Original Article

The Possible Effects of Protodioscin Inhibit Tumor Proliferation, Migration and Invasion in Colon Cancer Cell Lines

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Key Words
Protodioscin;
Colorectal cancer;
Anti-cancer;
Cell proliferation;
Apoptosis

Purpose. Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide. Protodioscin is a natural compound found in certain plants and has shown potential anti-cancer activity against a wide range of cancers; however, as there are no reports concerning the anti-cancer properties of protodioscin in CRC, this study investigated the possible anti-cancer effects of protodioscin in CRC.
Methods. Human colon cancer cell lines, SW620 and HCT116, were used in this study, and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was utilized to detect cell viability. Wound-healing and Matrigel cell invasion assays were performed for evaluation of cell migration and invasion, while western blot analyses of proteins related to cell proliferation, migration and angiogenesis were conducted. Flow cytometry was performed to investigate the effect of protodioscin on the apoptosis of colon cancer cells. Animal tests were conducted to evaluate tumor growth.
Results. In MTT assay, protodioscin showed reduction of cell viability in cell lines. As for cell migration and invasion, inhibition of both actions was noted in the study group that was tested by both wound-healing and Matrigel invasion assays. In flow cytometry, the study group showed a higher percentage of apoptotic cells with western blot analysis showing decreased N-cadherin, cyclin D1 and VEGF protein expression but increased E-cadherin and cleaved caspase-3. In the animal study, reduction in volume of the tumor was demonstrated.
Conclusions. Protodioscin shows promising anti-cancer effects in colon cancer cell lines, inhibiting tumor proliferation, migration and invasion while inducing apoptosis. These findings suggest protodioscin is a potentially valuable therapeutic agent for the treatment of CRC.

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Colorectal cancer (CRC) is a serious health issue, ranking among the third most commonly diagnosed cancers worldwide while being also the second-leading cause of cancer-related death globally, highlighting the need for effective treatment options.[1,2] The treatment options for CRC include elimination of underlying infections, surgery, cryosurgery, chemotherapy, radiation therapy and targeted therapy; nevertheless, surgery and chemotherapy remain the mainstay of treatment, where surgical resection involves

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the removal of the tumor and the surrounding mesentery, while chemotherapy on the other hand, uses medications to slow down the rate of growth of cancer cells.[3] These treatment options are effective in managing CRC and have shown promising results in improving patient outcomes; regardless, the overall recurrence rates of CRC are up to 15-20% (as 10-12% for stage II colon cancer and about 25-30% for stage III).[4]

Protodioscin is a naturally occurring compound found in certain plants, particularly in yam, asparagus, solanum, lily and onion families.[5] It belongs to the class of alkaloids that are known for their diverse biological activities, and has gained attention in the field of drug discovery due to its potential pharmacological activities, having been reported to possess various health benefits including antimalarial activity, antibacterial activity, antifungal activity and antioxidant properties.[6,7] In addition, protodioscin has shown promising results in terms of its potential anti-tumor and anti-inflammatory effects.[7,8] Functionally, protodioscin has been shown to have various biological activities; for example, it has been reported to have a cytotoxic effect on cell lines derived from leukemia and solid tumors, while also being found to have a strong growth inhibitory effect on HL-60 cells.[9] Protodioscin has shown potent anti-cancer activity against a wide range of cancer cell lines including leukemia, hepatocellular carcinoma cell, ovarian and breast cancers, and specific types such as cervical carcinoma, bladder cancer and renal cancer.[10-14] However, there is no report concerning the anti-cancer properties of protodioscin in CRC; accordingly, the purpose of this study is to investigate the possible anti-cancer effects of protodioscin in CRC.

Materials and Methods

Cell culture

Human colon cancer cell lines, SW620 and HCT 116, were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and seeded in a 24-well plate with approximately 3×10^4 cells in a 0.5 mL volume per well. All cell lines were incubated at 37 °C in an atmosphere of 5% CO_2 with saturated humidity.

Cell proliferation assay

To evaluate the effects of protodioscin on SW620 and HCT116 cell proliferation, we used an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to detect the cell viability, which was determined after a 24-h incubation with different concentrations (0, 1, 2, 3, 4, 5, 6, and 7 μM) of protodioscin.

In vitro migration assay

Cell migration was assessed using a wound-healing assay (ibidi, Martinsried, Germany; 80209). We used 24-well plates coated with a human colon cancer cell line and incubated at 37 °C for 12 hours. 70 μl cells were seeded at a density of 1×10^5 cell per well, then after 24 hours, the cells were washed twice with phosphate-buffered saline (PBS) and photographed. Cell migration was then determined after a 24-h incubation with 0 and 1 μM protodioscin.

