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Boehmenan, a Lignan From the Chinese Medicinal Plant *Clematis armandii*, Inhibits A431 Cell Growth via Blocking p70S6/S6 Kinase Pathway

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Abstract

Previously, we have shown that boehmenan, a natural product isolated from the dried stem of *Caulis clematidis armandii*, exhibits various biological activities. The current study investigated the effects of boehmenan on the growth of human epidermoid carcinoma A431 cells. Cell viability and 50% inhibiting concentration (IC_{50}) were assessed by CellTiter-Glo luminescent cell viability assay. Cell cycle arrest was measured by flow cytometry. Intracellular reactive oxygen species production and mitochondrial membrane potential ($\Delta\Psi_m$) collapse were analyzed by a fluorescence spectrophotometer. The activation of epidermal growth factor receptor signaling pathway was evaluated by Western blot. The results showed that boehmenan significantly inhibited the growth of A431 cells (IC_{50} = 1.6 μM) in a concentration- and time-dependent manner. This compound also blocked cell cycle progression at G2/M phase and modulated mitochondrial apoptosis-related proteins, as evidenced by upregulating p21, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase protein levels and by downregulating Bcl-2, pro-caspase-9 levels. In addition, boehmenan also markedly induced intracellular reactive oxygen species production and $\Delta\Psi_m$ depolarization in a concentration-dependent manner. Furthermore, boehmenan-attenuated epidermal growth factor mediated the phosphorylation of signal transducer and activator of transcription 3 (STAT3), p70 ribosomal protein S6 kinase (p70S6)/S6 in a concentration-dependent manner. Taken together, our results suggest that boehmenan-mediated antiproliferative property in A431 cells was mediated partially by modulation of mitochondrial function and inhibition of STAT3 and p70S6 signal pathways.

Keywords

boehmenan, A431, proliferation, cell cycle, p70 ribosomal protein S6 kinase

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Introduction

Squamous cell carcinoma (SCC) is the second most common nonmelanoma cancer of the skin.[1] The incidence of SCC has been steadily increasing during the past decades.[2] The treatment of SCC including surgery, radiotherapy, or chemotherapy, however, is still unsatisfactory. Therefore, there is a great need for more effective chemotherapeutic agents. Currently, novel therapies with traditional herbs or natural products are used for the treatment of cancer patients.[3]

Uncontrolled proliferation and reduced apoptosis are 2 critical hallmarks of tumor cells. Inducing cell apoptosis is one of the therapeutic strategies to manage the problem of tumor growth.[4,5] The mitochondrial pathway of apoptosis is mitochondria dependent,[6] involving changes of the ratio of Bax/Bcl-2 family proteins, which is followed by the activation of caspase-9 and -3, causing apoptosis.[7,8] The collapse of mitochondrial membrane potential ($\Delta\Psi_m$) is one of the most important mechanisms of mitochondrial pathway.[4] Meanwhile, excessive levels of intracellular reactive oxygen species (ROS) may be one of the major causes that triggers the apoptosis of cancer cells.[5] Epidermal growth factor

