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A chalcone derivative SBD-2 exerts anticancer effects in human colorectal cancer cells

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Abstract

Background In this study, the potential anticancer activity and mechanism of action of SBD-2, a chalcone isolated from *Shutteria involucrata*, was investigated in colorectal cancer (CRC) cells.

Results SBD-2 inhibited the proliferation of Caco-2 cells in a dose-dependent manner. It elicited the cells arrested in the G2/M phase and induced apoptosis. Mechanistically, SBD-2 inhibited Akt phosphorylation, which suppressed the anti-apoptotic protein Bcl-2 and cell cycle regulator Cyclin B1, leading to apoptosis and cycle arrest, respectively.

Conclusions The presented chalcone compound SBD-2 from *Shutteria involucrata* induced apoptosis and cell cycle arrest through inhibiting Akt pathway, highlighting the possibility to develop as a new agent for CRC treatment.

Keywords Chalcone, Akt, Colorectal cancer, Traditional Chinese Medicine

Background

As one of the most malignant types of tumors, colorectal cancer (CRC) is emerging with shifts in modern lifestyles and the aging of populations. According to the reports of the World Health Organization (WHO), CRC has become the fourth most common cancer globally. In 2020, adults under 50 years of age experienced a 42% mortality rate and a 32% incidence [3]. Although surgery is considered the most effective treatment, it is limited to early-stage CRC [2]. Consequently, chemotherapy

remains the primary approach for CRC management. However, patients endure severe side effects, diminishing their quality of life [5]. Therefore, identifying and developing natural products with low toxicity has become an appealing strategy for CRC treatment.

Shutteria involucrata belongs to the *Shutteria* genus of the Leguminosae family and grows in the south of Yunnan, China [19]. The plant, also called “Tong Qian Ma Huang,” has been used by the Yi people, an ethnic minority in China, to treat antipyretic colds, coughs, sore throat, chronic bronchitis, and pharyngitis for a long time [23]. As with most folk medicines, little is known about the effective ingredients of *Shutteria involucrata*. SBD-2 (2',3,4,4'-tetrahydroxy-2-prenylchalcone), a chalcone compound, was recently isolated from *Shutteria involucrata* [26]. Chalcone is a common chemical scaffold of many naturally occurring compounds with broad pharmacological activities [18]. Chalcone-based drugs such as metochalcone and sofalcone have been approved as cholagogues and anti-ulcer agents, respectively [10]. However, no biological activities have been reported

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regarding SBD-2. Therefore, in this study, we evaluate the anticancer activities and elucidate the mechanism of SBD-2 in CRC cells.

Methods

Cell culture

MDA-MB-231, MCF-7, and LoVo were cultured in RPMI-1640; HCT-116 was cultured in Dulbecco's modified eagle medium (DMEM); and U87 and Caco-2 cell lines were cultured in minimum essential medium (MEM). Fetal bovine serum (10%), penicillin (100 µg/mL), and streptomycin (100 µg/mL) were purchased from Gibco and supplemented with the culture media. Cells were grown in a 37 °C humidified incubator containing 5% CO₂.

Antibodies and reagents

Primary antibodies against human proteins: Cleaved Caspase-6 (Asp162; cat. no. CST-9761), Cleaved Caspase-9 (Asp315; cat. no. CST-9505), Bcl-2 (cat. no. CST-2876), Bax (cat. no. CST-2772), Akt (cat. no. CST-9272), Phospho-Akt (Ser473) (cat. no. CST-4051), Anti-β-Actin (cat. no. Sigma-A5411). Secondary antibodies anti-rabbit (cat. no. 211-035-109) and anti-mouse (cat. no. 715-035-150) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Sulforhodamine B (SRB), Hoechst 33,258, trichloroacetic acid (TCA), crystal violet, and NP-40 were purchased from Sigma Aldrich (St. Louis, MO, USA). Dr. Zhuya Yang provided SBD-2 from the Yunnan University of Chinese Medicine.