In vitro invasion assay

Transwell cell invasion assays were performed *in vitro*. Briefly, the cells were seeded at a density of 1×10^4 cells per insert, and the lower chamber of the Transwell was filled with 0.5 mL of medium containing 0.4 μM protodioscin. After 24 hours of incubation, cells remaining on the upper surface of the Transwell membrane were removed using a cotton swab, and the cells that had invaded the Transwell membrane into the bottom of the insert were fixed, stained, photographed and quantified by counting the cell number in six, random high-powered microscopic fields.

Flow cytometry

Cells were added to 6-well plates and incubated for 24 hours followed by treatment with protodioscin for 24 hours, then both detached and attached cells were centrifuged at 1000 rpm for 5 minutes followed

by washing once with PBS and fixing in PBS containing 70% ethanol at -20 °C. After 3 hours, the cells were harvested by centrifugation at 1000 rpm for 5 minutes at 4 °C; then, the cells were resuspended in PBS and analyzed using a Muse® Annexin V and Dead Cell Assay Kit (Millipore, Burlington, MA, USA; MCH100105). Cell cycle data were collected on a Muse® Cell Analyzer (Millipore), with 10,000 events collected per sample.

Western blot

All samples were lysed in 200 μl lysis buffer, and 50 μg of protein from each sample was loaded into the wells of an SDS-PAGE gel and electrophoresed at 80 V for 30 min and 120 V for 1 hour. The separated proteins were transferred to a polyvinylidene difluoride membrane, then after 1 hour in blocking buffer, the membranes were incubated with primary antibody N-cadherin (1:500, proteintech; 22018-1-AP), E-cadherin (1:500, proteintech; 20874-1-AP), Cyclin D1 (1:500, proteintech; 60168-1-Ig), VEGF (1:500, ABGENT; #AP6290b), Cleaved caspase-3 (1:500, cell signaling; #9661) and β-actin (1:10000, proteintech; 60008-1-Ig) for 2 hours at room temperature and secondary antibody for 90 minutes. ECL solution was used to detect specific bands with a MINICHEMI Chemiluminescent Imaging and Analysis System (Beijing Sage Creation Science Co., Ltd., Beijing, China).

Animal model of CRC

Six-week-old male NU/NU mice were obtained from BioLasco (Taipei, Taiwan). All animal experiments were conducted according to protocols approved by the Animal Care and Use Committee of Kaohsiung Medical University following the Guiding Principles for the Care and Use of Laboratory Animals. Mice were maintained under a 12 h/12 h dark/light cycle at 24 ± 1 °C with *ad libitum* access to food and water. Animals were injected subcutaneously with 1×10^5 HCT116 cells to initiate tumor growth, then starting on the following day, tumor model mice were administered 2 μM/kg or 4 μM/kg protodioscin once daily for 42 consecutive days. Tumor dimensions were measured three times per week and volume (in cm^3) was calculated as (length × $width^2$)/2 on days 7, 14, 21, 28, 35 and 42 post-seeding. The mice were then sacrificed 42 days after the injection of the tumor cells.

Data analysis

SPSS 19.0 (IBM Corp., Armonk, NY) was used for statistical analysis. The western blot results were analyzed using Lane 1D software (Beijing Sage Creation Science Co., Ltd.). A Student's t-test was used to compare the results of the invasion assay. A one-way ANOVA was used for the apoptosis. A two-way ANOVA was used to compare the results of the Western blot assays, migration assay and tumor size, with a p-value < 0.05 considered statistically significant.

Results

Protodioscin attenuated cell viability in colon cancer cell lines

The SW620 and HCT116 cells demonstrated reduced viability at doses of 1, 2, 3, 4, 5, 6 and 7 μM protodioscin in comparison to the 0 μM control group at 24 h (Fig. 1). These data indicated a correlation between protodioscin and reduced cell viability in the two colon cancer cell lines.

Protodioscin inhibited cell migration and invasion in CRC cells

To evaluate cell migration, we used a wound-healing assay and compared the results between control and protodioscin groups in SW620 and HCT116 (Fig. 2A). In both SW620 and HCT116 cells, protodioscin markedly inhibited the migratory capability at 24 h (Fig. 2B; all $p < 0.001$). These data suggested that protodioscin inhibited migration in colon cancer cells.