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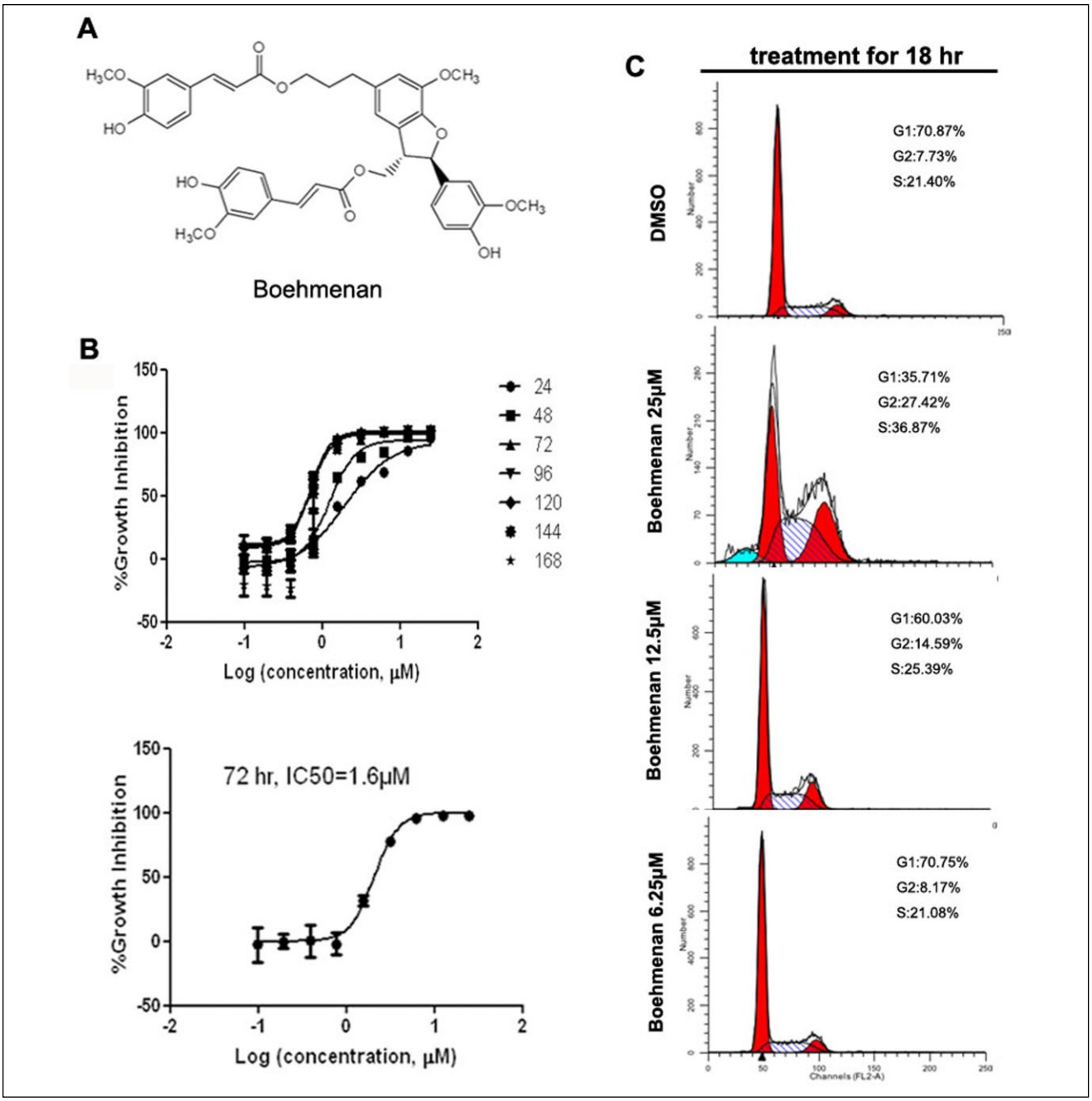

Figure 1. Effects of boehmenan on cell proliferation and cell cycle arrest. (A) The chemical structure of boehmenan. (B) A431 cells were treated with indicated concentrations of boehmenan for different periods; the proliferation of A431 cells was measured and IC_{50} was calculated after 72-hour treatment. (C) A431 cells were treated with indicated concentrations of boehmenan for 18 hours. The cell cycle distribution was measured by a flow cytometer; representative flow cytometric histograms of the distribution of cell cycle after treatment for 18 hours.

receptor (EGFR), a cell surface receptor for peptide growth factor, plays crucial roles in regulating cell survival, proliferation, migration, and differentiation.[9,10] Aberrant activation of EGFR and its downstream pathways is characteristic of various cancer types and an important step toward tumorigenesis or disease progression in various tumors.[11-14] Thus, targeting EGFR or their downstream pathways might be important therapeutic approaches to treat various tumors, including SCC.[14,15]

Boehmenan (Figure 1A), a lignan, has been isolated and identified from the dried stem of *Caulis clematidis armandii*. The dried stem of *C armandii* is listed as a popular

herb medicine named "Chuan-Mu-Tong" in Chinese Pharmacopoeia and has been long used in the treatment of inflammatory conditions, such as rheumatism, urinary tract infection, and so on.[16] Numerous studies have shown that boehmenan exhibited potent cytotoxic effects against many cancer cell lines.[17,18] However, the anticancer potential of boehmenan and underlying mechanisms on SCC cells are still unknown. To this end, in the present study, we investigated the growth inhibitory effects of boehmenan on A431 cells in vitro and the possible underlying mechanisms of action.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), MitoTracker Red CMXRos, and fetal bovine serum (FBS) were from ThermoFisher Scientific (Shanghai, China). Antibodies against total- and phosphor (p)-signal transducer and activator of transcription 3 (STAT3) (Tyr^{705}), total- and p-EGFR, total- and p-extracellular signal-regulated kinase 1/2 (ERK) (Thr^{202}/Tyr^{204}), total- and p-Akt (Ser^{473}), total- and p-mitogen-activated protein kinase (MEK), total- and p-p70 ribosomal protein S6 kinase (p70S6), total- and p-S6, p21, pro-caspase-9, active caspase-3, cleaved PARP-1, and Bcl-2 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). EGF and others chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO), if not stated otherwise. Boehmenan (Figure 1A), a lignin, was recently isolated and identified from the stems of *C armandii*, and the purity was more than 98%, as determined by high-performance liquid chromatography. Boehmenan was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was less than 0.1%, and the same amount of dimethyl sulfoxide was in the control cells as negative control.