In vitro cell proliferation assay

Cells were seeded into 96-well plates at a density of 1.0×10^4 cells per well and subsequently exposed to varying concentrations of SBD-2 for an incubation period of 72 h. Post-treatment, cell fixation was achieved by adding 50% (v/v) TCA at a volume of 50 µL per well and incubating for 1 h at 4 °C. The cells were stained with 0.4% (w/v) SRB solution (100 µL per well) for 10 min at room temperature. This was followed by a comprehensive wash with 1% acetic acid to eliminate unbound dye. After allowing the plates to air dry, the bound dye was solubilized using a tris base solution (pH 7.4, 10 mM) at a volume of 200 µL per well. Each well's optical density (OD) was measured at a wavelength of 515 nm using a microplate reader (Molecular Devices, Inc.; Sunnyvale, CA, USA). Based on these readings, cell viability and IC₅₀ values were calculated.

Colony formation assay

Caco-2 cells were seeded in 6-well plates at a density of 2000 cells per well. After a 7-day treatment with SBD-2,

the resultant colonies were stained using a 0.2% (w/v) solution of crystal violet in formalin for 10 min. Subsequent imaging of the stained colonies was performed using the "Gel Doc XR+" Imaging System.

Assessment of cell morphological changes

After exposure to SBD-2 for 48 h, cellular specimens were collected, rinsed with PBS, and subjected to staining using Hoechst 33,258 at a final concentration of 0.1 mg/mL (Sigma Aldrich). This staining process was conducted in buffered formalin solution supplemented with 5.6% NP-40 (Sigma Aldrich). Visualizing both viable and apoptotic cells was done under UV light using an upright fluorescence microscope (Leica DM2500 Fluorescence Microscope) at a magnification of $\times 400$.

Flow-cytometric analysis of apoptosis

The FITC Annexin V Apoptosis Detection Kit I quantified cell apoptosis using flow cytometry from BD Bioscience (Franklin Lakes, NJ, USA). Cells were initially plated in 6-well dishes at a density of 200,000 cells per well and were treated with varying concentrations of SBD-2. After an incubation period of 72 h, the cells were collected, washed with chilled PBS, and subsequently suspended in 1× binding buffer. The cells were then incubated with 2.5 µL of FITC Annexin V and 0.25 µg/mL of propidium iodide (PI) for 15 min at room temperature before analysis on the FACS Aria III flow cytometer, as detailed in the article, we published [6].

Western blotting

Cell lysis was performed using RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Basle, Switzerland). The cells were incubated on ice for 10 min before being disrupted. The resulting cell lysate was cleared through high-speed centrifugation to remove insoluble material. The total protein concentration and protein expression detection were carried out following the previously established protocol by our previously established method [6].

Plasmid transfections

The plasmid pT3-myr-AKT-HA, generously provided by Xin Chen (Addgene plasmid # 31789), was introduced into Caco-2 cells via Gene Jet Plus transfection (Signa-Gen Laboratories, Frederick, MD, USA), adhering to the manufacturer's guidelines. Subsequently, stable clones were established and corroborated through Western blot analysis.

Statistical analysis

Unless otherwise specified, all data are presented as mean values accompanied by their standard deviation (SD).

Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA), with significance levels set at * for $p < 0.05$ and ** for $p < 0.01$. When a significant F-value was obtained, Tukey's post hoc test was applied to explore the differences further. The statistical analyses were carried out using GraphPad Prism 8.0.2 software.

Results

SBD-2 inhibits the proliferation of CRC cells

The chemical structure of SBD-2 is depicted in Fig. 1A [26]. To evaluate the antiproliferative effect of SBD-2, we did SRB assays on a panel of cancer cell lines from different tissue origins. As shown in Fig. 1B, SBD-2 substantially inhibited the cancer cell lines with IC_{50} values ranging from 11.38 to 127.9 μ M. Compared to other cancer cell lines, CRCs were relatively sensitive to SBD-2 treatment (Fig. 1B). Thus, we focused on the anticancer activity of CRC and chose Caco-2, the most vulnerable CRC cell line to SBD-2, for the rest of this study. SBD-2 inhibited cell proliferation of Caco-2 dose-dependently with an IC_{50} of 11.38 μ M (Fig. 1C). By the long-term

colony formation assays, the antiproliferative effect of SBD-2 was confirmed (Fig. 1D).

