Botrychium schaffneri Underw. extract acts via DIABLO to induce apoptosis and inhibit proliferation of non-small cell lung carcinoma in vitro and in vivo

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Contributions: (I) Conception and design: YJ Liu, P Huang; (II) Administrative support: P Huang; (III) Provision of study materials or patients: YW Zhang, L Li; (IV) Collection and assembly of data: SL Zheng, XC Zheng; (V) Data analysis and interpretation: HY Ding, WX Xin, J Sun, L Li; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

# These authors contributed equally to this work.

Background: Botrychium schaffneri Underw. has been popularly consumed since ancient times as a traditional medicine in China to treat whooping cough, bronchial asthma, and febrile convulsive twitch disease. This led us to investigate whether Botrychium schaffneri Underw. extract (BSE) may be effective against lung cancer, especially non-small cell lung carcinoma (NSCLC).

Methods: In this study, we extracted the ethanolic root extract of the grass, Botrychium schaffneri Underw. In vitro study, the change of NCI-H1299 cell proliferation was observed with CCK8 and MTT assays. Cell apoptosis was assessed using a kit based on staining with FITC-conjugated annexin V.

In vivo study, we establish a stable animal model of NSCLC in nude mice, tumor volume and weight was measured twice a week. We conduct gene microarray screened for differentially expressed genes (DEGs), between NCI-H1299 cells treated by BSE or not. Then the DEGs were functionally annotated and path enriched.

Results: It was revealed that BSE significantly suppressed NSCLC cell proliferation (IC50 134 μg/mL) and induced apoptosis. It also slowed tumor growth without affecting body weight, and a dose of 25 g/kg led to significantly smaller tumors than in control animals (13.85±3.36 vs. 23.40±6.05, P=0.044). Apoptosis-related protein direct IAP Binding protein with low PI (DIABLO) expression was up-regulated by BSE, and DIABLO knockdown significantly attenuated the anti-tumor effects of the extract.

Conclusions: In conclusion, BSE reduces the viability of NSCLC cells and promotes apoptosis, and these effects may be mediated by DIABLO.

Keywords: Botrychium schaffneri Underw. extract (BSE); non-small cell lung carcinoma (NSCLC); apoptosis; direct IAP Binding protein with low PI (DIABLO)

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Introduction

Lung cancer is a major cause of cancer-related deaths worldwide. Non-small cell lung carcinoma (NSCLC) accounts for approximately 80% of lung cancer diagnoses and is associated with a 5-year survival rate of only 11% (1). Platinum-based chemotherapy and radiotherapy are widely used treatments, but NSCLC can develop resistance to both, leading to relapse when the primary tumor becomes metastatic (2,3). Therefore, a novel, efficient, and low-toxicity therapy for NSCLC is urgently needed.

An ethanolic extract of the entire root of the grass *Botrychium schaffneri* Underw. (BSE) has been used in China for centuries to treat whooping cough, bronchial asthma, and febrile convulsive twitch disease (4,5). More recently, BSE has been shown to have expectorant and anti-inflammatory effects. It has also been shown that BSE and its components exert anti-inflammatory and anti-allergic effects in mice, as well as inhibit the proliferation of skin papilloma (6,7). In our previous work, we showed that BSE may be effective against severe asthma (8). This led us to question whether BSE may be effective against lung cancer, in particular NSCLC.

In this study, we examined whether BSE exerted anti-tumor effects over NSCLC cell lines in vitro and over NSCLC xenografts in mice. We also examined whether anti-tumor effects of the extract involved altering the expression of DIABLO, also known as the “second mitochondria-derived activator of caspases” (Smac). The DIABLO protein is released from mitochondria and reactivates caspases that have been suppressed by inhibitors of apoptosis proteins (IAP) (9,10), and DIABLO down-regulation has been linked to cancer progression. These results suggest that DIABLO may help protect against cancer. Consistent with this, the Smac mimetic JP1201 sensitizes NSCLC to combination chemotherapy (11).

We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5409).

