Inhibitory effect of Aphidicolin - a tetracyclic diterpene - on the proliferation and apoptotic induction in human cervical cancer (HeLa) cells

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Summary

Purpose: The objective of the present work was to investigate the antitumor and apoptotic effect of aphidicolin against human cervical cancer (HeLa) cells.

Methods: Flow cytometry was performed to detect alterations in the mitochondrial membrane potential loss (ΔΨm) after aphidicolin treatment. Cell viability was assessed by the MTT assay while the apoptotic effects of the compound were examined by fluorescence microscopy and flow cytometry.

Results: Aphidicolin induced dose-dependent as well as time-dependent cytotoxic effects in HeLa cells. Chromatin condensation and formation of apoptotic bodies were observed by Hoechst 33258 staining after drug treatment. Aphidicolin induced both early and late apoptosis in HeLa cells which were correlated with strong concentration of the compound. ΔΨm was also observed following aphidicolin treatment at varying doses.

Conclusion: Aphidicolin is a potent antitumor and apoptotic agent against human cervical carcinoma and its effects are mediated via chromatin condensation and mitochondrial membrane potential loss.

Key words: anticancer activity, aphidicolin, apoptosis, cervical cancer, mitochondrial membrane potential

Introduction

Cervical neoplasia is one of the most common cancers in women and is linked with high-risk human papillomavirus (HPV) infection. Early-stage disease has virtually no symptoms. Later symptoms may include abnormal vaginal bleeding, pelvic pain or pain during sexual intercourse. Cervical cancer is the second most frequent malignancy in women and more than 80% of the cases are found in developing countries [1]. There are several treatment methods used for cervical cancer, but each one of them has obvious drawbacks. Although radiotherapy constitutes an effective treatment option, one third of the patients will develop advanced or recurrent cancers, the pelvis being the most common site of failure [2]. The recurrence rate of cervical cancer ranges between 11 and 22% in FIGO stages Ib-IIa and between 28 and 64% in FIGO stages IIb-IVa [3]. HPV infection plays a crucial role in the development of more than 85% of the cases. However, most people who have had HPV infections do not develop cervical cancer. Other risk factors include smoking, weak immune system and birth control pills [4]. Cervical cancer has various histological types including squamous cell carcinomas, which comprise about 90% of the cases, followed by adenocarcinoma accounting for remaining 10% of the cases. Classically, cervical cancer treatment consists of
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a combination of surgery, chemotherapy and radiotherapy. In the United States, 5-year survival is around 65% and this rate largely relies on how promptly the tumor is diagnosed. Bigger sized early-stage tumors may be treated with radiotherapy and cisplatin-based chemotherapy, hysterectomy plus adjuvant radiation therapy, or neo adjuvant cisplatin-based chemotherapy followed by hysterectomy [5,6]. Hycamtin and cisplatin are the two drugs which are prescribed for patients with late-stage cervical cancer. However, this combination treatment is associated with severe side effects including neutropenia, anemia, and thrombocytopenia.

The objective of the current study was to investigate the antitumor and apoptotic effects of aphidicolin - a tricyclic diterpene natural product against HeLa cervical cancer cells in vitro. Natural products have always played a significant role in anticancer drug discovery and around 60-65% of the anticancer drugs have been isolated from natural sources [7,8]. The anticancer activity of this diterpene against cervical cancer cells has not been reported so far and as such our current findings constitute the first report over this issue.

Methods

Cell line, culture condition and drug treatment

HeLa cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). MTT was purchased from Sigma Chemical Co., (St. Louis, MO, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Lonza Biologics, Singapore), 100 U/mL penicillin and 100 μg/mL streptomycin (Vega Pharma Limited, Zhejiang, China). The cells were kept at 37°C in a humidified atmosphere containing 5% CO₂. In cell proliferation experiments, HeLa cells were treated with aphidicolin, or vehicle alone for 12, 24 and 48 hrs. Aphidicolin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in DMSO (Sigma Chemical Co.).

Cell viability testing by MTT assay

Inhibition of cell proliferation by aphidicolin was assessed by the MTT assay. Briefly, cells were plated in 96-well culture plates (1×10⁵ cells/well). After 24-hr incubation, cells were treated with aphidicolin (0, 10, 20, 40, 80 and 120 μM, 8 wells per concentration) for 12, 24 and 48 hrs; MTT solution (10 mg/mL) was then added to each well. After 4-hr incubation, the formazan precipitate was dissolved in 100 μL dimethyl sulfoxide, and then the absorbance was measured in an ELISA reader (Thermo Molecular Devices Co., Union City, USA) at 570 nm. The cell viability ratio was calculated by the following formula:

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\text{Inhibitory ratio} (\%) = \frac{\text{optical density/OD control} - \text{OD treated}}{\text{OD control} \times 100%}.
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Cytotoxicity was expressed as the concentration of aphidicolin inhibiting cell growth by 50% (IC₅₀ value).