SBD-2 induces cell cycle arrest

Next, we checked whether SBD-2 altered the cell cycle progression of Caco-2 cells, which is the known event for reduced cell proliferation. After 24 h of treatment, SBD-2 significantly increased the distribution of Caco-2 cells in the G2/M phase in a concentration-dependent manner (Fig. 2A, B).

SBD-2 induces mitochondria-mediated apoptosis of Caco-2 cells

The induction of apoptosis is a vital cellular process that contributes to the antiproliferative effects of compounds. To assess the impact of SBD-2 on apoptosis in Caco-2 cells, flow cytometry analysis was conducted following annexin V-FITC/PI staining. After 72-h treatment with SBD-2, there was a significant increase in the percentage of apoptotic cells (annexin V positive) to 72.96% at 50 μ M, 47.27% at 25 μ M, and 33.74% at 12 μ M, compared to the control group at 10.69% (Fig. 3A, B). The nuclei of

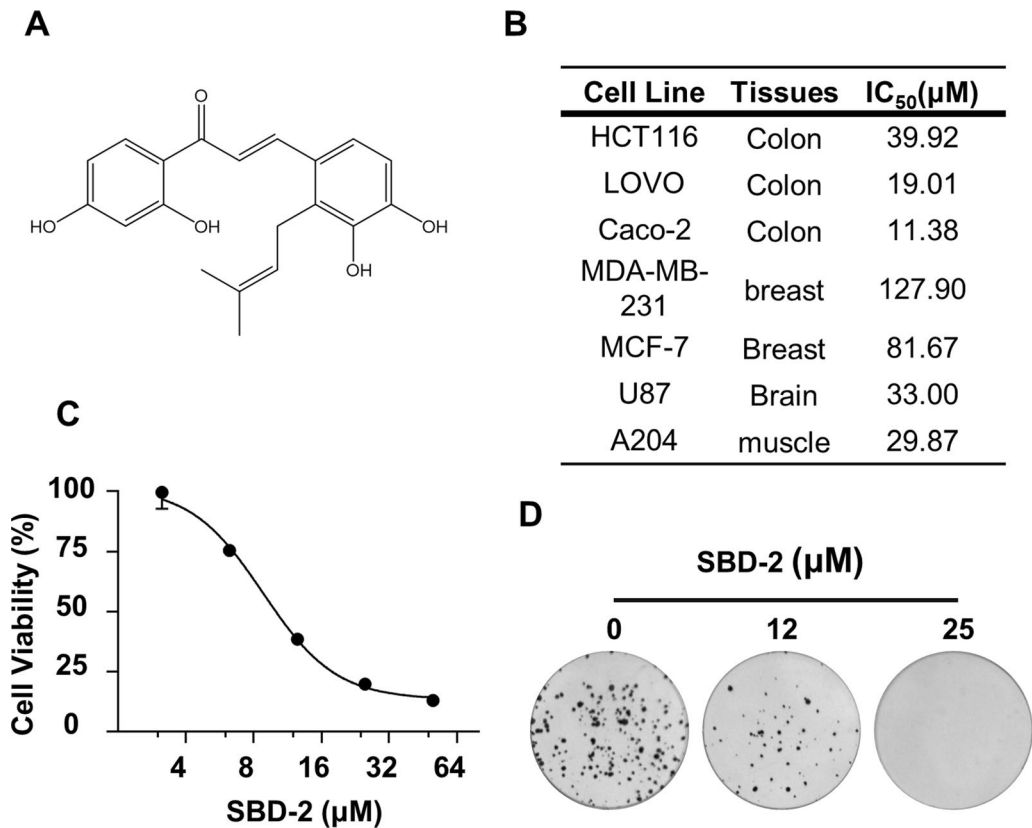


Fig. 1 SBD-2 inhibits the proliferation of CRC cell lines. **A** The chemical structure of SBD-2. **B** The IC_{50} of SBD-2 on different cell lines for 72 h. **C** Caco-2 cell proliferation in the presence of SBD-2 after 72 h exposure measured by the SRB. **D** Dose-dependent colony forming assay of SBD-2 of Caco-2 cells

