Novel effects of piperlongumine on uterine fibroid tumor: An in vitro mouse model study

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Abstract

Purpose: To investigate the in vivo anti-tumor effect of piperlongumine (PL) in a mouse model of leiomyoma xenograft and in leiomyoma cell lines.

Methods: The anti-proliferative effect of PL on ELT-3 cells was determined using MTT assay. Human and rat leiomyoma cells were used for the in vitro investigations. Rat leiomyoma cell lines were treated with various PL concentrations (50 - 100 μM) for 48 h. Immunodeficient mice were subcutaneously injected with varying doses of estrogen or progesterone, and xenografted with explanting human leiomyoma cells in vivo experiments. Proliferation assessment, caspase-3 expression, analysis of tumour samples, insertion of pellets of oestrogen-progesterone, tissue treatment and implantation, and immuno-histochemical analyses were carried out using appropriate procedures.

Results: Piperlongumine (PL) produced significant and dose-dependent increase in caspase-3 activity, apoptosis and suppression of cellular proliferation (p < 0.01). Moreover, Western blot data demonstrated that PL decreased phosphorylation of Akt signaling pathway. The results showed significant (p < 0.01) inhibition of tumor growth, including in ultra-sound in vivo studies, when compared with 30-day control and animals treated with PL (100 μg/g). Immuno-histochemical studies showed that PL decreased the expression of proliferation marker in xenografted tumor tissues (p < 0.02).

Conclusion: These results suggest that piperlongumine has potentials as a therapeutic agent for the management of uterine leiomyoma. However, additional studies using human cell lines are required to understand its genetic and molecular mechanisms.

Keywords: Uterine fibroids, Immune-deficiencies, Xenograft animal model, Tumor, Leiomyoma cells, Estrogen, Progesterone

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INTRODUCTION

Uterine leiomyomas, also known as fibroids, are abnormal growths in the smooth muscles of the uterus. These growths cause symptoms such as formation of abdominal mass, depression, abdominal pains, infertility, spontaneous abortions, dysmenorrhea, and irregular menstruation [1]. They are commonly benign tumors present in the reproductive system of females.
Piperlongumine, a natural alkaloid derived from the fruit of *Piper longum* which grows in southern regions of India and Asia, is known for its anticancer properties [2]. In addition, several studies have reported that PL exhibited cytotoxicity against cancer cell lines, and inhibited tumour growth in xenograft mouse models through a variety of mechanisms such as increase in levels reactive oxygen species (ROS), and suppression of LMP-1 gene expression [3]; inhibition of the activity of telomerase reverse transcriptase [4], promotion of autophagy [5], inhibition of NF-κB expression [6], activation of the ERK pathway [7], as well as phosphorylation of AMPK [8] and C/EBP homologous proteins [9]. Piperlongumine is also known to exhibit antitumor activity in breast carcinoma [10], and in various other carcinomas e.g., ovarian carcinoma. Various findings suggest the anticancer activities of PL in cancer cell lines through many mechanisms such as induction of apoptosis [11] and induction of necrosis with autophagy [12]. *In vitro*, PL induces cell-specific cytotoxic effects without affecting other cells. Studies have revealed that PL induces anticancer activity through a mechanism involving elevation of ROS, leading to cell apoptosis, although the exact pathway through which PL induces apoptosis is yet unknown [13].

The present study investigated the *in vivo* therapeutic effect of PL in a mouse leiomyoma xenograft model, as well as its effects on tumour size and volumes.

**EXPERIMENTAL**

**Cells**

Eker rat leiomyoma cell line (ELT-3) was provided by Cancer Institute and Research Centre, China. The cells were maintained in DF8 medium after complete characterization [14]. The ELT-3 cells were incubated in a 5 % CO₂ atmosphere below 37 °C until they attained 70-80 % confluence.

**Materials**

Piperlongumine (PL) was obtained as a gift from Adamas Pvt Ltd (Shanghai, China). Caspase-3 assay kit and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay kit were obtained from Merck, USA. Protease inhibitors without EGTA were purchased from Roche, USA. Monoclonal antibodies (anti-phospho Akt and anti-total Akt) as well as rabbit polyclonal antibodies (anti-Ki67) were products of Cell Signaling (USA).

**Proliferation assay**

The effect of PL on cell proliferation was determined using MTT assay. The ELT-3 cells were seeded in 96-well plates and cultured in the presence of PL at concentrations of 6.25, 12.5, 25, 50 and 100 µM for 48 h. The MTT assay was carried out as reported earlier [15].