Cell Culture

The human epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection (Manassas, VA) and routinely maintained in DMEM containing 1800 mg/L $NaHCO_3$, supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Cell Viability

Cell viability was measured using a CellTiter-Glo kit (Promega, Madison, WI) following the manufacturer's protocol. Briefly, cells seeded at 3×10^4 cells/mL in a 96-well plate were incubated with indicated concentrations of boehmenan for indicated periods. After 0 to 168 hours (in 24-hour intervals), luciferin and Ultra-Glo recombinant luciferase were then added to each well, and the plate was incubated at room temperature for 10 minutes with constant shaking. Luminescence intensity was read using a microplate reader (Safire, TECAN, Austria GmbH, Vienna, Austria), taking the luminescence reading of vehicle control as the 100% value.

Cell Cycle Analysis Using Flow Cytometry

Cells were seeded on a 6-well plate of 1.2×10^5 cells/mL and then switched to serum-free media to synchronize the cell cycle. After 24 hours, the media were changed to complete media containing boehmenan. Cells were harvested after 18 hours by trypsinization and washed with cold phosphate-buffered saline (PBS) and fixed in ice-cold ethanol (70%) overnight. Finally, the cells were centrifuged and cell pellets were resuspended in PBS containing 5 μg/mL RNase A (Sigma) and 50 μg/mL propidium iodide (Sigma) for analysis. Cell cycle analysis was performed using the FACScan Flow Cytometer (Becton Dickson, San Jose, CA) according to the manufacturer's protocol. The Modfit software was used to calculate the cell cycle phase distribution from the resultant DNA histogram, and expressed as a percentage of cells in the G1 (G0/G1), S, and G2 (G2/M) phases. Each assay was performed in triplicate, and the standard deviation was determined.

Intracellular ROS Measurement

The production of intracellular ROS is evaluated using a H_2DCF-DA probe as described previously.[19] Briefly, A431 cells were plated in 24-well plates (1×10^6 cells per well) and incubated in the presence or absence of boehmenan (6.25-25 μM) for 16 hours. After removing boehmenan, the cells were incubated with H_2DCF-DA (10 μM) for 30 minutes, and the fluorescence intensity was measured by a fluorescence spectrophotometer (M1000, TECAN, Austria GmbH, Vienna, Austria) using excitation/emission of 485/530 nm, and the relative ROS level is normalized to the control values (set as 1).

$\Delta\Psi_m$ Assessment

The $\Delta\Psi_m$ loss is evaluated using a MitoTracker Red probe as described previously.[4] In brief, A431 cells were plated in 24-well plates (1×10^6 cells per well) and incubated in the presence or absence of boehmenan (6.25-25 μM) for 16 hours. Cells were incubated with MitoTracker Red (100 nM) for 30 minutes before being fixed (4% paraformaldehyde), rinsed twice with PBS, and detected by a fluorescence spectrophotometer (M1000, TECAN, Austria GmbH)

using excitation/emission of 579/599 nm. The relative $\Delta\Psi_m$ level is normalized to the control values (set as 1).

Growth Factor Stimulation and Western Blot

After starvation for 24 to 48 hours, cells were then pretreated with the desired concentration of boehmenan in serum-free medium for 24 hours, then stimulated for 30 minutes with 100 ng/mL EGF. The reaction was stopped by the addition of ice-cold PBS.

For Western blot analysis, equal amounts of protein extract were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare), which was then blocked for 1 hour. The membrane was further incubated overnight at 4°C with primary antibodies. Then, membranes were incubated for 1 hour at room temperature with IRDye 800CW conjugated anti-mouse or anti-rabbit IgG as the secondary antibody (Licor, Lincoln, NE) for infrared detection by Odyssey Infrared Detection System (Licor). The density of the signals was quantified with the AlphaEase software.

Statistical Analysis

Results of the experimental studies were expressed as mean ± standard error of the mean (SEM). The computer software PRISM (version 4, GraphPad, Inc) was used to make graphs and all statistical analyses. Differences between mean values of multiple groups were analyzed by one-way analysis of variance with Tukey's test for post hoc comparisons. Statistical significance was considered at $P < .05$.

Results

Boehmenan Inhibited the Proliferation of A431 Cells

We examined the effect of boehmenan treatment on the viability of A431 as determined by CellTiter-Glo kit assays. The A431 cells were treated with boehmenan at concentrations ranging between 1 and 50 μM for 24 to 168 hours, and the number of viable cells was determined. The results revealed that boehmenan markedly inhibited the proliferation of A431 cells in a concentration- and time-dependent manner (Figure 1B). The inhibitory rate at 72 hours was significantly higher compared with others times, and the 50% inhibiting concentration (IC_{50}) was 1.6 μM at 72 hours.

Boehmenan Induced G2/M Phase Cell Cycle Arrest

To further investigate the effect of boehmenan on cell growth, we analyzed the effect of boehmenan on the cell cycle distribution of A431 cells. As shown in Figure 1C, compared with untreated control cells, boehmenan (6.25-25 μM) induced an accumulation of cells in the G2/M phase, accompanied by a decrease in the number of cells in the G1 phase in a concentration-dependent manner. This finding suggested that G2/M phase arrest by boehmenan is, at least in part, due to profound alterations in the expression of regulatory cell cycle–related factors.