Methods

Cell culture and transfection

The human NSCLC cell line NCI-H1299 (CellCook Biotech, Guangzhou, China) was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS; GE Healthcare Life Sciences Hyclone Laboratories, Chicago, IL, USA), penicillin (100 units/mL), and streptomycin (100 mg/mL) at 37 °C in 5% CO₂. A day before transfection, cells were seeded into 6-well plates, cultured overnight, then transfected with a short interfering RNA against DIABLO (si-DIABLO) or a negative control RNA (si-NC) (Biomics Biotechnologies, Jiangsu, China). Transfections were performed for 6 h using Lipofectamine® 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer’s protocol. We chose 3 different si-DIABLO sequences (Table 1); si-DIABLO-3 showed the highest efficiency and was chosen for subsequent assays (Figure S1). After transfection, the medium was replaced with fresh RPMI-1640 containing 10% FBS and antibiotics, as described above.

*Botrychium schaffneri* Underw. extract

In this study, we used the ethanolic root extract of the grass, *Botrychium schaffneri* Underw., which is known as *Sceptridium ternatum*, Yin Di Jue, or Xiao Chun Hua. *Botrychium schaffneri* Underw. was collected from Lishui in the Zhejiang Province, China (118°41′E-120°26′E, 27°25′N-28°57′N; voucher specimen ZJ-20120926), and the species was confirmed by Professor Xi-Lin Chen (Zhejiang Chinese Medicine University) to meet the specifications in the Chinese Pharmacopoeia. *Botrychium schaffneri* Underw. (10 kg) was mixed with 10 volumes of 70% (v/v) aqueous ethanol and subjected to reflux extraction under heat. A total of 3 cycles of extraction were performed for 2 hours each time. The filtrates were combined and concentrated under reduced pressure, and the resulting ethanol extract was diluted in water to a density of 2 g/mL, representing the crude drug extract.

Cell viability assay

Cells were seeded into 96-well plates, cultured overnight, then treated for 72 h with different doses of BSE. The cell viability was determined using Cell Counting Kit-8 and MTT assay. CCK-8 solution (10 μL) was added to each well, and the plates were incubated for 1 h at room temperature; MTT solution (20 μL) was added to each well and incubated for a further 4 h at 37 °C, then aspirated and 100 μL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Cell survival was quantified in terms of the number of viable cells by measuring absorbance at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). Average IC₅₀ values were calculated from at least
3 independent experiments using the “log it” method (12).

**Apoptosis assay**

Cells were cultured overnight in 6-well plates and treated for 24 h with BSE. Apoptosis in the cultures was assessed using a kit based on staining with FITC-conjugated annexin V (catalog no. C1062S, Beyotime, Shanghai, China) and flow cytometry (Beckman Coulter, Brea, CA, USA). Cells were suspended in a mixture of 1X annexin V-FITC binding buffer (195 μL) and annexin V-FITC (5 μL), incubated at room temperature for 10 min, and centrifuged at 1,000 ×g for 5 min. The pellet was resuspended in binding buffer (190 μL), and propidium iodide (PI) working solution (10 μL) was added before the samples were analyzed using flow cytometry.

**DAPI assay**

Cells were cultured overnight in 6-well plates, treated with BSE for 24 h, washed twice with phosphate-buffered saline (PBS), then incubated for 30 min with 4’,6-diamidino-2-phenylindole (DAPI, 1 mg/mL). Stained nuclei were observed under a fluorescence microscope (DMI 4000 B, Leica, Wetzlar, Germany). Cells with nuclei showing fragmentation and chromatin condensation were considered apoptotic.

**Western blotting**

Protein extracts were resolved by 8–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% bovine serum albumin (BSA) for 1 h and incubated with primary antibodies against the following proteins: intact poly (ADP-ribose) polymerase (PARP) or cleaved PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), full-length or cleaved caspases-3 and -9 (Cell Signaling Technology, Danvers, MA, USA), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or DIABLO (Proteintech, Rosemont, IL, USA). Membranes were then blotted with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA), followed by enhanced chemiluminescence detection (Biological Industries, Beit HaEmek, Israel).

**Quantitative real-time polymerase chain reaction**

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to measure levels of DIABLO messenger RNA (mRNA). Total RNA was extracted and reverse-transcribed into complementary DNA (cDNA) using SuperScript (Thermo Fisher, Waltham, MA, USA). The DIABLO mRNA levels were quantified relative to levels of GAPDH mRNA using SYBR Green (Thermo Fisher, USA) and the $2^{-\Delta\Delta Ct}$ method. The primers used in this assay are shown in **Table 2**.

