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Original Article

Piperlongumine Increases the Apoptotic Effect of Doxorubicin and Paclitaxel in a Cervical Cancer Cell Line

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ABSTRACT

Objective: Piperlongumine (PL) is an alkaloid derived from the edible pepper (Piper longum L) and it has been described to have various biologic activities including anticancer effects. Our aim in this study was to assess the cytotoxic role of PL on a cervical cancer cell line (HeLa) and to evaluate the effects of PL/doxorubicin and PL/paclitaxel combination therapies on apoptotic cancer cell death. **Material and Methods:** The cytotoxicity, IC50 doses by MTT assay confirmed by fluorescent imaging, and apoptotic cell rates by Annexin V staining using flow cytometry were determined for PL, doxorubicin, paclitaxel, and for their combinations. **Results:** It was shown that the PL by itself induced the apoptosis in HeLa cells. PL in combination with doxorubicin and paclitaxel increased apoptotic cell death compared to either chemotherapeutic agent alone. **Conclusion:** We conclude that the PL inhibits cancer cell growth by inducing apoptosis and has a potential anticancer activity in cervical cancer, especially when combined with doxorubicin and paclitaxel.

Keywords: *Apoptosis, doxorubicin, HeLa, paclitaxel, piperlongumine*

Introduction

Piperlongumine (PL) is an alkaloid derived from the edible pepper (Piper longum L) and its extract form has been described to have various biologic activities including cytostatic, cytotoxic, apoptotic, immune modulatory, antiinflammatory, and anticancer effects.[1-3] PL has also been reported to have cancer cell-specific cytotoxicity with relative sparing of normal cells *in vitro*. Increased reactive oxygen species (ROS) and apoptotic cell death have been proposed to be the main mechanisms responsible for the anticancer effects of PL although the exact mechanism by which this compound induces cancer cell death remains obscured.[4,5] Despite of recent advances in therapeutic agents, late-stage cervical cancer remains to be one of the leading causes of cancer-related death.[6,7] Anthracycline and taxanes are among the commonly used chemotherapeutic agents against advanced cervical cancers; however, therapy response and survival rates are far from satisfactory.[8,9] Novel therapeutic agents which can act synergistically with existing therapies can improve the outlook in late-stage disease. Although the cytotoxic effect of PL has been demonstrated in several cancer cell lines, its effect on HeLa, which is the most commonly used cervical cancer cells, has been inadequately studied. There are also several studies in the literature which have reported synergistic cytotoxic activity of the PL with doxorubicin and paclitaxel. Both of these agents are currently included in chemotherapy regimens for the treatment of advanced cervical cancers.

We aimed to evaluate the cytotoxic effect of PL on HeLa cells and also to study whether it increases the effectiveness of these chemotherapeutic agents. We

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hypothesized that piperlongumine has antiproliferative properties against HeLa cancer cells and has synergistic anticancer effects when used in conjunction with chemotherapeutic agents.

Materials and Methods

HeLa cell (ATCC number: CCL-2) was used in order to determine the cytotoxic and apoptotic effect of the PL (Sigma Aldrich) alone and in combination with doxorubicin and paclitaxel. Cytotoxicity and IC50 doses were determined by MTT assay confirmed by live/dead cell staining using fluorescent microscope. Combinatory effects of the PL, doxorubicin, and paclitaxel on apoptotic cell death were detected using the Annexin V assay by Flow Cytometry.

Cell culture medium and conditions

Cells were grown in the medium [Dulbecco's modified Eagle's medium (DMEM); Cat#41965062; Gibco] supplemented with 1% penicilin-streptomisin (PS; Cat#: 15140122, Gibco), 15% fetal bovine serum [(FBS); Cat#10082147)], and 1% L-glutamine (Cat# 25030081, Gibco). Cells were detached from the culture vessel surfaces using trypsin-EDTA, and then stained with Trypan blue and counted with a Neubauer Chamber. Approximately, 3.2×10^4 cells were placed in each well in a 96-well plate for MTT analysis, 9.5×10^5 cells in each well in 6-well plate for applications, and 8×10^5 cells in 35-mm Petri dishes for flow cytometry evaluations. All cultures were incubated for 24 h in order for cells to attach and proliferate.