Boehmenan Induced Intracellular ROS Production and $\Delta\Psi_m$ Depolarization

Increased intracellular ROS generation and $\Delta\Psi_m$ collapse are involved in the induction of apoptosis through various pathways. Therefore, the formation of intracellular ROS production in A431 cells was determined to evaluate the possible mechanism of boehmenan-mediated antitumor activity. As shown in Figure 2A, treatment with boehmenan for 16 hours concentration-dependently induced intracellular ROS generation. Meanwhile, boehmenan treatment for 16 hours also significantly induced $\Delta\Psi_m$ depolarization in a concentration-dependent manner (Figure 2B).

Boehmenan Modulated p21 and Expression of Apoptosis-Related Proteins

In order to investigate the possible mechanisms of boehmenan on A431 cells, cell cycle protein p21 and apoptosis-related proteins (p53, pro-caspase-9, cleaved-caspase-3, and cleaved-PARP) were analyzed by Western blot (Figure 3A). We first analyzed the effect of boehmenan on p21 expression in A431 cells. Consistent with cell cycle arrest, the expression level of p21 concentration-dependently increased after boehmenan treatment for 8 hours, but not 18 hours or 24 hours (Figure 3B). Meanwhile, boehmenan treatment markedly induced degradation of caspase-9 and significantly increased cleaved-caspase-3 and cleaved-PARP, a known target of caspase-3, in time- and concentration-dependent manners (Figure 3C, D, and E). In addition, anti-apoptotic protein Bcl-2 expression was downregulated by boehmenan treatment (Figure 3F).

Boehmenan Inhibited EGF-Induced EGFR and Its Related Signaling Activation

To identify the possible signaling mechanism of boehmenan-mediated antitumor activity, we analyzed the activation of EGFR and its downstream signaling pathways. EGF (100 ng/mL) stimulation markedly induced EGFR (Figure 4A), STAT3 (Figure 4B), MEK (Figure 4C), and ERK (Figure 4D) phosphorylation, and boehmenan only concentration-dependently suppressed the EGF-mediated STAT3 phosphorylation, while it showed no effects on EGFR, MEK, or ERK phosphorylation (Figure 4).

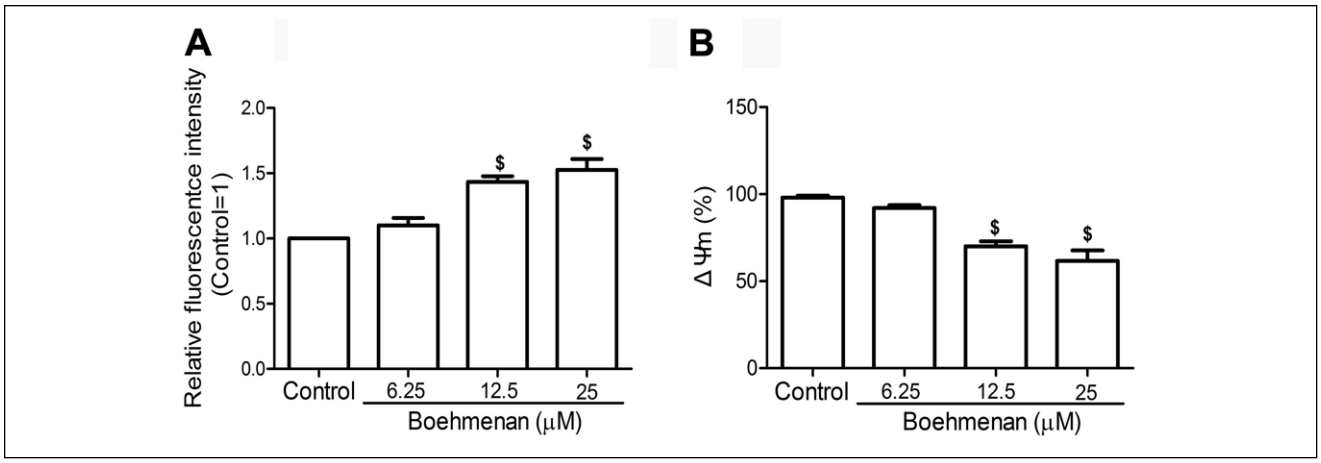

Figure 2. Boehmenan-induced intracellular ROS production and $\Delta\Psi_m$ depolarization A431 cells were treated with indicated concentrations of boehmenan for 16 hours, and the intracellular ROS production and $\Delta\Psi_m$ depolarization were analyzed. Quantitative analysis of intracellular ROS production (A) and $\Delta\Psi_m$ loss (B). Data shown are means ± SEM. $^{\$}P < .05$, compared with control cells; Data were from at least 3 independent experiments, each performed in duplicate.