To evaluate cell invasion, we used a Matrigel invasion assay and compared the results obtained between control and protodioscin groups in SW620 and HCT116 (Fig. 3A). In both the SW620 and HCT116

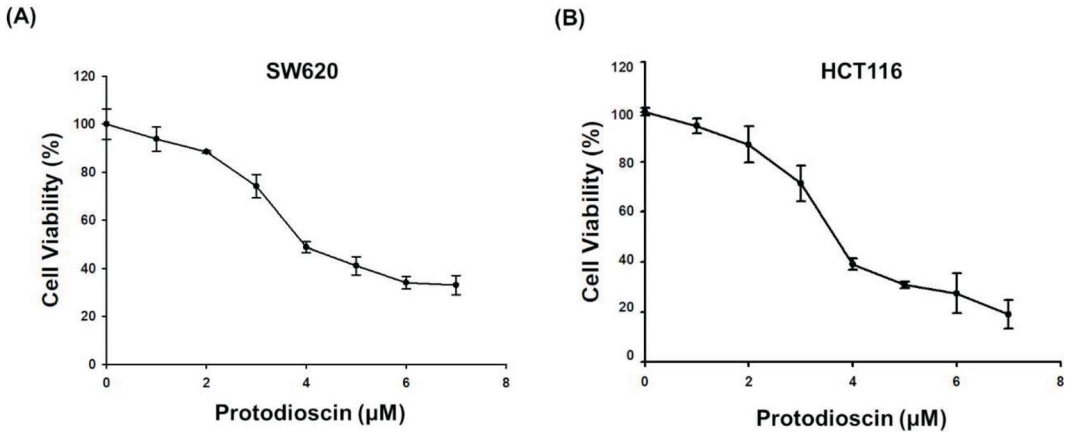

Fig. 1. The synthetic triterpenoid protodioscin dose-dependently reduces HCT116 and SW620 cell proliferation. Cells were cultured for 24 h in the indicated protodioscin concentrations and viable cell number estimated by MTT assay. Results expressed as mean ± SEM relative to untreated controls.