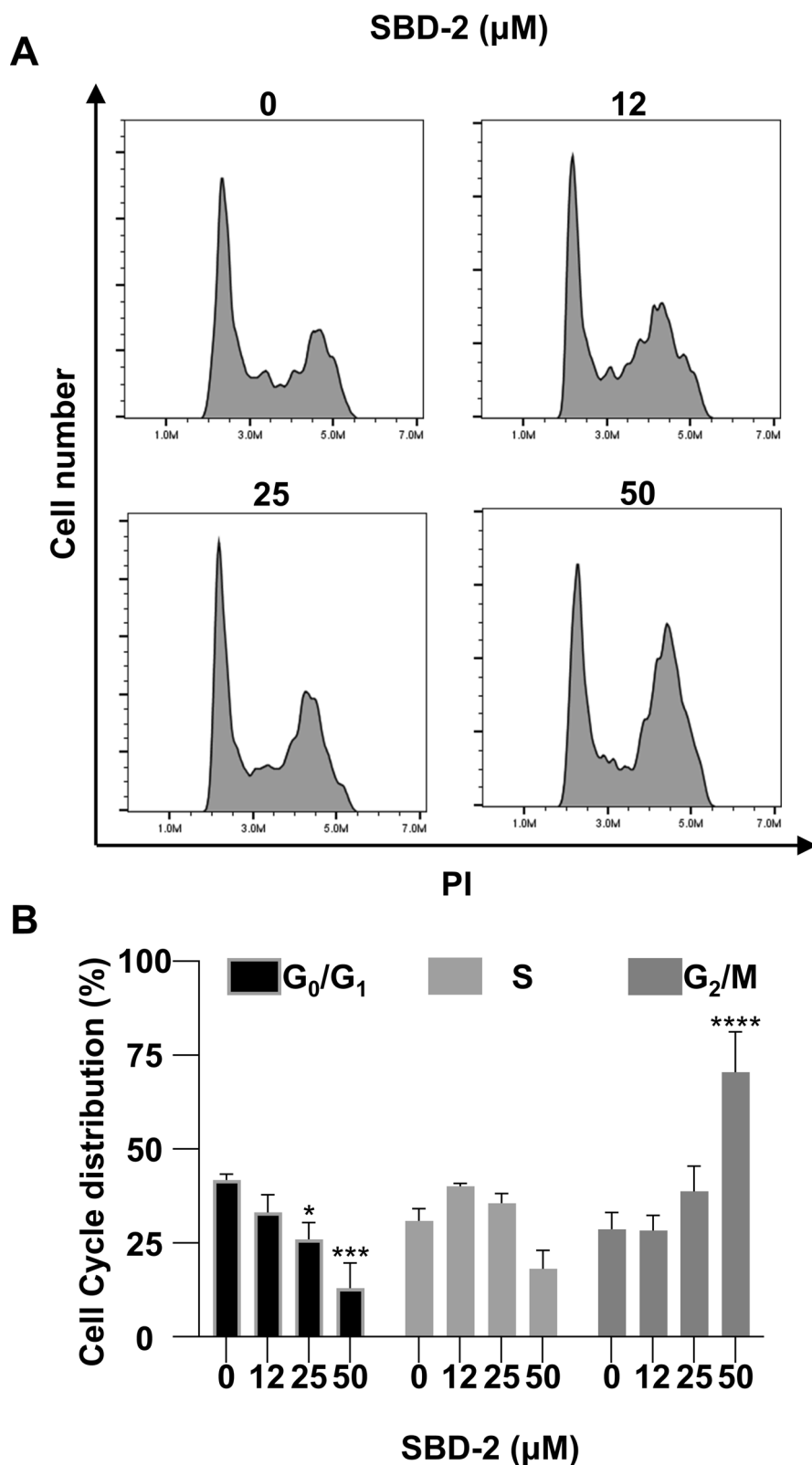


Fig. 2 SBD-2 induces cell cycle arrest in Caco-2 cells. Caco-2 Cells were treated with SBD-2 for 24h. **A** Flow cytometry was used to detect cell cycle distribution. **B** Analyze the cell population in the three phases of the cell cycle ($G_0/1$, S, and G_2/M) by FlowJo software