Furthermore, we also examined the effects of boehmenan on Akt/70S6 pathway activation, which is an important oncogenic signaling pathway involving growth and migration of cancer cells.[20] As shown in Figure5A to C, Western blot results revealed that boehmenan concentration-dependently inhibited EGF-induced 70S6 and S6 phosphorylation, but did not affect the Akt phosphorylation.

Discussion

SCC is the second most common type of nonmelanoma cancer of the skin and many efforts have been made by researchers during past several decades to develop novel chemotherapeutic agents. The present study revealed that boehmenan, a bioactive lignin isolated from the dried stem of *C armandii*, strongly exerted potential anti-proliferative activity on A431 cells. Boehmenan caused G2/M cell cycle arrest, which was accompanied by an increase in p21 protein level; the cell death was due to the induction of intracellular ROS generation and $\Delta\Psi_m$ depolarization. In addition, boehmenan also modulated various apoptosis-related proteins involved in the apoptotic pathway, especially Bcl-2, caspase-3, caspase-9, and PARP. We further demonstrated that induction of anti-proliferative capacity by boehmenan involved inactivation of EGF-mediated 70S6/S6 and STAT3 signal pathways.

Proliferation and apoptosis are 2 critical hallmarks of tumor cells, and suppression of proliferation and induction of apoptosis are 2 principal mechanisms of antitumor drugs. The present study clearly demonstrated that boehmenan exerted anti-proliferative activity on A431 cells, with an IC_{50} value of 1.6 μM. Tumor suppressor p21 is known to be involved in cell cycle arrest and apoptosis. The upregulation of p21 resulted in G_0/G_1 or G_2/M phase cell cycle arrest.[21,22] Consistent with the reports, treatment with boehmenan resulted in arresting the A431 cells at the G_2/M phase, manifested by induction of p21 expression. It is well known that the process of apoptosis can be induced either by the extrinsic pathway or by the intrinsic pathway.[23] The intrinsic signaling pathway of apoptosis involves mitochondrial dysfunction.[6] Mitochondrial dysfunction triggers intracellular ROS production and $\Delta\Psi_m$ loss, which further causes degradation of caspase-9 and caspase-3 and subsequent cleavage of specific cellular death substrates (eg, PARP), ultimately leads to cell apoptosis.[24] In the present study, boehmenan treatment significantly induced production of intracellular ROS, loss of $\Delta\Psi_m$, degradation of caspase-9, and cleavage of caspase-3 and PARP. In addition, our results also demonstrated that boehmenan time- and concentration-dependently decreased the anti-apoptotic factor Bcl-2 expression. Collectively, the present data demonstrated that boehmenan exerted antitumor activities in A431 cells partly via activation of the intrinsic apoptotic pathway.

EGFR is an intracellular domain with tyrosine kinase activity, and aberrant activation of EGFR is implicated to be the driver in a variety of cancer types.[12,25] Once bound with EGF, EGFR undergoes a series of regulated processes to activate distinct downstream kinases, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase (PI3K)/Akt, and STAT3. Previous studies have shown that the STAT3 signaling pathway is overactivated and contributes to tumor progression and resistance to apoptosis in malignancies.[26,27] Phosphatidylinositol 3-kinase (PI3K)/Akt is downstream of EGFR signaling and plays a central role in the regulation of cell growth, survival, and proliferation.[14,28] Its dysregulation is thought to be an important step toward tumorigenesis or disease progression in various tumors.[29]