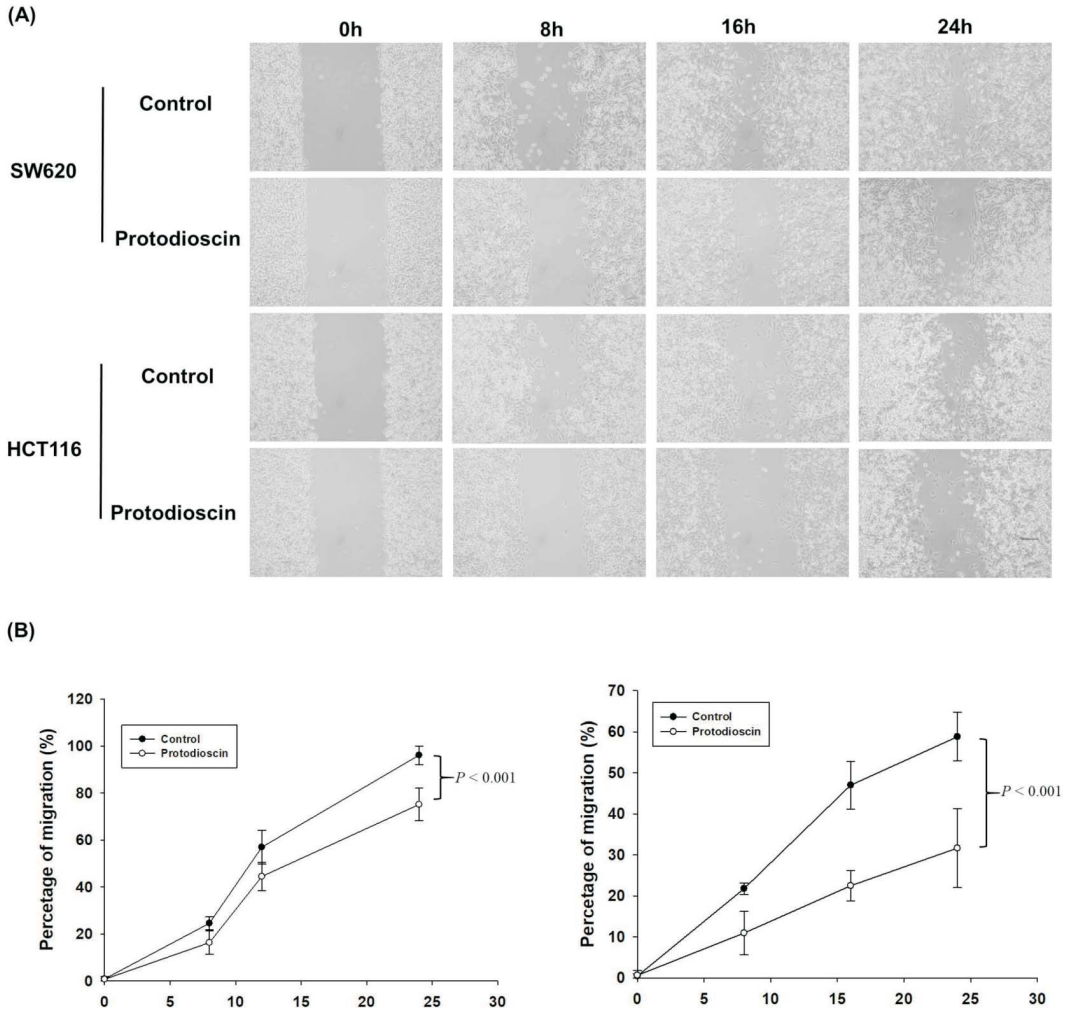

Fig. 2. Protodioscin suppresses SW620 and HCT116 cell migration. Wound-healing assays were conducted in the presence of the indicated protodioscin dose. The number of migrating cells was counted 0, 16, 24, and 48 h after protodioscin treatment (A). Results expressed as mean ± SEM relative to untreated controls (B; $p < 0.001$).

cells, protodioscin markedly inhibited the invasive capability at 24 h (Fig. 3B; all $p < 0.001$). These data suggest that protodioscin inhibited invasion in colon cancer cells.

Protodioscin induced apoptosis in CRC cells

To investigate the effects of protodioscin on the apoptosis of colon cancer cells, we used flow cytometry to detect the percentage of apoptotic SW620 and HCT116 cells following protodioscin treatment (Fig. 4A). In SW620, the results revealed that the percentage of apoptotic cells in the control group were less than the protodioscin group in the 2 μM, 4 μM and 6 μM treatments (Fig. 4B Left; all $p < 0.05$). For HCT116 cells, the percentage of apoptotic cells in the control group were also less than the protodioscin group in the 2 μM, 4 μM, and 6 μM treatments (Fig. 4B Right; all $p < 0.001$). Therefore, protodioscin induced the apoptosis of CRC cells.

Protodioscin treatment reduced protein expression in colon cancer cells

To evaluate the expression of proteins related to cell proliferation, migration, and angiogenesis, Western blot analyses of N-cadherin, E-cadherin, vascular endothelial growth factor (VEGF), cyclin D1 and cleaved caspase-3 were conducted. N-cadherin is a marker of metastasis (where E-cadherin is important in maintaining the epithelial phenotype and regulating homeostasis of tissues by modulating various signaling pathways),[15] VEGF is a marker of angiogenesis, cyclin D1 regulates cell cycle while cleaved caspase-3 relates to induction of apoptosis. In both the HCT116 and SW620 cells, Western blotting revealed that protodioscin led to the downregulation of N-cadherin, cyclin D1 and VEGF expression and upregulation of E-cadherin (Fig. 5; all $p < 0.001$). In addition, these data suggested that protodioscin not only decreased N-cadherin, cyclin D1, and VEGF protein expression, but also increased E-cadherin and cleaved caspase-3 protein expression in CRC cells.

Protodioscin attenuated colon tumor growth in mice

As per the above cell culture findings, daily intraperitoneal injection of protodioscin significantly reduced the rate of tumor growth in nude mice implanted