Caco-2 cells treated with SBD-2 exhibited characteristic apoptotic features such as fragmentation and intense staining with Hoechst 33,258 (Fig. 3C, D). Additionally, pretreatment with Q-VD-OPH, a broad-spectrum caspase inhibitor, protected Caco-2 cells from SBD-2-induced apoptotic cell death (illustrated in Fig. 3E, F). Western blot analysis revealed that SBD-2 treatment triggered the activation of initiator caspase-9 and effector caspase-6 in Caco-2 cells in a dose-dependent manner (shown in Fig. 4A). Furthermore, pretreatment with Q-VD-OPH significantly restored Caco-2 cell proliferation as determined by the SRB assay (Fig. 3G).

SBD-2 inhibits cell proliferation by regulating AKT

To investigate the possible mechanism of SBD-2 inhibited cell proliferation of Caco-2, we analyzed the protein levels in key pathways regulating cell cycle progression and apoptosis, including AKT, mTOR, MYC, and ERK [4, 22]. Among these proteins, only phospho-Akt (Ser473), the active form of Akt, was significantly reduced by SBD-2 (Fig. 4A). Through the regulation of CDC2 and cyclin B1, Akt promotes cell cycle progression [3, 13]. In line with the induction of cell cycle arrest (Fig. 2), SBD-2 significantly decreased cyclin B1 and phospho-CDC2 (Fig. 4A), which are essential proteins for cell cycle progression and whose insufficiency causes G2/M arrest [24]. At the same time, Akt induced the anti-apoptotic protein, Bcl-2 expression, which subsequently forms heterodimers with BAX, thus preventing BAX-mediated apoptosis [8]. Consistent with the point, SBD-2 reduced Bcl-2 protein levels dose-dependently (Fig. 4A).

Our findings suggested that SBD-2 inhibits cell proliferation through Akt-mediated cell cycle and apoptosis pathways. To verify this mechanism, we transfected a construct of Myr-Akt, the constitutively active form of Akt, into Caco-2 cells. Myr-Akt consists of a myristoylation sequence ligated to Akt and is tenfold more active than the wild-type counterpart [11]. As noted, after transfection, Caco-2 cells stably expressed high levels of phospho-AKT, which reversed the protein levels of Bcl-2 and cyclin B1 after SBD-2 treatment (Fig. 4B). Furthermore, Myr-Akt rescued SBD-2 induced apoptosis as visualized by Hoechst 33258 staining (Fig. 4C, D). Cell cycle arrest was also rescued as measured by flow cytometry

(Fig. 4E, F). Finally, the expression of Myr-Akt rescued cell proliferation was inhibited by SBD-2 (Fig. 4G).

Discussion

Chalcone is a molecular scaffold with good drug ability, convenient synthesis, and versatile binding properties [18]. Chalcones exhibit potential anticancer activities, including angiogenesis inhibition, tubulin assembly inhibition, apoptosis induction, and anti-estrogenic activities [7]. Generally, chalcones have received less research attention compared to other constituents found in traditional medicinal plants. Nevertheless, their structural flexibility allows them to interact with numerous drug targets, resulting in diverse pharmacological activities. The efficacy of clinically approved chalcone derivatives, such as metoprolol and sofosbuvir, highlights their potential as promising precursor compounds. SBD-2 is a novel chalcone derivative isolated from *Shutteria involucrata*. In this study, we reported the anticancer activities of SBD-2 for the first time, highlighting its potential implications as a lead compound against CRCs.

This study has exclusively focused on the in vitro anticancer evaluation of SBD-2. This initial investigation yielded prompt and direct insights into the compound's pharmacological properties. Nevertheless, additional validation through in vivo studies is imperative. Another concern of SBD-2 is its bioavailability, a common issue with chalcones, leading to suboptimal efficacy concentrations or restricted access to target proteins upon administration [16]. Additional pharmacokinetic assessments for SBD-2 are warranted, and exploring structural modifications to enhance its bioavailability should also be considered.