**Animal experiments**

A total of 20 female BALB/c nude mice (4-6 weeks old) were purchased (Shanghai Sippr-BK Laboratory Animals, Shanghai, China) and allocated to 4 groups (5 mice per group). The NCI-H1299 cells were injected subcutaneously into the left side of the posterior flank. Once tumor volumes reached about 100 mm$^3$, control group mice were treated with 0.9% normal saline and the others were treated with a different dose of BSE (1, 5, or 25 g/kg), injected into the caudal vein. Tumor volume was measured twice a week and mice were euthanized at 25 days after implantation. Animal
experiments were approved by the Animal Experimental Ethics Committee of the Center for Drug Safety Evaluation and Research of Zhejiang University (ZJU) (No. IACUC-17-163) and were in compliance with the guidelines of Institution Animal Care and Use Committee of Center for Drug Safety Evaluation and Research (CDSER) of Zhejiang University (ZJU) for the care and use of animals. A protocol was prepared before the study without registration.

**Gene microarray analysis**

Total RNA was extracted using TRIZOL Reagent (Cat#15596-018, Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions and checked to inspect RNA integrity. Qualified total RNA was further purified by RNeasy mini kit (Cat#74106, QIAGEN, GmBH, Hilden, Germany) and labeled. Each slide was hybridized with 600 ng Cy3-labeled cRNA using Gene Expression Hybridization Kit (Cat#5188-5242, Agilent Technologies, Santa Clara, CA, US), then slides were scanned with default settings. The original data from the gene microarray were adjusted using the limma package (13) and screened for differentially expressed genes (DEGs), defined as genes whose expression differed between NCI-H1299 cells which treated by BSE or not by $2^{(\log_{2}\text{fold change})}\geq 1$ with $P<0.05$. The database for annotation, visualization, and integrated discovery (DAVID 6.8; https://david.ncifcrf.gov/) was used to analyze DEGs for Gene Ontology (GO) (14). We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) knowledge database to identify enrichment of biochemical pathways among DEGs, and R Profiler (3.6.0) was used to annotate and visualize the KEGG pathways of DEGs (15). We selected GO terms and pathways enriched by DEGs based on a cut-off criterion of $P<0.05$.

**Gene set enrichment analysis (GSEA)**

GSEA (https://software.broadinstitute.org/gsea/index.jsp) was used to assess DIABLO related pathways and molecular mechanisms in NSCLC samples (16). Enriched gene sets with $P$ value of $<0.05$ were considered statistically significant.

**Statistical analysis**

All experiments were performed in at least three biological replicates. Data were presented as mean ± SD. Inter-group differences were assessed for significance using the 2-tailed Student’s t-test.

**Results**

**BSE suppressed proliferation and promoted apoptosis in NSCLC cell cultures**

It was shown that BSE significantly inhibited proliferation of NCI-H1299 cells in a dose- and time-dependent manner by CCK8 and MTT assays (Figure 1A,1B). It also induced apoptosis (Figure 1C,1D), which was associated with increases in levels of the cleaved forms of PARP and caspases-3 and -9 (Figure 1E). Moreover, when treated with Caspase inhibitor Z-VAD-FMK, the percentage of cell apoptosis was decreased as shown in Figure 1D. All of these results suggested that BSE induced apoptosis in NCI-H1299 cells mediated via caspase signal pathway.

**BSE suppressed growth of NCI-H1299 xenografts in nude mice**

Without exerting obvious toxicity on xenograft-bearing mice (Figure 2A), BSE reduced tumor volume relative to control tumors in a dose-dependent manner (Figure 2B). At 25 days after treatment, relative tumor volume was significantly smaller in animals treated with 25 g/kg BSE (13.85±3.36) than in control animals (23.40±6.05, $P=0.044$; Figure 2C). A corresponding dose-dependent decrease in tumor weight was also observed (Figure 2D,2E).

**BSE promoted apoptosis of NSCLC cells in vivo**

The above anti-tumor effects correlated with an increase in apoptosis based on immunostaining of tumor sections for cleaved forms of PARP and caspase-3 (Figure 3A,3B). In fact, BSE doubled the proportion of tumor cells containing cleaved PARP, from 20% to 40%.