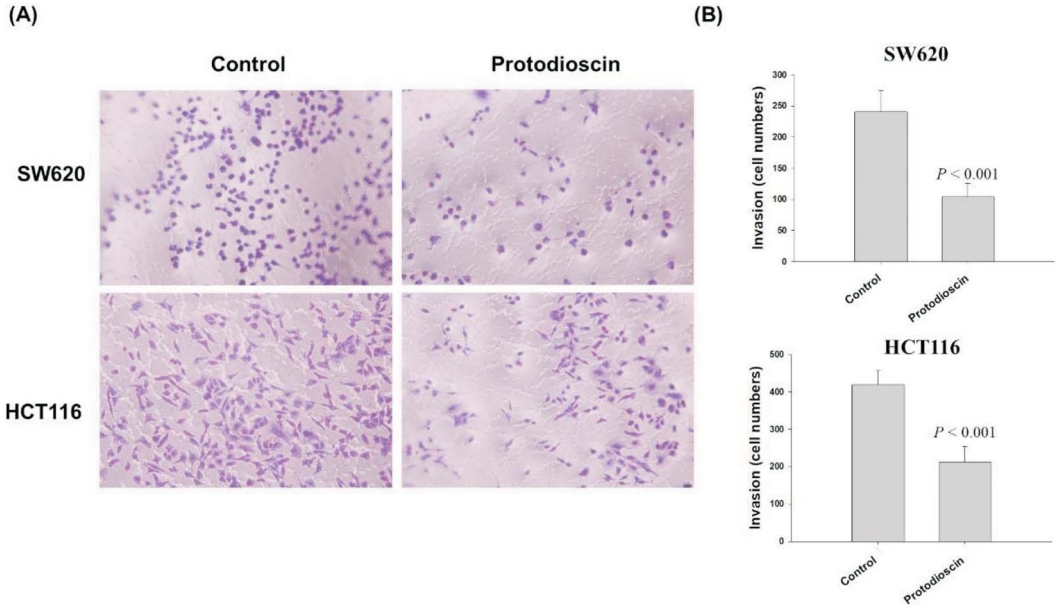

Fig. 3. Protodioscin suppresses SW620 and HCT116 cell invasion. Transwell invasion assays were conducted in the presence of protodioscin, and the number of invading HCT116 or SW620 cells was counted after 24 h (A). Results expressed as mean ± SEM compared to controls (B; all $p < 0.001$).