**Caspase-3 assay**

Caspase-3 assay was carried out using quantitative fluorometric method (Thermo Scientific, Pierce), as described earlier [15]. The cells were seeded in 96-well plates. After 24 h, the culture medium (Smooth Muscle Growth Medium) was replaced with medium containing human fibroblast and epidermal growth factors (0.3 and 0.4 %), fetal bovine serum (5 %), gentamicin or amphotericin B (0.3 %) and insulin (0.2 %). These media contained PL at concentrations of 6.25, 12.5, 25, 50 and 100µM, respectfully. After 48 h, the cells were lysed. Equal volumes of lysates added to a reaction mixture containing caspase-3 substrate (Z-DEVD-R110). Fluorometric measurement of caspase-3 activity was completed in approximately 60 min.

**Studies using tumor samples**

The approval of the Ethics Committee was obtained from the Human Ethics Committee of Cancer Institute and Research Center, China (approved protocol no. EC/CIRC/043_UF/20) and informed consent was obtained from patients (preferring hysterectomy) to gather (if not discarded) leiomyoma samples and shifted to the experimental laboratory immediately after sterilization [14]. Two samples of two distinct patients were collected and analyzed at two different time intervals.

**Animals**

The animal study using female mice was approved by the Ethics Committee for Care and Use of Animals of Medical Centre, Beijing (approval no. EC/28/013/20). The animals were treated according to the guidelines of this committee. This study used a modified animal model of xenograft leiomyoma previously described [16]. The in-house cancer center provided 6-week-old female mice with multiple immunodeficiencies (NOG). The numerous and combined immune defects make the mice suitable as xenograft models for human studies [17]. Sterility was maintained when performing each experiment, and the mice were housed in adequate environmental conditions.
Estrogen-progesterone pellet insertion

The rats were anaesthetized using isoflurane (1.5 percent). Subcutaneous injection of 1, 17β-estradiol (50 µg/kg/day in 0.2 mL of safflower oil), or progesterone (50 mg/kg/day in 0.1 safflower oil) for 21 days, was given to each mouse before 5 days of placing the xenograft leiomyoma. For pain relief, buprenorphine (50-100 µg/kg) was administered in an injection volume of 1 mL per kg via the subcutaneous route, twice daily or as required [16].

Tissue processing and implantation

Tumors (leiomyoma) obtained from patients were immediately processed under sterilized conditions. The tumor was also cut into 2 x 2 x 2 mm cylindrical slices using biopsy tool, and the slices were immersed before loading into the matrix membrane. Sterility of the leiomyoma tumor was ensured during processing. Twenty mice were injected with the tumour through subcutaneous incisions on the flanks (sides) of the skin, which were later sealed using sterile surgical staples. Two or three tumor slices were given for each mouse). The mice were continuously monitored for any symptoms of discomfort or illness during the procedure. Treatment began after a week of xenograft insertion, only when staples were removed. The mice (n = 16) were randomly assigned to either the treatment (n = 8) or control (n = 8) groups.

Animal treatments

Subcutaneous injection of PL at a dose of 100 µg/gm body weigh/day, or vehicle control, was carried out for 30 days. The mice were closely observed daily, and a caliper was used to measure the tumor size for 30 days. Three-dimensional tumor size analysis was done using a 30 - 50 MHz high-resolution ultrasonic probe (Visual Sonics, China). Tumor volume (V) was computed as shown in Eq 1.

\[ V = 0.52lwh \]  …………… (1)

where \( l \) is length, \( w \) is width and \( h \) is height of the tumor.

The mice were euthanized with isoflurane injection after an ultrasound scan, followed by cervical dislocation. The tumors were excised through skin incision. A caliper was used to measure the weight of and size of each wet tumor. The tumours were maintained in a fixative solution of 10 % formalin at 4 °C prior to immunostaining to preserve the visibility of specific tissues. The personnel who conducted the experiments and measured the size of tumors were naïve to the treatments given to the mice groups.

Immuo-histochemical evaluation

Tumor specimens obtained from the mice were embedded in 10 % buffered formalin, and tissue sections were prepared in blocks. The morphologies of the tissue sections were determined using hematoxylin and eosin (H&E) staining. The immuno-peroxidase method using chromogen-DAB was employed to obtain immuno-stained proliferation marker Ki67, and the degree of immuno-staining was quantified using Image-Pro Plus software (Media Cybernetics, USA). After 48 h of treatment with PL, fluorometric caspase-3 activity assay was carried out on ELT-3 cells. The results were obtained after normalizing to control, following three replicated experiments.