Akt, a pivotal survival factor in cellular processes, plays a significant role in human cancer [1]. Activation of Akt is a common alteration in cancer, influencing cell growth and proliferation. Akt activates mTOR kinase, regulates ribosomal biogenesis, and controls protein translation [17]. Akt modulates cyclin/CDK kinases in cell growth regulation, including cyclin B1 [13]. Additionally, Akt demonstrates anti-apoptotic activity by up-regulating Bcl-2 expression in a CREB-dependent manner [25]. Its involvement extends to tumor invasion and metastasis, promoting matrix metalloproteinase secretion and

(See figure on next page.)

Fig. 3 SBD-2 induces the Apoptosis in Caco-2 cells. Caco-2 cells were treated with SBD-2 for 72 h. **A** Representative flow cytometry plots using Annexin V-FITC/PI staining for apoptosis. Caco-2 cells were treated for 72 h and stained with Annexin V-FITC/PI for flow cytometric analysis. **B** Quantification data of apoptotic cells. **C** Visualization of apoptotic morphological changes by fluorescent microscope with Hoechst 33,258 staining. Representative pictures are shown (400x). **D** Percentage of apoptotic cells treated with the three agents together or respectively. **E, F** Representative flow cytometry plots using Annexin V-FITC/PI staining for apoptosis, Q-VD-OPH was able to partially rescue apoptotic cells. **G** Treated with pan-caspase inhibitor Z-VAD(OMe)-Fmk and Q-VD-OPH, and assay about the cell viability