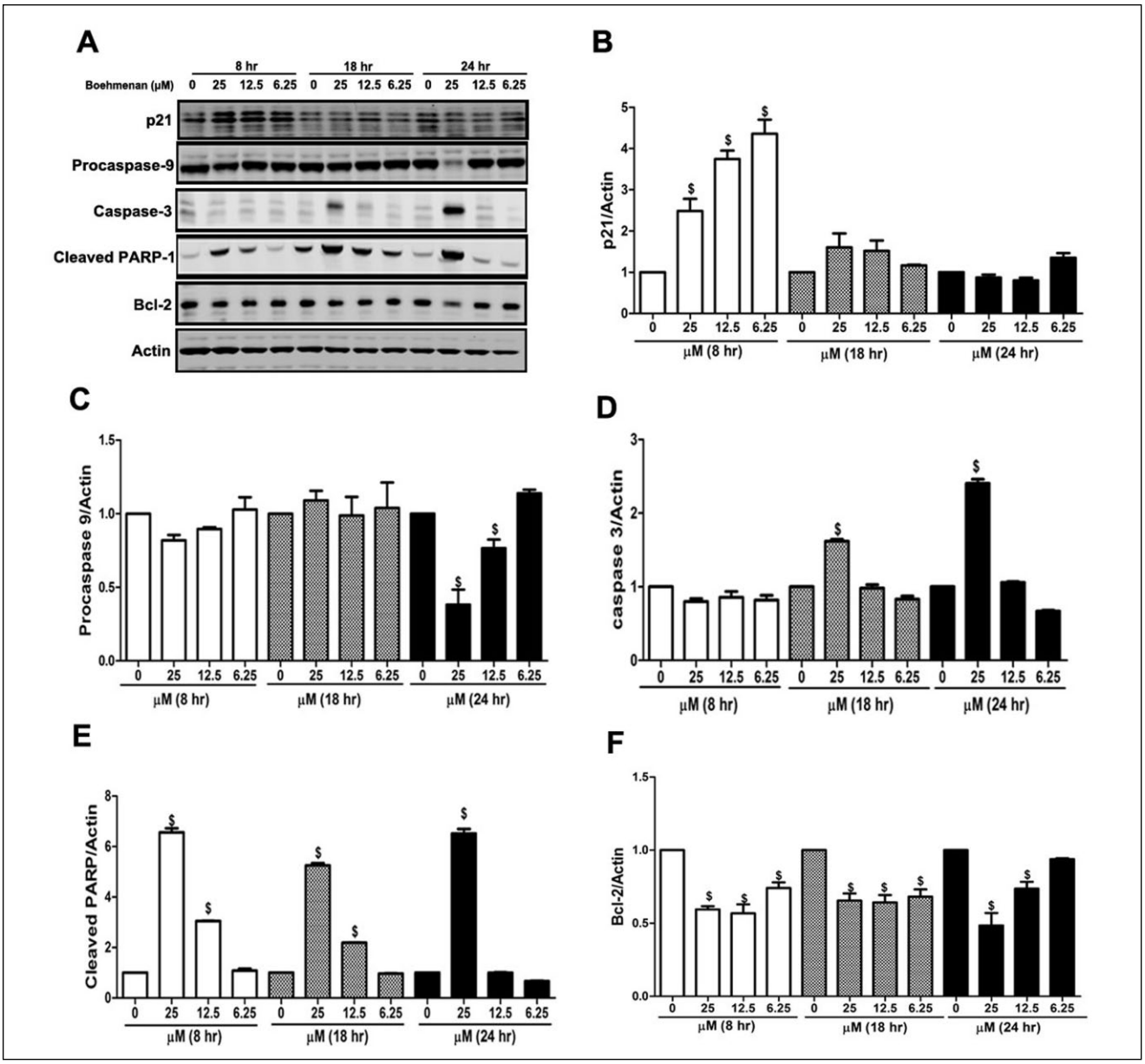

Figure 3. Boehmenan modulated p21 and apoptosis-related proteins expression. A431 cells were treated with indicated concentrations of boehmenan for different periods; the levels of p21, pro-caspase-9, cleaved caspase-3, cleaved PARP, and Bcl-2 were analyzed by Western blot. (A) Representative picture showed bands of p21, pro-caspase-9, cleaved-caspase-3, cleaved PARP, and Bcl-2. Bar graph shows quantitative analysis of p21 (B), pro-caspase-9 (C), cleaved caspase-3 (D), cleaved PARP (E), and Bcl-2 (F). β-Actin was used as loading control. Data shown are means ± SEM. $^{\$}P < .05$, compared with control cells. Data were from at least 3 independent experiments, each performed in duplicate.

Therefore, inhibition of STAT3 or Akt is associated with induced cell cycle arrest and conveyed antitumor effects.[30] Hence, the efficacy of proliferation inhibition by boehmenan through STAT3 activation was investigated. We observed a significant inhibition of STAT3 phosphorylation in A431 cells by boehmenan signifying their growth inhibitory potential. p70S6, a serine/threonine kinase, is a downstream signal of the PI3K/Akt/mammalian target of rapamycin and mitogen-activated protein kinase cascades.[13] On activation, p70S6 subsequently phosphorylates ribosomal protein S6, which, in turn, promotes protein translation and cell growth.[13] In addition, we also found that boehmenan remarkably inhibited the expression of p-Akt, p-70S6, and p-S6, which is critical for cell survival and resistant to apoptosis.[29]