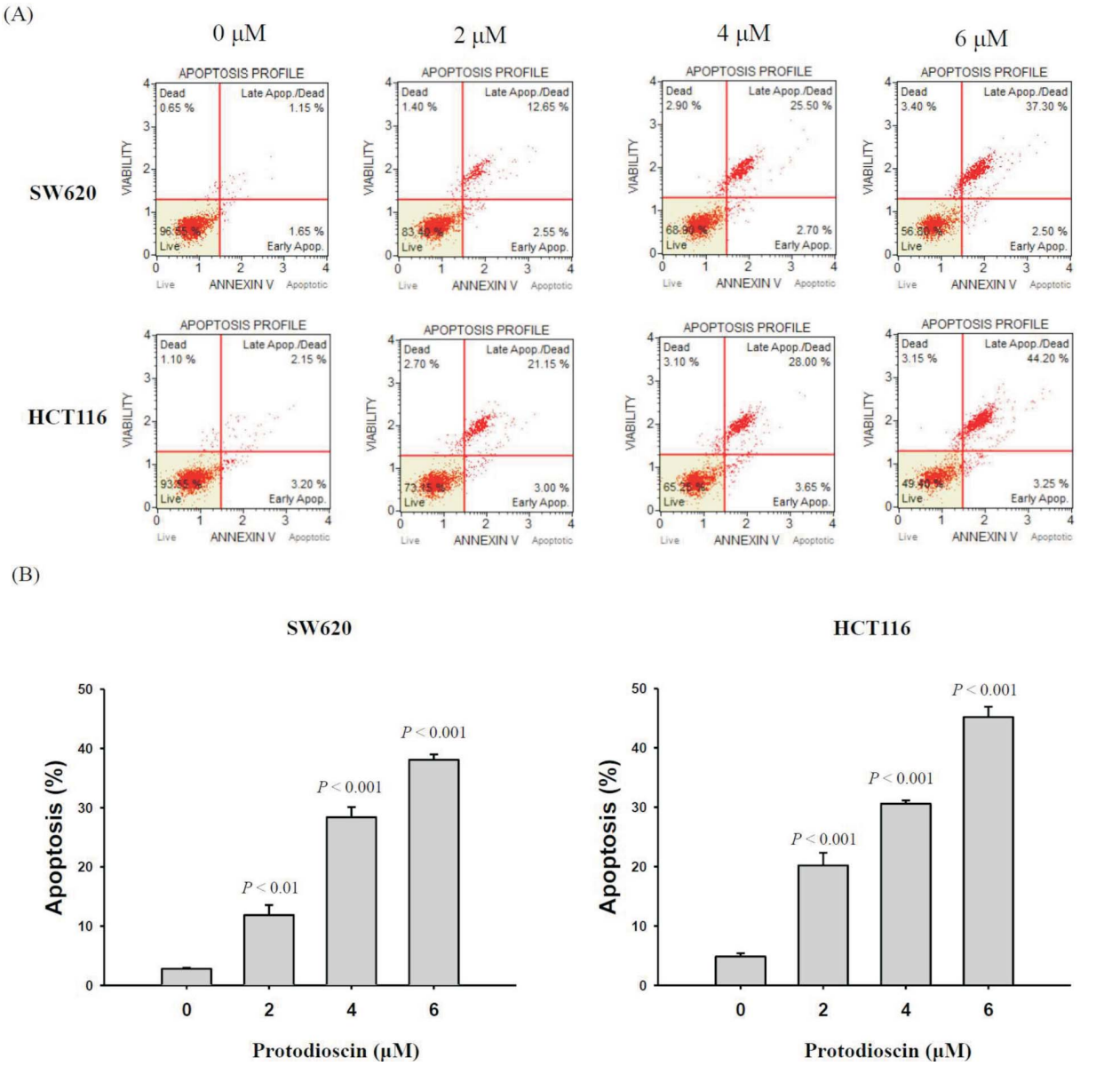

Fig. 4. Flow cytometry analysis of SW620 and HCT116 cells following protodioscin treatment. Representative images following 0, 2, 4 and 6 μM protodioscin-treating SW620 and HCT116 (A). The percentage of apoptosis increased significantly with concentration in SW620 and HCT116 (B; all $p < 0.05$).

with 1×10^5 HCT116 cells at 6 weeks of age (Fig. 6A). Moreover, this antitumor effect was dose-dependent, as 2 μM protodioscin daily reduced final tumor volume from 88.10 ± 11.560 mm^3 in control model mice to 22.41 ± 4.000 mm^3, while 4 μM daily reduced tumor volume to 7.39 ± 1.020 mm^3. The tumor burdens were analyzed and mice that received protodioscin treatment had significantly smaller cancer lumps than those in the control group (Fig. 6B; all $p < 0.001$).

Discussion

Protodioscin has been shown to exhibit cytotoxicity against a wide range of cancer cell lines, including leukemia, solid tumors and ovarian cancer.[10-14] These findings highlight the broad spectrum of anticancer activity that protodioscin possesses, making it a potentially therapeutic agent for multiple types of cancer.[10-14] In our results, protodioscin inhibited tu-