**Identification of DEGs as a result of BSE treatment**

To identify other proteins that may help mediate the anti-tumor effects of BSE on NSCLC, we profiled the transcriptomes of NCI-H1299 cells in cultures exposed to BSE (100 μg/mL) and compared to these cultures undergoing no treatment. After applying stringent cut-offs, we were able to identify 10 up-regulated genes (SNX29,
**Figure 1** Effects of BSE on proliferation and apoptosis of the NSCLC cell line NCI-H1299 in vitro. (A) Viability of BSE-treated cells based on the CCK-8 assay (left), MTT assay (right); (B) change in cell numbers; (C) fluorescence microscopy of DAPI-stained cells after treatment with BSE; (D) flow cytometry of cells after treatment with BSE, Z-VAD-FMK and staining with annexin V/PI. The numbers in parentheses refer to the percentages of the total population falling within the gating; (E) Western blotting of apoptosis-related proteins in cells following treatment with BSE. BSE, *Botrychium schaffneri* Underw. extract; NSCLC, non-small cell lung cancer; CCK-8, Cell Counting Kit-8; MTT, Methyl Thiazolyl Tetrazolium; Z-VAD-FMK, Caspase inhibitor; DAPI, 4’,6-diamidino-2-phenylindole; PI, propidium iodide.

**Figure 2** Assessment of anti-tumor effects of BSE in nude mice with NCI-H1299 xenografts measured up to 25 days after treatment with extract. (A) Body weight; (B) tumor volume; (C) relative tumor volume; (D) tumor weight; (E) representative photographs of tumors. BSE, *Botrychium schaffneri* Underw. extract.
Liu et al. *Botrychium schaffneri* Underw. inhibit proliferation of NSCLC

**Table 3** Differential expression of genes in NCI-H1299 cells after treatment with BSE (100 μg/mL)

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BSE, *Botrychium schaffneri* Underw. extract; SNX29, Sorting Nexin 29; DIABLO, direct IAP Binding protein with low PI; GPR152, G Protein-Coupled Receptor 152; MTRNR2L2, MTRNR2 Like 2; HLA-J, Major Histocompatibility Complex, Class I, J (Pseudogene); SHISA7, Shisa Family Member 7; KCP, Kielin Cysteine Rich BMP Regulator; AZGP1, Alpha-2-Glycoprotein 1, Zinc-Binding; C6orf99, Chromosome 6 Putative Open Reading Frame 99; LRRC39, Leucine Rich Repeat Containing 39; ZNF546, Zinc Finger Protein 546; ANK2, Ankyrin 2; FAM186B, Family With Sequence Similarity 186 Member B; B4GALT6, Beta-1,4-Galactosyltransferase 6; FBXW12, F-Box And WD Repeat Domain Containing 12.

**Table 3** Differential expression of genes in NCI-H1299 cells after treatment with BSE (100 μg/mL)

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**Figure 3** Apoptosis-related protein expression in NCI-H1299 xenografts in mice after treatment with BSE. Tumor sections were stained with hematoxylin-eosin, then with antibodies against the cleaved forms of PARP and caspase-3. (A) Reactivity assessment in tissue slices; (B) semi-quantification of cleaved forms of PARP and caspase-3 immunostaining intensity. The intensity was scored as follows: −, negative; +, weak; ++, moderate; ++++, strong. BSE, *Botrychium schaffneri* Underw. extract; PARP, poly (ADP-ribose) polymerase.

**Role of DIABLO in the anti-tumor effects of BSE on NSCLC cells**

In an effort to elucidate the molecular pathways by which BSE exerts anti-tumor effects, we investigated the genes of which the expression was altered by BSE treatment. We began by examining DIABLO, which has been previously linked to cancer progression. We found that BSE significantly up-regulated DIABLO mRNA levels in NCI-H1299 cultures (Figure 5A) and xenografts in mice (Figure 5B).