Tissue area, staining intensities, and the percent of stained cells relative to the total number of cells, were evaluated using Image-Pro Plus software in ten isolated high-power fields (10) on each slide. The observer (pathologist) interpreting data from the slides had no idea which sample belonged to the treatment and control groups.

Statistical analysis

Results are expressed as mean ± SEM. Results from in vitro studies were compared amongst control and PL groups using One-way analysis of variance (ANOVA), followed by Post-hoc Bonferroni analysis (GraphPad Prism 8.0 version). Tumor sizes and immunohistochemistry values for control and PL groups were compared using Student’s t-test. Values of \( p < 0.05 \) were considered indicative of significant differences.

RESULTS

Apoptosis in leiomyoma cells

Results from fluorometric assays show that PL treatment for 48 h dose-dependently caused significant differences in apoptotic cell death in ELT-3 cells, relative to control. One-way ANOVA results showed significant variations between control and each of the PL groups (\( p < 0.004 \)). Bonferroni's post-hoc analysis revealed significant differences between the PL-treated groups (50 and 100 µM) and the control group (\( p < 0.01 \); Figure 1).
Figure 1: Effect of PL treatment on leiomyoma cells on apoptosis following fluorometric caspase-3 activity assay. The data was standardised to control and shown as the mean±SEM of three independent assays. DMSO stands for dimethyl sulfoxide, and PL stands for piperlongumine. ** P < 0.01 in comparison to control; ***P < 0.001 in comparison to control

Proliferation of leiomyoma cells

The effect of PL on the proliferation of leiomyoma cells was assessed using MTT assay. After treatment of ELT-3 cells with PL for 48 h, results of one-way ANOVA indicated significant difference in proliferation between groups (p = 0.031). Bonferroni’s post-hoc analysis revealed that 100 μM PL significantly suppressed cell proliferation, when compared to control group (p < 0.05; Figure 2).

Figure 2: Effect of PL treatment on leiomyoma cells for 48 hours on cellular proliferation following a (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. The data was standardised to control and shown as the mean ± SEM of three independent assays. DMSO stands for dimethyl sulfoxide, and PL stands for piperlongumine. *P < 0.05 vs Control

PL Inhibited Akt signaling pathway in leiomyoma cells

Results from Western blotting showed that PL markedly and dose-dependently down-regulated the phosphorylation of Akt protein (p < 0.001). These results are shown in Figure 3.

Figure 3: Effect of PL treatment on leiomyoma cells with the specified doses for 48 hours on activation of the Akt cellular signalling system, accompanied by western blot analysis of cell lysates for activated (phosphorylated) Akt and total Akt. The data was standardised to control and shown as the mean ± SEM of three independent assays. DMSO stands for dimethyl sulfoxide, and PL stands for piperlongumine. **P < 0.01 in comparison to control; ***P < 0.001 in comparison to control

Piperlongumine (PL) inhibited growth of xenograft leiomyoma in mice

The sizes of tumors in PL-treated groups were 29.4 and 40.5 % smaller than the corresponding sizes of tumors in control group at week 3 and week 4. Furthermore, the mean tumor size in the treated group was 45.7 % lower than that in the control group (p = 0.043; Figure 5), as recorded using ultrasound scanning. The mean weight of tumor in the treatment group was 14.5 % lower than that in the control group, although the difference was not significant (p = 0.14; Figure 4).

PI Inhibited proliferation of leiomyoma cells in xenografted tumor

Figure 5 shows that PL treatment significantly reduced the expression of the proliferation marker by 47.2 percent (p = 0.015).
Figure 4: Effect of piperlongumine (PL) treatment on tumour volume (A) and tumour size (B) in a patient-derived leiomyoma xenograft animal model (B). For 30 days, treated animals (n = 8) were given 100 g/g body weight/day subcutaneously, while controls (n = 8) were given vehicle. Calipers were used to quantify tumour volume (mm$^3$) in the control and PL groups on a weekly basis. The weight of the tumours in the control and PL groups was measured shortly after euthanasia. The data is presented as a mean ± standard error of the mean (SEM)

