liver cancer cell proliferation and resulted in apoptosis *in vitro* and *in vivo*. AB4 exhibited durable (both short-term and long-term) cytotoxicity in both cell lines and efficiently inhibited xenograft tumor outgrowth. Indeed, these pharmacological effects are interesting, suggesting AB4 as a novel candidate for liver cancer treatment. Notably, biochemical experiments have demonstrated that AB4 protects human 293 cells from platinum-induced injuries by increasing reactive oxygen species scavenger activity (31). In an adenine-induced kidney injury rat model, AB4 administration markedly reduces blood urea nitrogen and creatinine levels, suppresses the expression of pro-inflammatory cytokines, and attenuates collagen deposition in the renal interstitium (32). These findings highlight a kidney-protective effect of AB4 and may indicate important clinical significance. Since most cytotoxic agents are excreted through the kidney, the kidney-protective properties of AB4 could compensate chemotherapeutic agent-induced kidney injury. Therefore, it is reasonable to hypothesize that the combination of AB4 with other chemotherapeutic agents may reduce renal toxicity.

Another notable finding of the present study was the observation of the involvement of Notch signaling in the anticancer activity of AB4. The present study focused on Notch signaling because the GSI DAPT has similar anticancer efficacy compared with AB4. This finding was in compliance with the notion that Notch signaling is hyperactivated in liver cancer, thereby driving carcinogenesis and tumor progression (33). However, targeting Notch via a conventional pharmacological approach is technically challenging and severe adverse effects could occur upon non-selective inhibition of Notch (34). The present study highlighted that AB4 treatment readily inhibited Notch signaling in liver cancer *in vitro* and *in vivo*. This finding not only elucidated the mechanism underlying the anticancer efficacy of AB4, but also elicited important clinical significance. Firstly, AB4 could be considered as a novel Notch inhibitor and it was speculated that AB4 may exert broad spectrum anticancer activity, particularly in cancers driven by Notch. Secondly, a combinational strategy would result in more robust anticancer efficacy. For example, our recent study suggested that AB4 also inhibits the PI3K/Akt/mTOR signaling pathway (35). Therefore, it is reasonable to hypothesize that AB4 could be combined with chemotherapeutic agents to kill cancer cells through inhibition of multiple signaling pathways. Furthermore, the combinational strategy is expected to be well tolerated. Since AB4 possesses antioxidant and anti-inflammatory properties, combination therapy could reduce cytotoxic agent-induced hepatological and renal toxicity. In addition, increasing lines of evidence have demonstrated that Notch is a predictive biomarker for immunotherapy. Patients with non-small cell lung cancer carrying Notch mutations tend to have a higher response rate and longer progression-free survival (36). It was predicted that disrupting Notch signaling by AB4 would facilitate with immunotherapy. Therefore, it would be interesting to investigate the anticancer efficacy of AB4 and anti-PD-1 combination therapy.

In conclusion, the present study reported the anticancer efficacy of AB4 in liver cancer. AB4 treatment readily inhibited liver cancer cell proliferation and induced cell apoptosis *in vitro* and *in vivo*. Furthermore, the present study demonstrated that Notch is an important pharmacologic target for AB4. Further experiments evaluating the combinational strategy of AB4 would be interesting.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ and QM designed and supervised the research. ZX and YL performed the majority of the experiments. GL, SX, XL and ZZ analyzed the data. LS, BL, YZ and TM provided technical support in conducting the experiments. All authors wrote the manuscript, read the final manuscript and approved the submission.

Ethics approval and consent to participate

All experiments were approved by the Ethic Committee of The Hospital 971 of The Navy of Chinese People's Liberation Army (Qingdao, China) and were performed in accordance with Animal Ethics Guidelines (#401LL-2017010).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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