Fluorescence microscopic study of aphidicolin-induced cell apoptosis

Morphological observation of nuclear changes was assayed with Hoechst 33258 using fluorescence microscopy. HeLa cells (1×10⁶ cells/ml) were seeded in 6-well plates and were exposed to varying concentrations (0, 20, 40 and 80 μM) of aphidicolin for 48 hrs at 37°C. The cells were collected, washed, fixed in 5% paraformaldehyde for 25 min and then stained with 10 μg/mL Hoechst 33258 (Hoechst Staining Kit, Beyotime, China) for 20 min at room temperature. Fluorescence microscopy (Model IX51; Olympus, Japan) was used to detect and measure cell shape captured from different random visual fields. The ratio of apoptotic cells to total cell number was calculated.

Annexin V binding assay for the quantification of apoptotic cell death

Annexin V binding assay was performed using flow cytometry to confirm the extent of apoptosis. Briefly, HeLa cells were treated with aphidicolin (0, 20, 40 and 80 μM) extract for 48 hrs. Next, treated and untreated cells were harvested by trypsinization. Harvested cells were then incubated in annexin V-FITC (50 ng/ml) and propidium iodide (PI) (10 μg/ml), at room temperature for 20 min in the dark, and analysed using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA) taking a minimum of 22,000 cells in each sample.

Measurement of mitochondrial membrane potential (ΔΨm)

Rhodamine-123 as a cationic fluorescent probe has been used for the determination of mitochondrial membrane potential. HeLa cells were seeded at 1×10⁶ cells/well into 6-well plates. After 24-hr incubation, cells were exposed to different concentrations (0, 20, 40 and 80 μM) of aphidicolin for 48 hrs. Untreated controls and treated cells were harvested and washed twice with PBS. The cell pellets were then resuspended in 5 mL of fresh incubation medium containing 5% CO₂. In cell proliferation experiments, HeLa cells were treated with aphidicolin, or vehicle alone for 12, 24 and 48 hrs. Aphidicolin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in DMSO (Sigma Chemical Co.).

Statistics

SPSS11.5 statistical software package was used for data analyses. The data are presented as mean ± SD for the three experiments performed in triplicate. Stu-
dent’s t-test and one-way analysis of variance (ANOVA) were performed for analyses and a value of p<0.05 was considered to be statistically significant.

**Results**

**Effect of aphidicolin on the viability of HeLa cells**

HeLa cells were treated with different concentrations (0, 10, 20, 40, 80 and 120 μM) of aphidicolin for 12, 24, and 48 hrs and cell viability was evaluated using an MTT assay. Figure 1 shows the dose-dependent as well as the time-dependent cytotoxic effects of aphidicolin on HeLa cells. The percentages of growth inhibition at various concentrations in these cells were determined as the percentage of viable treated cells in comparison with viable cells of untreated controls. At lower doses of aphidicolin, time periods of 12, 24 and 48 hrs had a lower effect on cancer cell growth inhibition. However, at higher doses, exposure of cancer cells to longer time resulted in higher growth inhibition and 48-hr exposure at 120 μM dose led to more than 90% growth inhibition.

**Fluorescence microscopy investigation of aphidicolin-induced apoptosis**

With the purpose of confirming whether aphidicolin induced apoptotic death in HeLa cells, we observed the effect of aphidicolin on nuclear morphology using Hoechst 33258 staining involving a fluorescence microscope. HeLa cells were treated with different doses of aphidicolin (Figure 2 A-D). Figure 2 A shows untreated cells with normal nuclear morphology without any indication of chromatin condensation, Figure 2 B-D depicts 20, 40 and 80 μM doses of aphidicolin respectively. Aphidicolin-treated cells showed dose-dependent chromatin condensation which rises from A-D. The nuclei of untreated control HeLa cells were stained in less bright blue and homogeneous color. On the other hand, after treatment with different concentrations of aphidicolin for 48 hrs, most of the cells revealed very intense staining of condensed and fragmented chromatin.

**Quantification of apoptosis induced by aphidicolin**

The extent of apoptosis induced by aphidicolin was examined by Annexin V/FITC assay. HeLa cells were labeled with FITC-conjugated Annexin V and PI for flow cytometry analysis. The results of the flow cytometry study with Annexin V/FITC and PI showed that, within 48 hrs of incubation, approximately 17.8, 28.5 and 46.7% of the cells underwent early apoptosis after treatment with 20, 40 and 80 μM of the compound, respectively. Similarly, 6.3, 14.2 and 32.4% of the cells underwent late apoptosis after treatment with 20, 40 and 80 μM of the extract, respectively. The effect of aphidicolin on the apoptotic death (early and late apoptosis) is shown in Figures 3 and 4. The divisions of the plots distinguish necrotic cells (Q1) from early apoptotic cells (Q4) and late apoptotic cells (Q2), while Q3 shows viable cells. The plots in the Figures are representative of 3 independent experiments.