Figure 5: Photomicrographs of the control and PL-treated animals (40x magnification). Tumor tissues acquired after death are placed in a 10% formalin buffered solution and then prepared to obtain tissue slices. The proliferation marker Ki67 was then employed as a chromogen for immunostaining, with (3, 3'-diaminobenzidine) as a chromogen. The number of positively stained cells and staining intensity in high-power fields (20) in each slide were then used to grade expression using image analysis software. The results are presented as the mean SEM of 20 individual treatment and 20 individual control group slides

DISCUSSION

The results obtained in this study showed that piperlongumine exerted apoptotic effects in vitro, suppressed the proliferation of leiomyoma cells, and caused leiomyoma tumors in mouse xenograft models. It also inhibited the growth of leiomyoma tumor in an animal model of a patient-derived xenograft.

Mice used in this study had endogenous sex steroids, because they were not ovariectomized. It has been reported that animal models of leiomyoma xenograft showed cell necrosis only in the absence of endogenous steroids [14]. The exact therapeutic effect of PL due to estrogen and progesterone is not yet known since no studies have been reported so far. Thus, there is need for further studies in this area.

Several significant findings were made in the present study. In the first place, piperlongumine was effective in the management of uterine leiomyoma. Secondly, modification of protocols used for establishing animal model in an earlier study [14] resulted in active growth and consistently preserved uterine leiomyoma. The modification entailed substitution of oestrogen pellets with oestrogen-progesterone injection, since progesterone has been shown to be essential for the survival and development of uterine leiomyoma [14]. Another advantage is that it was easier and more accurate to carry out calculations on tumors in the subcutaneous layer. The total volume of tumors after four weeks was 195 percent of the initial volume. This is in agreement with the findings in an earlier study [14].

There are various mechanisms involved in the antitumor effect of PL [2]. A study has shown that PL inhibited tumour growth in vivo by activating the transcription factor fork head box O3A (FOXO3A-BIM) axis [18]. The phosphorylation and activation of Akt kinase inhibited activated FOXO3A. In a study conducted on cancer cell bladder cells, piperlongumine increased ROS concentrations and cell growth inhibition, blocked the cancer cells at G2/M phase, and also suppressed migration of the cancer cells [19]. Piperlongumine inhibited cell growth and Ras proteins. A level of P13 K which resulted in the suppression of Akt/NF-kB activation did not cause toxicity [15]. Furthermore, research has shown that PL inhibited phosphorylation of the AKT signaling pathway of growth factors, which in turn inhibited cell proliferation. Moreover, PL showed reversal of resistance to chemotherapy and regulation of gene expressions in tumor cells, along with decreased toxicity and increased activity. The PL-induced changes in Akt/Foxo3/NRF2/P-gp signaling pathway inhibited Cis efflux in A549/Cis cells. Phosphorylation of BAD was decreased by dephosphorylated Akt, which inactivated NRF2 due to reduction of Bcl-xL. The generation of the heterodimer, BAD-Bcl-xL promotes apoptosis. Other mechanisms may also operate due to the different complexities present in in vivo tumor microenvironment, relative to cell culture environmental conditions. As a result, further research is required in various in vivo leiomyoma mouse models to understand the mechanisms involved in different antitumor activities of PL.
This study has some limitations. No myometrial control was used to determine myometrial toxicity or the effect of PL on the leiomyoma-treated mice or control group. Moreover, PL levels were not investigated in the plasma and tumor. In further studies, the effect of PL on human cell lines will be investigated to correlate with aspects of the current research findings. Tumors beneath the skin are different from tumors in the usual uterine microenvironment region susceptible to steroids such as growth and inflammatory factors. Thus, development of models of orthotopic xenograft leiomyoma is of substantial benefit in leiomyoma studies.

CONCLUSION

Piperlongumine exerts therapeutic effect on uterine leiomyoma. However, further studies are required to understand the mechanism of this activity. Piperlongumine was found to be effective in regulating expression of the tumor cell gene with reversal of resistance to chemotherapy, which resulted in a decrease in toxicity.

DECLARATIONS

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Conflict of interest

Authors have no conflicts of interest with respect to the publication of this study.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors contributed equally to the publication of this research work. The manuscript was written by Mei Zhang. The materials were collected by Mei Ye, Qingxiang Hou. Bin Yan and Chao Yang offered assistance during revision of the manuscript. All experiments were performed by Mei Zhang, Mei Ye, Qingxiang Hou, Bin Yan, and Chao Yang under supervision of Li Ma.

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