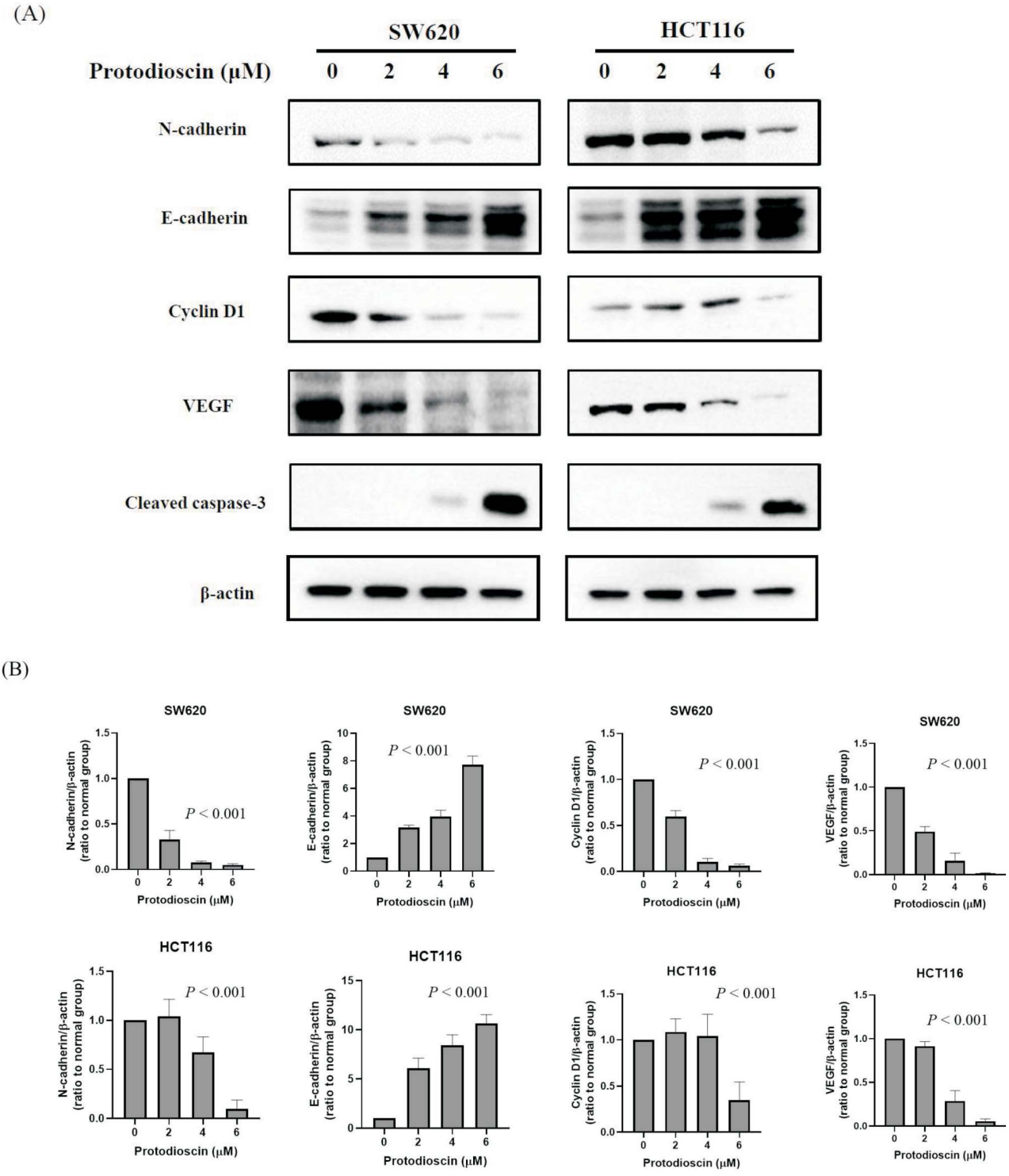

Fig. 5. Protodioscin suppresses the epithelial–mesenchymal transition of HCT116 and SW620 cells. Western blots showing upregulation of E-cadherin and downregulation of N-cadherin. β-actin was used as the gel loading control for all experiments (A). The expressions of all proteins were significantly inhibited (B; all $p < 0.05$).

mor progression including proliferation, invasion and migration in colon cancer cells. The mechanism of anti-cancer included the arrest of the cell cycle, induction of apoptosis, and inhibition of angiogenesis.

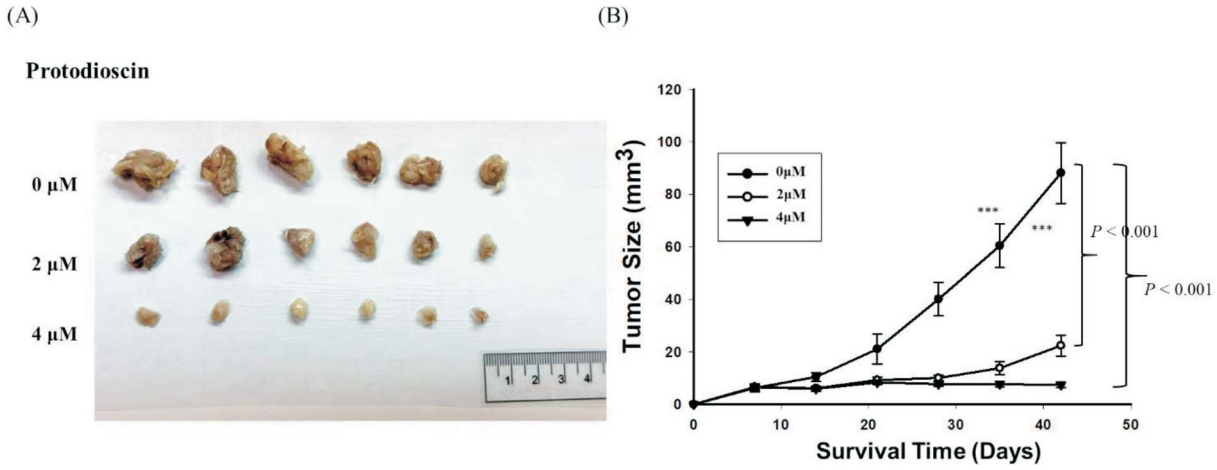

Fig. 6. Protodioscin reduces the volume of tumor in nude mice. Isolated tumors showing volume reduction by protodioscin treatment (A). Tumor burdens were analyzed. Mice that received protodioscin treatment had significantly smaller cancer lumps than those in the control group (B; all $p < 0.001$).

Cyclin D1 is a protein that plays a crucial role in the regulation of cell cycle progression and proliferation.[16] It acts as a key component of the cyclin-dependent kinase complex, which is responsible for driving cells through different phases of the cell cycle.[17] Research studies have shown that dysregulation of cyclin D1 expression can contribute to uncontrolled cell proliferation, a hallmark characteristic of cancer.[18] Our results revealed that protodioscin led to the downregulation of cyclin D1, which implies its effect on arrest of the cell cycle.