Cell viability and IC50 doses detected by MTT

The viability test was carried out with the commercial MTT kit (Vybrant MTT Cell Proliferation Assay, Cat#V13154, Thermo Fisher Scientific) according to the manufacturer's instructions. MTT analyses were performed using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Mindray MR 96 A, PRC). A 12-mM MTT stock solution was prepared by adding 1 mL of sterile phosphate-buffered saline (PBS) to a 5 mg vial of MTT. After removing cell culture medium, 90 µL of fresh culture medium and 10 µL of MTT stock solution were added per well and incubated at 37°C for 2 h, protected from light. Afterward, 50 µL of dimethyl sulfoxide was added to each well and mixed thoroughly with a pipette, then incubated at 37°C for an additional 10 min prior to its photometric measurement at the 570 nm wavelength. The viability percentages were calculated by comparison of the photometric measurement of the groups treated with different doses to the untreated control group. The concentrations required to inhibit growth by 50% (IC50) were calculated from survival curves. IC50 doses were determined for each compound by technically and experimentally duplicated assays.

Live/dead cell staining by acridine orange (AO) and propidium iodide (PI)

AO/PI stain prepared with 10 g sodium-ethylenediaminetetraacetic acid, 4 mg PI, and 50 ml FBS, 4 mg AO (dissolved in 2 ml 99% ETOH) mixed well and sterile distilled water added to reach a 200 ml final volume. With the nucleic acid binding dyes AO and PL, we have determined cell viability *in situ*. AO is an intercalating dye that can permeate both live and dead cells. AO stains all nucleated cells and generates green fluorescence. PI can only enter dead cells with poor membrane integrity, and stain all dead nucleated cells to generate red fluorescence. Cells stained with both AO/PI; all live nucleated cells fluoresce green and all dead nucleated cells fluoresce red. AO/PI stained plates were visualized by a fluorescent microscope (Leica). Micrographs obtained after AO/PI staining are presented in Figure 1.

Apoptosis by flow cytometry

Apoptotic and death cell rates were detected as experimentally duplicated assays by FITC Annexin V Apoptosis Detection Kit (Biolegend) using a flow cytometry (BD FACS Calibur). The experiments and the data collection were performed according to the manufacturer's protocol. HeLa cells were treated by various doses of doxorubicin, paclitaxel, and PL which had been determined by MTT during the initial studies. Apoptosis was also studied for doxorubicin and PL, and paclitaxel and PL combinations under their IC50 doses. Staurosporine (CAS 62996-74-1) was used as positive control to induce the apoptosis in HeLa cells.

Statistics

The results of all tests were expressed as the mean (SD) of at least two independent experiments. All statistical analysis was conducted using SPSS software (Version 18.0, SPSS Inc., Chicago, IL, USA). Mann–Whitney U test was applied for comparison of two independent samples. $P < 0.05$ was accepted as statistically significant.

Results

MTT detected IC50 doses of doxorubicin, paclitaxel, and PL for HeLa cells

The IC50 values of doxorubicin, paclitaxel, and PL for HeLa cells determined by MTT assay were 1695 nM for doxorubicin, 169 nM for paclitaxel, and 171 µM for PL. Live/dead cells staining by acridine orange/propidium iodide confirmed the MTT results *in situ* by fluorescent microscope [Figure 1].

