Hexane fraction of *Pluchea indica* root extract inhibits proliferation and induces autophagy in human glioblastoma cells

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Abstract. *Pluchea indica* (L.) Less. is a perennial plant known for its versatile uses in traditional medicine. Previous findings have shown that the extracts of *Pluchea indica* possess significant anti-inflammatory, anti-ulcer and anti-tuberculosis activity. The aim of this study was to demonstrate the anticancer activity of the hexane fraction of *P. indica* root extract (H-PIRE) in human glioblastoma cells using flow cytometric and western blot analysis. The results shoewd that, H-PIRE suppressed the growth of glioblastoma cells in a dose-dependent manner. H-PIRE treatment markedly decreased the population of cells in S and G2/M phases. The significant upregulation of acidic vesicular organelle (AVO) was detected during H-PIRE treatment. The expression levels of microtubule-associated light chain 3-II (LC3-II) protein, phosphorylated JNK and phosphorylated p38 were significantly increased, confirming the occurrence of autophagy during the process. Finally, the combination treatment of H-PIRE and LY294002, a pan PI3K inhibitor, further decreased cell viability, suggesting an additive anticancer effect. Taken together, our results suggest that H-PIRE suppresses the proliferation of glioblastoma cells by inducing cell cycle arrest and autophagy.

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Introduction

Glioblastoma multiforme (GBM) is among the most common malignant cancers of the brain (1). Although the main cause of this disease remains unknown, possible risk factors include genetic disorder (2), viral infection (3), alcohol consumption (4) and ionizing radiation exposure (5). Standard treatments for GBM include surgicael resection, chemotherapy (e.g., temozolomide therapy) and radiotherapy (6,7). As GBM spreads rapidly and has a high potential for relapse even the most aggressive treatment rarely achieves survival >3-years and the median survival time is approximately 1-year (7,8). Complementary and alternative therapies commonly used in GBM patients include vitamin and nutrient supplements as well as herbal extracts (9). Some clinical evidence shows that treatment with herbal extracts can reduce mortality in GBM patients (10).

Pluchea indica (L.) Less. (Asteraceae) is a perennial shrub with medicinal properties that are widely recognized in many Asian countries (11). *P. indica* is traditionally used to heal minor wounds because it has astringent, antipyretic and anti-inflammatory activities (11,12). The leaves of *P. indica* contain α-glucosidase inhibitors that could help treat diabetes mellitus by suppressing carbohydrate digestion (13). Methanol extracts of the *P. indica* root are known to neutralize snake venoms (14) and contain anti-amoebic activities (15). The methanol fraction of *P. indica* root and leaf extracts has revealed potent anti-ulcer (16) and anti-tuberculosis effects (17). The aqueous extract of *P. indica* has antiviral activity against human immunodeficiency virus type 1 (18). Quinic acid esters from the leaves of *P. indica* showed inhibitory activities towards collagenase, MMP-2 and -9 (19). *In vitro* studies also revealed that crude aqueous extracts and ethanol extracts of *P. indica* root effectively suppress human malignant glioma cancer cells and human cervical cancer cells (20,21).

Therefore, the aim of this study was to investigate the underlying mechanisms of the anticancer effects of the hexane fraction of *P. indica* root extract (H-PIRE) in human U87 GBM cells and primary GBM cells.

Materials and methods

Plant material and extraction. The *P. indica* plants used in experiments in this study were collected and verified as described in a previous study (20). The roots of *P. indica* were prepared as a dried powder and deposited at the National Sun Yat-sen University (Kaohsiung, Taiwan, R.O.C.) and the H-PIRE was extracted using the method described by Kao *et al* (21). Briefly, the *P. indica* extract was prepared by immersing the root powder in 95% (v/v) ethanol at room temperature overnight, filtering with 0.45 μm filters, then partitioning the water and ethyl acetate (v/v, 1:1). The ethyl acetate fraction was further partitioned with a 1:1 (v/v) mixture of 75% ethanol and hexane. The hexane layer was collected and concentrated to obtain powdered H-PIRE. The powdered H-PIRE was then dissolved in dimethyl sulfoxide (DMSO) to desired concentrations in subsequent experiments.