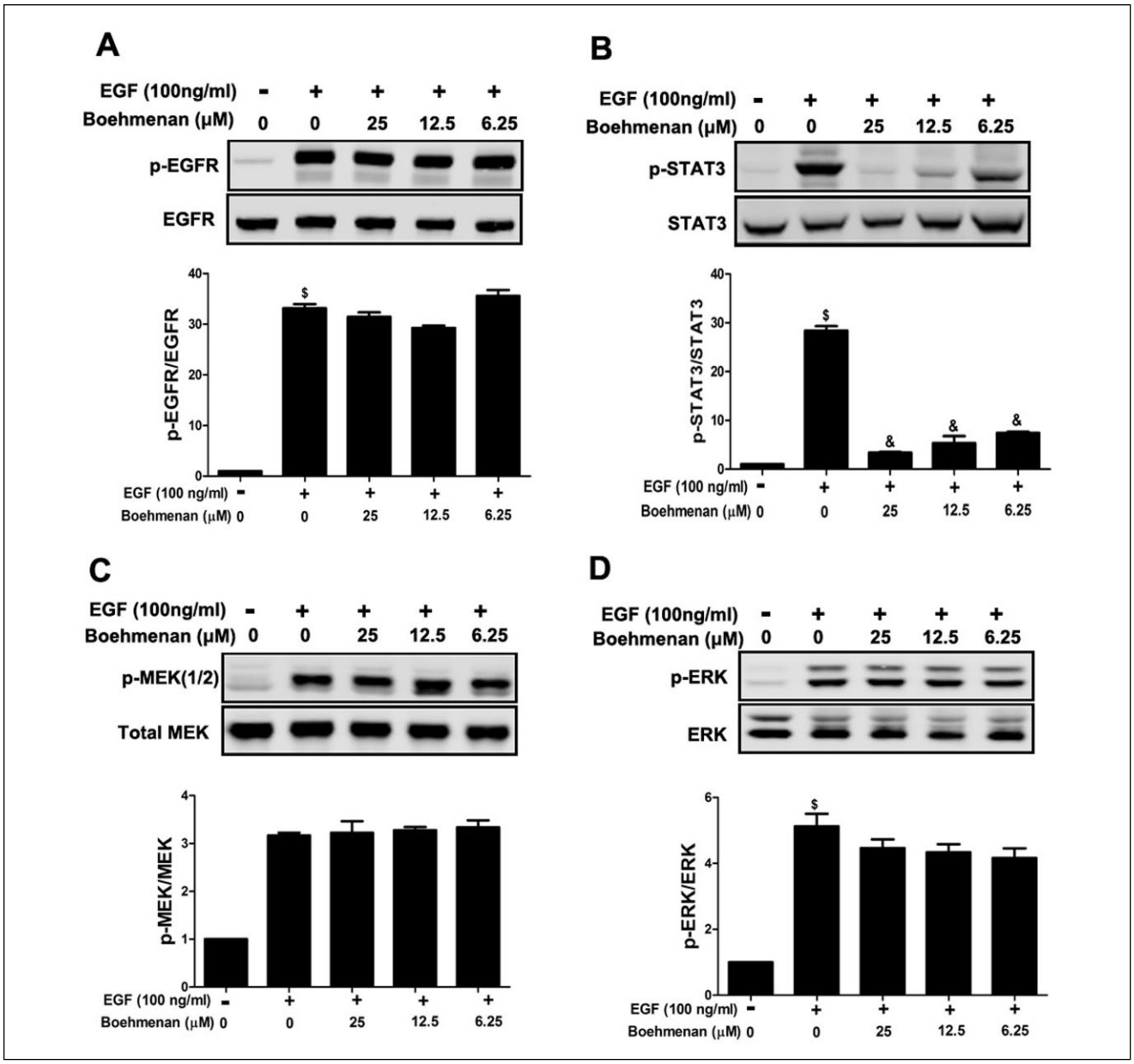

Figure 4. Boehmenan inhibited EGF-induced STAT3 activation. A431 cells were pretreated with boehmenan (6.25-25 μM) for 4 hours and then stimulated with EGF (100 ng/mL) for 30 minutes; the expression of phosphor (p)- and total-EGFR, MEK, ERK, and STAT3 was analyzed by Western blot. Bar graphs and quantitative analysis for p-EGFR/EGFR (A), p-STAT3/STAT3 (B), p-MEK/MEK (C), and p-ERK/ERK (D). Data shown are means ± SEM. $^{\$}P < .05$, compared with control cells; $^{\&}P < .05$, compared with EGF-stimulated cells. Data were from at least 3 independent experiments, each performed in duplicate.

Thus, suppression of STAT3 and PI3K/Akt pathway contributed to cell apoptosis and inhibition of cell survival triggered by boehmenan. A limitation of our study was the absence of a suitable positive control when interpreting our findings. We suggest that this limitation might be caused by the unclear target of boehmenan. This limitation could be circumvented through further research on the target, which will be the next chapter in this story.

Conclusion

The present study showed that boehmenan has cell proliferation inhibitory effects in A431 cells via induction of cell cycle arrest and intrinsic apoptotic pathway. Additionally, specific inhibition of STAT3 and 70S6/S6 activation by boehmenan also contributed to its anti-proliferative property on A431 cells (Figure 6). Therefore, boehmenan might be a promising candidate for treating SCC.