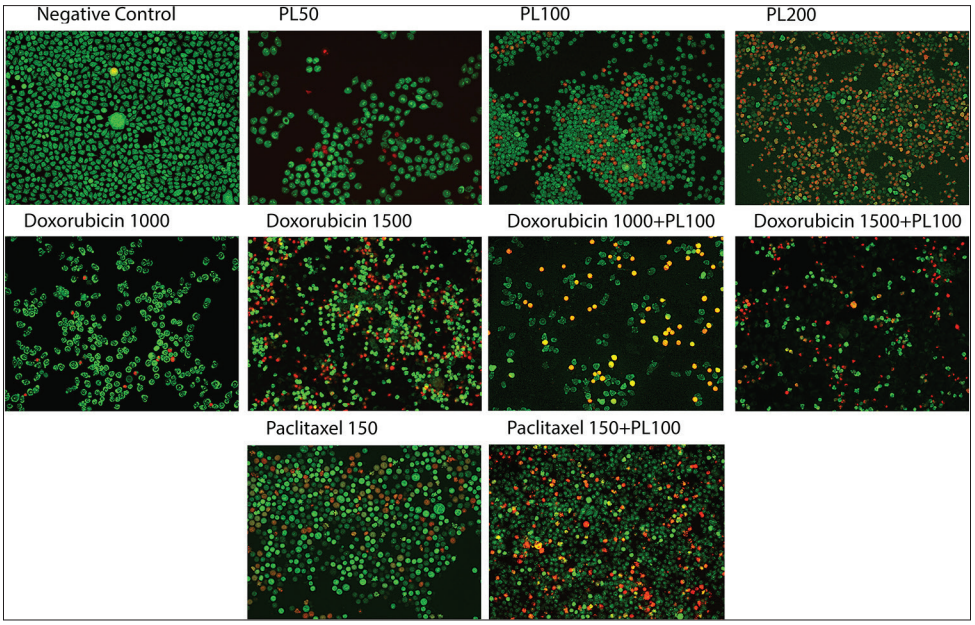

Figure 1: Fluorescent microscope images for live/dead cell staining by AO/PI of the HeLa cells treated by various concentrations of PL, doxorubicin, and paclitaxel

Flow cytometry provided quantitative analyses for apoptotic effect of the PL, doxorubicin, and paclitaxel

Apoptosis was measured by flow cytometry in HeLa cells treated with PL (50 μM, 100 μM, 200 μM), and 100 μM PL combined with 1000 μM and 1500 μM of doxorubicin, and 150 μM of paclitaxel. Apoptotic cell death percentages were 5.65 (0.44) at the negative control cultures, 43.61 (0.16) at the 1000 μM of doxorubicin, and 48.40 (0.12) at the 1500 μM of doxorubicin. When the 100 μM of PL combined with 1000 μM and 1500 μM of doxorubicin doses administered, apoptosis percentage rates were 58.02 (0.23) and 64.50 (0.24), respectively [Figure 2g]. The percentage of apoptosis was 12.88 (0.13) at the 150 μM of paclitaxel applied cultures, while it was 5.65 (0.44) at the negative control cultures. When the 150 μM of paclitaxel administered in combination with 100 μM PL, apoptotic cell percentage was 36 (1.41) [Figure 2h]. Our results also showed that the PL by itself induced the apoptosis in HeLa cells. Apoptotic cell percentages measured by flow cytometry in HeLa cells were 22.17 (0.13) for 50 μM of PL, 38.85 (0.16) for 100 μM of PL, and 53.40 (0.57) for 200 μM of PL [Figure 2a-f]. We have demonstrated that PL can increase doxorubicin and paclitaxel-induced apoptotic cell death of HeLa by 1.33 and 2.82 fold, respectively [Figure 2i].

Discussion

Treatment of late-stage cervical cancer remains a challenge due to limited number of therapeutic agents that has proven to be effective. Disease progression is inevitable due to rapidly evolving resistance of cancer cells to the available chemotherapeutics. Therefore, there is an ongoing need for discovery and development of new agents that can inhibit the development of chemoresistance. Agents that can promote anticancer effects of already established chemotherapeutics may play a role to overcome the chemoresistance. As a pyridone alkaloid that is naturally occurring in the fruit of Piper longum, PL has been reported to have selective cytotoxic properties against several types of cancer cell *in vitro*. It has been shown that the PL has synergistic properties when used simultaneously with chemotherapeutic drugs such as cisplatin.[10] The enhancement of apoptotic cell death was observed when PL was used in combination with either paclitaxel or doxorubicin when both of the chemotherapeutic drugs were used at concentrations below their predetermined IC50 values. When the combinations were compared to each other, doxorubicin and PL combination seems to be relatively more efficient for induction of apoptosis than paclitaxel and PL combination.