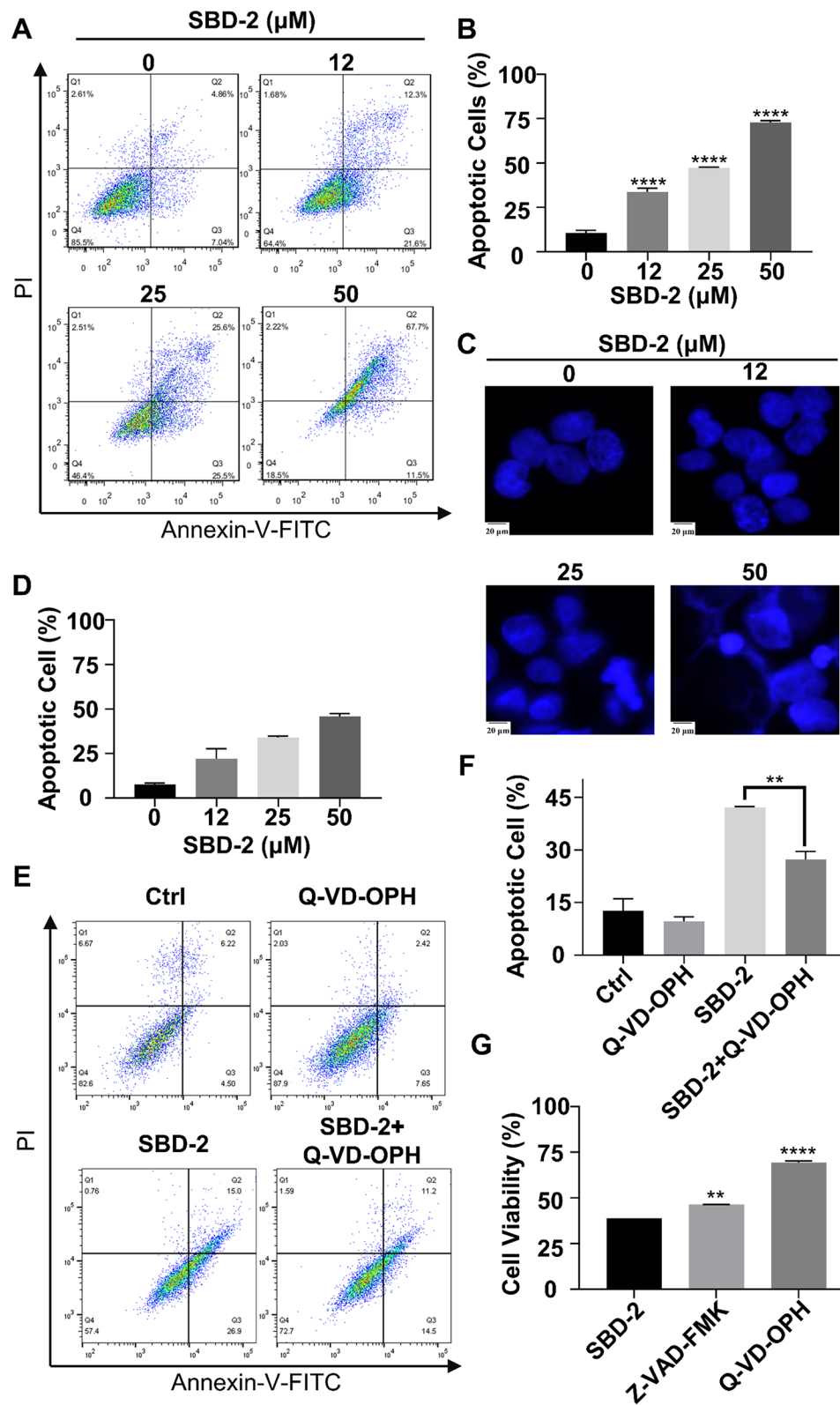


Fig. 3 (See legend on previous page.)