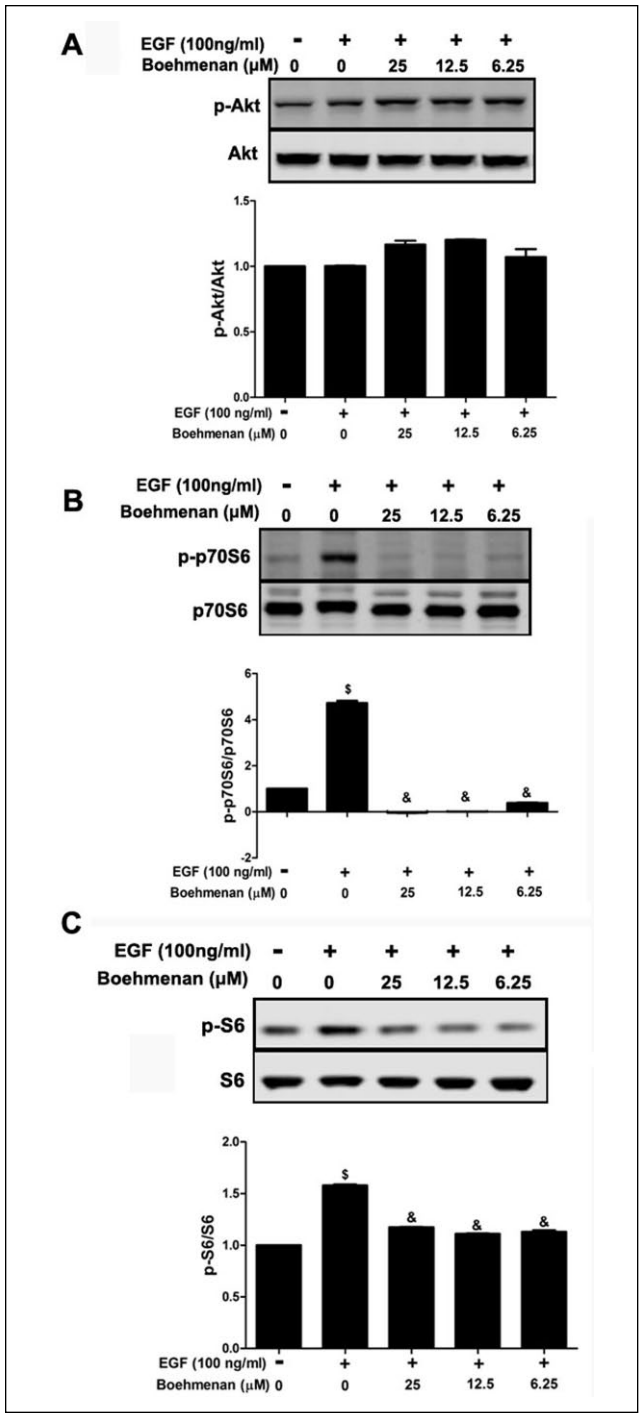

Figure 5. Boehmenan modulated EGF-mediated 70S6/S6 signaling activation. A431 cells were pretreated with boehmenan (6.25-25 μM) for 4 hours and then stimulated with EGF (100 ng/mL) for 30 minutes; the expression of p- and total-Akt, 70S6, and S6 was analyzed by Western blot. Bar graphs and quantitative analysis for p-Akt/Akt (A), p-70S6/70S6 (B), and p-S6/S6 (C). Data shown are means ± SEM. $^{\$}P < .05$, compared with control cells; $^{\&}P < .05$, compared with EGF-stimulated cells. Data were from at least 3 independent experiments, each performed in duplicate.

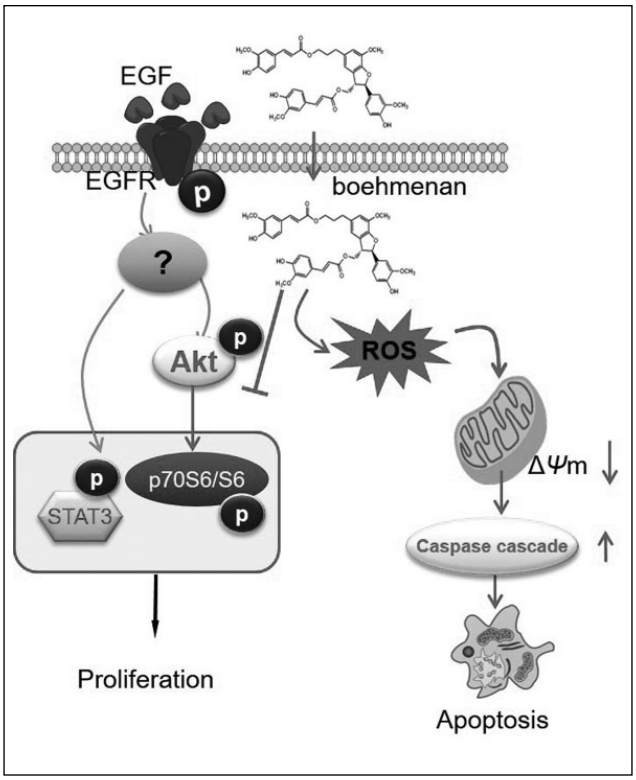

Figure 6. Proposed working model by which boehmenan exerted anti-proliferative activities in A431 cells. Boehmenan executed its anti-proliferative activities in 2 ways: by inhibiting STAT3 and p70S6 signaling pathways that ultimately suppressed EGF-mediated cell growth; and by disrupting mitochondrial function leading to apoptosis that indirectly repressed the proliferation of A431 cells.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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