Several cellular mechanisms have been proposed to explain the synergistic activity of PL with chemotherapeutic agents. It is reported that caspase-3 activation by paclitaxel induces mitochondria-mediated intrinsic apoptosis pathway and upregulated survivin expression is a key factor for resistance to apoptotic cell death in paclitaxel-treated HeLa cells.[11] Several studies have reported that PL can effectively decrease survivin expression levels and reverse drug resistance in cancer cells which can explain how PL can increase the cytotoxicity of paclitaxel.[12,13] Interestingly, some

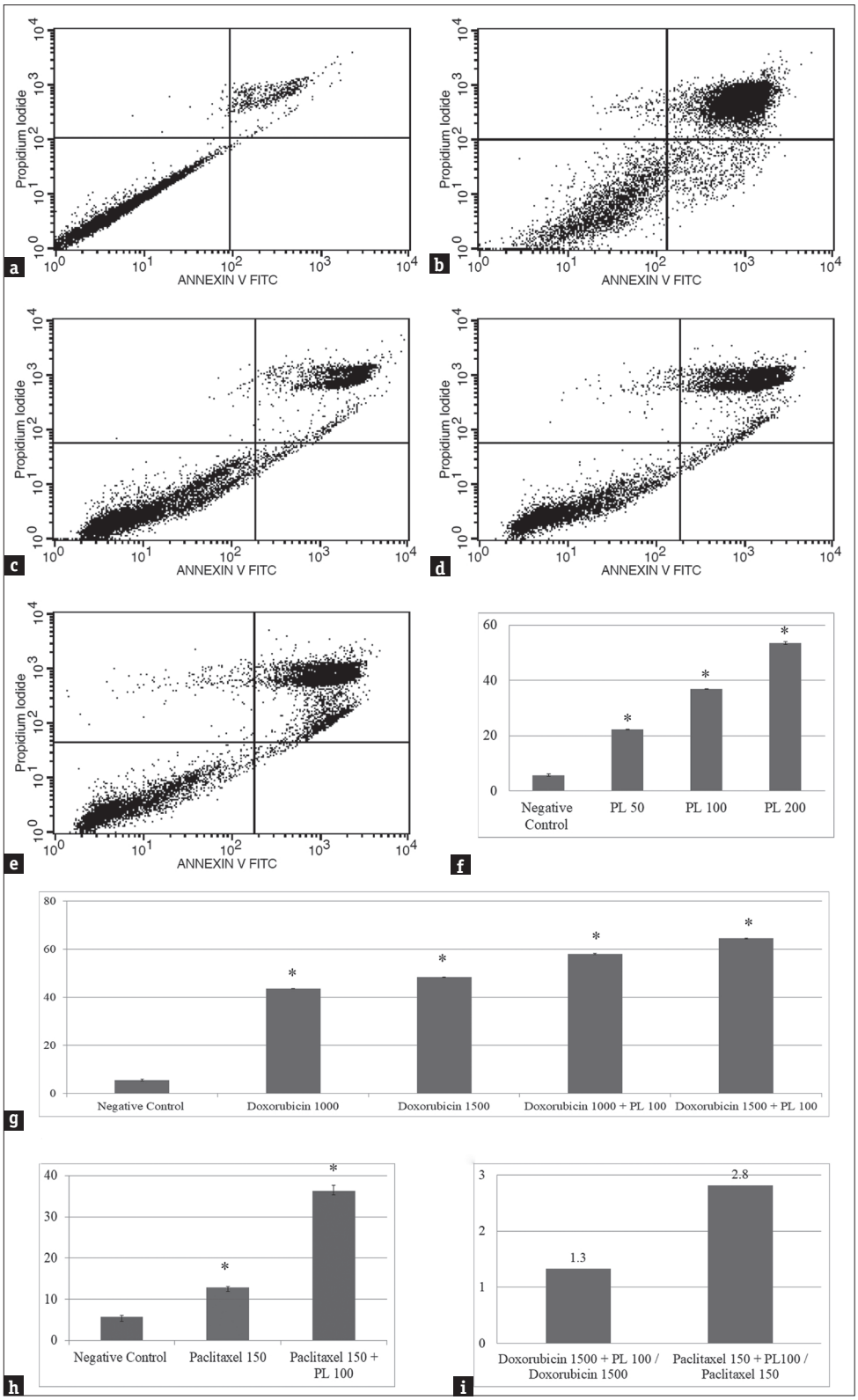

Figure 2: Flow cytometry results showing apoptosis rates in HeLa cells. (a) Apoptosis not induced (negative control), (b) apoptosis induced by staurosporine (positive control), (c) PL 50 μM, (d) PL 100 μM, and (e) PL 200 μM. (f) Apoptotic cell percentages after 24 h exposure to 50 μM, 100 μM, and 200 μM of PL. (g) Doxorubicin 1000 nM, 1500 nM, 1000 nM + PL 100 μM, 1500 nM + PL 100 μM. (h) Paclitaxel 150 nM and paclitaxel 150 nM + PL 100 μM. (i) Increased apoptotic effect seen with doxorubicin/PL and paclitaxel/PL. *Indicates a significant difference compared to control ($P < 0.001$)

studies in the literature reported that PL can outperform paclitaxel in terms of inducing apoptotic cell death. Our study results also demonstrate that PL has a greater apoptotoic effect on HeLa cancer cells compared to paclitaxel. It has been suggested that PL can more effectively lead to accumulation of radical oxygen species (ROS) in tumor cells compared to paclitaxel therefore can exert a higher apoptotic cell death efficacy.[14,15]

The result of this study is in line with other studies in the literature which have demonstrated increased synergistic activity against various cancer cell types when doxorubicin and PL is used in combination. Concomitant use of PL and doxorubicin may enable lower doses of doxorubicin to be used in treatment and therefore minimize risks related to cumulative doxorubicin dose. Various mechanisms have been proposed for the chemosensitizing effect of PL on tumor cells, which enhances the apoptotic effect of doxorubicin. PL shows greater synergy with doxorubicin against cancer cells. Administration of this agent decreased doxorubicin resistance in leukemia cells by the way of suppressing the expression of ATP-dependent drug efflux proteins, MDR1 and MDP.