**Aphidicolin induces mitochondrial membrane potential loss in HeLa cells**

Mitochondrial membrane potential (MMP,
Figure 2. Impact of aphidicolin on chromatin condensation in HeLa cells. The cells were treated with 0 μM (A, untreated control), 20 μM (B), 40 μM (C) and 80 μM (D), respectively for 48 hrs and stained with Hoechst 33258. The nuclear morphology was observed by fluorescent microscope (magnification ×400). Arrows show nuclear chromatin condensation and apoptotic body formation.

Figure 3. Effect of aphidicolin on the extent of apoptosis in Hela cells evaluated by Annexin V-FITC assay. Cells after exposure to 0, 20, 40 and 80 μM concentration of aphidicolin for 48 hrs and flow cytometry analysis with Annexin V-FITC vs PI. The divisions of the plots distinguish necrotic cells (Q1) from early apoptotic cells (Q4) and late apoptotic cells (Q2), while Q3 shows viable cells. The plots in the Figure are representative of 3 independent experiments.
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ΔΨm) indicates the normal mitochondrial function which is closely related with mitochondrial membrane permeability. In order to assess the effects of aphidicolin on the mitochondrial membrane potential, the ΔΨm in HeLa cells treated with different concentrations of aphidicolin was

**Figure 4.** Increase in the number of Annexin V positive cells (apoptotic cells) with increase in the dose of aphidicolin. The results are mean ± standard error of the mean (SEM) of three independent experiments. * p<0.05, ** p<0.01 vs 0 µM of aphidicolin-treated cells (control).

**Figure 5.** Loss of mitochondrial membrane potential induced by aphidicolin treatment. Mitochondrial membrane potential of HeLa cells after treatment with aphidicolin by using Rh-123 staining. (A) Treatment with 0 µM aphidicolin; (B) treatment with 20 µM aphidicolin; (C) treatment with 40 µM aphidicolin; (D) treatment with 80 µM aphidicolin; The experiment was repeated three times and representative graphs are shown.
measured using rhodamine-123 as fluorescent probe. The fraction of cells with depolarized mitochondria increased with aphidicolin dose. The percentage of ΔΨm disruption increased from 8.1% in control cells to 14.2, 34.2 and 52.3% in 20, 40 and 80 μM doses of aphidicolin, respectively. Figures 5 and 6 show the flow cytometric histogram and the graphical representation of the loss of mitochondrial membrane potential, respectively, after treatment with different doses of aphidicolin.

Discussion

Apoptosis is a highly regulated cellular suicide program that exterminates unwanted, faulty and potentially dangerous cells during the development and maintenance of cell homeostasis [9]. Inducing apoptosis is a crucial approach to eliminate cancer cells without encouraging an inflammatory reaction. Regulation of apoptotic signaling pathways incorporates a complicated system consisting of several pathways. Numerous conventional anticancer drugs are presently used in anticancer chemotherapy which are supposed to induce cell apoptosis via stimulation of these elements [10]. As a result, the ability of chemotherapeutic agents to induce the apoptotic program has been recognized as one of the major mechanisms which might serve for the development of novel approaches to treat cancer. There are two apoptotic pathways which have been recognized, the intrinsic pathway being principally controlled by the members of the Bcl-2 family proteins [11,12]. The second pathway is the extrinsic pathway. Several anticancer drugs function primarily to induce apoptosis in cancer cells and inhibit tumor development [13,14]. The morphological alterations of apoptosis witnessed in most cell types initially start with a reduction in cell volume and condensation of the nucleus. In many cases extensive DNA damage leads to activation of cell cycle checkpoints and results in cell cycle arrest and apoptosis. Dysregulation in the cell division and apoptosis are connected to the development of most cancers [15,16]. In this study, we investigated the antitumor and apoptotic effects of aphidicolin against human cervical cancer (HeLa) cells. The results of our experiments revealed that aphidicolin induced dose-dependent as well as time-dependent cytotoxic effects in HeLa cells. Chromatin condensation and formation of apoptotic bodies was observed by Hoechst 33258 staining after drug treatment. Aphidicolin induced both early and late apoptosis in HeLa cells in a concentration-dependent manner. Mitochondrial membrane potential loss was also observed following aphidicolin treatment at varying doses.

In conclusion, aphidicolin may be developed as a potent antitumor and apoptotic agent against human cervical carcinoma should further investigations involving in vivo and other mechanistic studies be performed.
References