In 2018, Lin et al. showed that protodioscin induces apoptosis in cervical cancer cells through the up-regulation of caspases 8, 3 and 9, as well as the down-regulation of Bcl-2 expression;[19] similarly, Chen et al. demonstrated protodioscin increased the expression of cell apoptosis-related proteins, cleaved-PARP and cleaved-caspase 3, in human bladder cancer cells in 2022.[10] In addition, protodioscin has been found to induce cell cycle arrest at the G2/M phase in both bladder cancer and cervical cancer cells.[10,20]

The association between N-cadherin and E-cadherin has been a subject of extensive research in the field of cancer metastasis.[21] Numerous studies have shown that E-cadherin acts as a metastasis suppressor, while N-cadherin and cadherin-11 promote metastasis.[22] This association between N-cadherin and E-cadherin in cancer metastasis has been observed in various types of cancers, including breast cancer, esophageal squamous cell carcinoma and liver metastasis of breast cancer.[21,24,25] In breast cancer, N-cadherin has been identified as an important biomarker of epithelial-mesenchymal transition, a process involved in cancer cell motility and invasion.[23] Furthermore, the loss of E-cadherin is believed to enable metastasis by disrupting intercellular contacts and allowing for the invasion of cancer cells into surrounding tissues. Another adhesion molecule, N-cadherin, has been found to be correlated with increased invasion of cancer cells.[26] Studies have shown that overexpression of N-cadherin in breast cancer is associated with invasiveness due to N-cadherin-mediated interactions between cancer cells and the stromal environment.[26] This interaction promotes motility and invasion in breast cancer cells, without a reduction in E-cadherin expression.[26]

In our result, protodioscin induced apoptosis and inhibited cell proliferation following up-regulation of cleaved caspase-3 and down-regulation of cyclin D1. Moreover, protodioscin attenuated the ability of migration, invasion and angiogenesis following up-regulation of E-cadherin and down-regulation of N-cadherin and VEGF. These results support protodioscin being a promising candidate for the development of effective anti-cancer drugs.

In conclusion, protodioscin has shown promising anti-cancer effects in colon cancer cells. It inhibits tumor proliferation, migration, and invasion while inducing apoptosis. These findings suggest that protodioscin has the potential to be a valuable therapeutic

agent for the treatment of colorectal cancer.

Acknowledgements

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Authors' Contributions

All authors contributed equally to the writing of the manuscript. All authors also reviewed any revisions that were made and provided their final approval of the manuscript.

Consent for Publication

This article refers to animal and cell line studies, and does not involve human study, so there is no need for signing of any subject consent form.

Competing interests

The authors declare that they have no competing interests.

Sources of Financial Support

Nil.

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原　著

原薯蕷皂苷可抑制結直腸癌之腫瘤增殖、遷移與侵襲

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目的　結直腸癌目前為全球診斷第三常見的癌症。原薯蕷皂苷是存在特定植物中的一種天然化合物，顯示出對多種癌症具有潛在的抗癌能力。然而，關於原薯蕷皂苷在大腸直腸癌中的抗癌效用仍無相關研究。在這項研究中，我們探討了原薯蕷皂苷在大腸直腸癌中可能的抗癌效應。

方法　於本研究，我們使用了人類結腸癌細胞株 SW620 和 HCT116 以探討原薯蕷皂苷在大腸直腸癌中可能的抗癌效應。我們採用 MTT 試驗來檢測細胞存活率、創傷癒合試驗以評估細胞遷移性和細胞移行實驗以評估細胞侵襲能力。亦使用西方墨點法分析與細胞增殖、遷移和血管生成相關的蛋白質、流式細胞儀用研究對結腸癌細胞凋亡的影響以及進行動物實驗以評估腫瘤生長。

結果　在 MTT 試驗中，原薯蕷皂苷對結腸癌細胞的存活率有降低作用。至於細胞遷移和侵襲，創傷癒合試驗和細胞移行實驗中均顯示其對腫瘤有抑制作用。在流式細胞儀實驗中，治療組有更高比率的凋亡腫瘤細胞。此外，西方墨點顯示在治療組中，腫瘤細胞產生較少的 N-cadherin、cyclin D1、VEGF；同時觀測到較多的 E-cadherin 以及 cleaved caspase-3。在動物實驗中，治療組的腫瘤體積亦有減少。

結論　原薯蕷皂苷在結腸直腸癌細胞中展現其可能的抗癌效果。它不但抑制了腫瘤細胞增殖、遷移和侵襲，也促進了細胞凋亡。以上結果顯示其為一種有潛力的結腸直腸癌的治療方式。

關鍵詞　原薯蕷皂苷、結直腸癌、抗癌、細胞增殖、細胞凋亡。