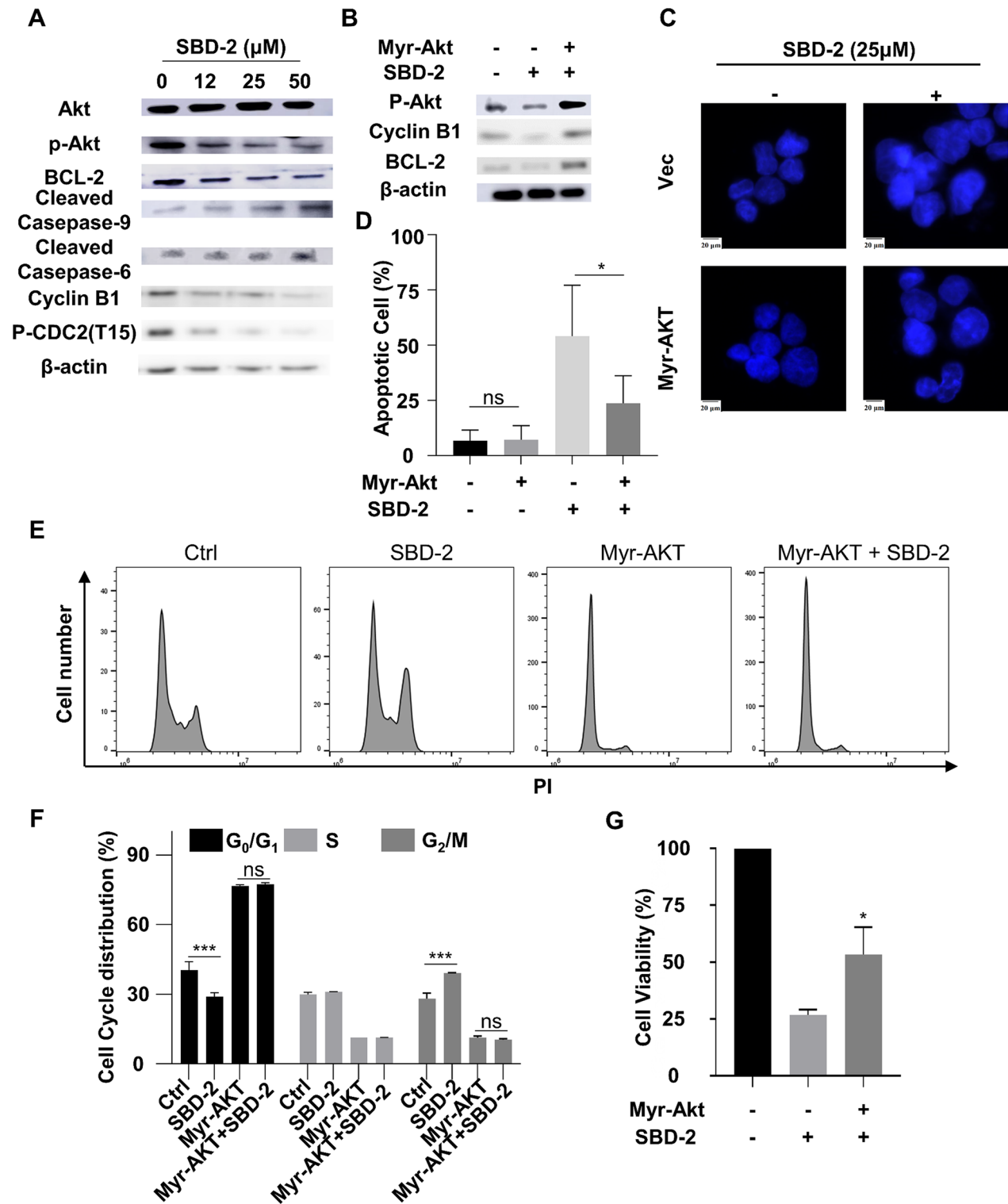


Fig.4 The SBD-2 decreases the phosphorylation of AKT and induces the Caco-2 cell's apoptosis and cell cycle arrest. **A** Western-blot analysis for Caco-2 cells. Caco-2 cells were exposed to 0–50 μM of SBD-2. **B** Overexpression of constitutively activated Akt (Myr-Akt) rescues cyclin D1 and Bcl-2 expression. **C, D** Myr-Akt rescues cell apoptosis in SBD-2 Caco-2 cells. Cell apoptosis was analyzed by fluorescent microscope with Hoechst 33,258 staining. Reprehsive pictures are shown (400x), and the percentage of apoptotic cells treated. **E, F** Myr-Akt rescues cell cycle arrest in SBD-2 treated Caco-2 cells. Cell cycle distribution was analyzed by flow cytometry, and the total cell population in the three phases of cell cycle. **G** SRB assay indicates that Myr-Akt rescues cell viability in the SBD-2 treated Caco-2 cells

inducing epithelial–mesenchymal transition (EMT) [12, 21]. Given its crucial functions, targeting Akt is a promising therapeutic strategy in cancer treatment [15].

However, no Akt inhibitor has been approved for clinical use after 30 years of its discovery [9]. Most Akt inhibitors, such as ipatasertib, uprosertib, and afuresertib, are in preclinical or clinical examination status [14]. It is known that AKT is regulated upstream by either the PI3K kinase or the mammalian target of rapamycin complex 2 (mTORC2) kinase [20]. However, after treatment with SBD-2, we did not observe a change in the PKC protein, the well-known target of mTORC2 (data not shown). So, we speculated that the possible target of SBD-2 may be PI3K or PDK-1, the upstream regulators of Akt in this signaling pathway. Experiments are ongoing to determine the direct target of SBD-2 in our laboratory.

Conclusions

SBD-2 (2',3,4,4'-tetrahydroxy-2-prenylchalcone), the chalcone from *Shutteria involucreata*, induces G2/M cell cycle arrest and apoptosis in Caco-2 cells by inhibiting Akt Phosphorylation, exhibits potent anticancer activity in colorectal cancer cells. Our data suggest that SBD-2 is worthy of further development as a potential chemotherapeutic agent for CRC as a PI3K/Akt inhibitor considering its excellent drug ability of chalcone compounds.

Abbreviations

CRC	Colorectal cancer
DMEM	Dulbecco's modified eagle medium
EMT	Epithelial–mesenchymal transition
MEM	Minimum essential medium
mTORC2	Mammalian target of rapamycin complex 2
PI	Propidium iodide
SAR	Structure–activity relationship
SRB	Sulforhodamine B
TCA	Trichloroacetic acid

Author contributions

All individuals meeting the criteria for authorship are acknowledged as authors, and each author confirms their substantial involvement in the study, enabling them to assume public responsibility for the content. This involvement encompasses participation in the manuscript's conceptualization, design, analysis, writing, and revision. Bowen Zheng and Wanjun Lin contributed equally to this work.

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Availability of data and materials

The data underpinning this study's findings are accessible upon reasonable request from the corresponding author.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have approved the manuscript for publication.

Competing interests

The authors have no conflicts of interest to declare.

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