[16] It has also been shown that PL can interfere with the metabolism of doxorubicin through interaction with cytosolic enzymes responsible for biotransformation of doxorubicin.[17]

However, studies specifically aimed at evaluating the synergy between PL and the chemotherapeutic drugs actively used in the treatment, doxorubicin and paclitaxel, in terms of cytotoxicity against cervical cancer cells are lacking. In this study, we demonstrated that the PL is by itself effective against HeLa cells and use of PL in combination with either doxorubicin or paclitaxel at concentrations below IC50 of both chemotherapeutic agents resulted in an increased proapoptotic activity against cervical cancer cells compared to use of doxorubicin and paclitaxel alone.

In the literature, the IC50 values of PL after 72 h exposure range from 1 μM to 100 μM in a series of cancer cell lines.[10,13] We describe in this study that the IC50 dose of 24 h of PL is 171 μM. The IC50 dose we described in HeLa cells is higher than the other cancer cell lines. This may result from the higher resistance of HeLa cells to cytotoxic agents than other cancer cell lines reported.

Conclusion

In conclusion, the present study demonstrates that the PL appears to be an attractive bioactive phytochemical for cervical cancer treatment. We have shown that PL has the ability to enhance the cytotoxicity of the two commonly used chemotherapeutic agents, doxorubicin and paclitaxel, against cervical cancer cells through increasing apoptosis of cancer cells. The therapeutic potential of this agent in cancer treatment warrants further investigation.

Ethical Approval

This study does not contain any studies with human participants or animals performed by any of authors.

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Conflicts of interest

There are no conflicts of interest.

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