To obtain further direct evidence showing that DIABLO may help mediate the effects of the BSE, we knocked down its expression using siRNA. This knockdown substantially weakened the extract's anti-tumor effects (Figure 5C,5D) and its ability to up-regulate apoptosis-related proteins.
Figure 4  Differential expressions of genes in NCI-H1299 cells after treatment with BSE. (A) Cluster heat map of differentially expressed genes; (B) GO enrichment analysis of DEGs; (C) analysis of KEGG pathway enrichment among DEGs; (D) GSEA showing high-DIABLO groups are associated with cell apoptosis-related pathways; BSE, Botrychium schaffneri Underw. extract; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; GSEA, gene set enrichment analysis.
**Figure 5** Effects of BSE on DIABLO expression in NCI-H1299 cells *in vitro* and *in vivo*. (A) DIABLO mRNA levels in cultures after treatment; (B) DIABLO mRNA levels in xenograft tumors in mice after treatment; (C) DIABLO levels in NCI-H1299 cultures after transfection with siRNAs against DIABLO (si-DIABLO) or negative control siRNA (si-NC); (D) proliferation of NCI-H1299 cells previously transfected with si-NC or si-DIABLO, followed by BSE; (E) Western blotting of apoptosis-related proteins in cells previously transfected with si-NC or si-DIABLO, followed by BSE (or not); (F) anti-DIABLO immunostaining of NCI-H1299 xenografts from mice treated with BSE (or not); (G) DIABLO levels in NCI-H1299 treated with BSE, Z-VAD-FMK. **, P<0.01; ***, P<0.001. BSE, *Botrychium schaffneri* Underw. extract; mRNA, messenger RNA; siRNAs, short interfering RNAs; DIABLO, direct IAP Binding protein with low PI; Z-VAD-FMK, Caspase inhibitor.

(Figure 5E,5F). When treated with caspase inhibitor Z-VAD-FMK, the expression of DIABLO was significantly decreased (Figure 5G).

**Discussion**

Despite rapid developments in treatment during the past decade, NSCLC continues to be the leading cause
of tumor-related deaths worldwide (17). Management of patients diagnosed with NSCLC includes surgery, chemotherapy, and radiation therapy. Current tumor targeting therapies have demonstrated excellent efficacy in NSCLC, but drug toxicity and resistance tend to limit the use of chemotherapy. Given that, an efficient and low-toxicity, good toleration and safety therapy for NSCLC is urgently needed. The present study provides in vitro and in vivo evidence that BSE, already used in traditional Chinese medicine, may be effective as chemotherapy against NSCLC because it can inhibit cell proliferation and induce apoptosis of tumor cells. We have also shown that these anti-tumor effects may involve up-regulation of the apoptosis regulator DIABLO.

BSE has been used as a traditional Chinese medicine for their therapeutic properties in lung diseases for centuries. The present work extends our previous study, in which we found that BSE can promote pulmonary vascular remodeling and mitigate pulmonary inflammation and further relieve pulmonary arterial hypertension (18). In the present study, we found that an ethanolic extract from the same plant can significantly reduce the volume of NSCLC xenografts. This finding, together with centuries’ long experience of using this BSE extract in traditional medicine, means that it may be more tolerable to patients than other chemotherapy drugs. These advantages are very important in the therapy of advanced NSCLC patients. Our results in vitro and in vivo suggest that BSE could be an effective treatment strategy in NSCLC.

Our finding that DIABLO helps mediate the anti-tumor effects of BSE is consistent with previous work showing a significant association between higher DIABLO expression in NSCLC tissue and longer tumor-free and overall survival (19). Further research is needed to clarify the details of how the DIABLO protein exerts anti-tumor effects. In addition, other DEGs that we identified in the NSCLC xenografts. This finding, together with centuries’ long experience of using this BSE extract in traditional medicine, means that it may be more tolerable to patients than other chemotherapy drugs. These advantages are very important in the therapy of advanced NSCLC patients. Our results in vitro and in vivo suggest that BSE could be an effective treatment strategy in NSCLC.

In conclusion, we present in vitro and in vivo evidence that BSE inhibits NSCLC cell proliferation and induces apoptosis, and these effects appear to involve the up-regulation of DIABLO. We will further identify the mechanism by which DIABLO was regulated in NSCLC and biological process involved on DIABLO expression is up- or down-regulated by BSE. Our findings provide the foundation for future efforts to discover improved therapies against NSCLC.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://dx.doi.org/10.21037/atm-21-5409

Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-5409

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-5409). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were approved by the Animal Experimental Ethics Committee of the Center for Drug Safety Evaluation and Research of Zhejiang University (ZJU) (No. IACUC-17-163) and were in compliance with the guidelines of Institution Animal Care and Use Committee of Center for Drug Safety Evaluation and Research (CDSER) of Zhejiang University (ZJU) for the
care and use of animals.

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References


Figure S1 DIABLO levels in NCI-H1299 cultures after transfection with 1 of 3 short interfering RNAs against DIABLO or a negative control siRNA (si-NC). **, P<0.01; ***, P<0.001. DIABLO, direct IAP Binding protein with low PI; siRNAs, short interfering RNAs.