Preliminary phytochemical analysis. Total phenolic compound content was determined with Folin-Ciocalteu reagent and presented as gallic acid equivalents in mg/g of extract. Total flavonoid content was determined by aluminum chloride colorimetric method and expressed as catechin equivalents in mg/g of extract (22). Condensed tannin (proanthocyanidin) content was analyzed by vanillin assay method as described by Butler *et al* (23) and presented as catechin equivalents in mg/g of extract. All assays were performed in triplicate.

Cell culture. Primary GBM cells were enzymatically dissociated from fresh glioblastoma samples as described by Pavon *et al* (24), according to a protocol approved by the Kaohsiung Medical University Institutional Review Board (Kaohsiung, Taiwan, R.O.C.). The U87 and primary GBM cell lines were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (all from HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 200 mM l-glutamine (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin (10,000 U/ml)/streptomycin (10,000 U/ml) (HyClone; GE Healthcare Life Sciences Hyclone), 1X non-essential amino acids and 1 mM sodium pyruvate (both from Invitrogen Life Technologies; Thermo Fisher Scientific, Inc.). The cells were cultured in 5% CO_2 at 37˚C in a humidified incubator. Institutional board approval was obtained before experimental use of primary GBM cells derived from patients.

Cell viability assays. Cell viability was determined using the WST-1 colorimetric assay method. The cells were cultured overnight in 96-well plates ($2x10^3$ cells/well) and treated with 0-1,000 μg/ml H-PIRE or DMSO (negative control) for 24 to 72 h. The medium was then carefully removed and replaced with fresh medium containing the cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) and the cells were incubated for an additional 3 h at 37˚C. Absorbance was measured at 450 nm with an ELISA plate reader (Micro Quant; BioTek Instruments, Inc., Winooski, VT, USA). The IC_{50} values were determined as previously described (20) and presented as mean ± standard deviation (SD) (n=3).

Table I. Phytochemical content of H-PIRE.

Phytochemical content	Mean ± SD
Total phenols	196.7±0.7 mg GAE/g DW
Total flavonoids	113.6±0.6 mg CE/g DW
Proanthocyanidins	28.947±0.0062 mg CE/g DW

Mean ± SD (N=3); H-PIRE, hexane fraction of *Pluchea indica* root extract; CE, catechin equivalent; GAE, gallic acid equivalent; DW, dry weight; SD, standard deviation.

Flow cytometric analysis. Apoptosis was detected with an fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit I (BD Pharmingen, BD Biosciences, Franklin Lakes, NJ, USA). After 24 and 48 h of incubation with H-PIRE or DMSO, $1x10^5$ cells were collected and suspended in 100 μl staining buffer containing 5 μl of FITC Annexin V and 5 μl propidium iodide (PI) at room temperature in the dark. After 15 min, 400 μl 1X binding buffer was added and flow cytometric analysis was performed as previously described (20).

Acridine orange staining. Cells were cultured in 60-mm dishes until 60% confluent and then treated with 300 μg/ml H-PIRE for 24 h. The cells were then stained with 1 mg/ml acridine orange (AO) for 15 min and washed with phosphate buffer saline (PBS) to detect acidic vesicular organelles (AVOs). A fluorescence microscope (Olympus IX70; Tokyo, Japan) with an excitation filter set to 490 nm and a 515 nm long-pass filter was used for cell analysis and digital imaging.

Western blot analysis. The cells were cultured in 60-mm dishes until 60% confluent and then treated with 300 μg/ml H-PIRE for 0 to 24 h. After incubation, the cells were washed with PBS and lysed in T-PER protein extraction reagent (Pierce). Cell lysates containing 20 μg total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Antibodies against p-p38 (1:1,000, rabbit monoclonal, Cell Signaling Technology cat. no. 4511), p-JNK (1:1,000, rabbit monoclonal, Cell Signaling Technology, cat. no. 4668), LC3B (1:1,000, rabbit monoclonal, Cell Signaling Technology cat. no. 3868) and β-actin (1:10,000, mouse monoclonal, Sigma-Aldrich,) were used for immunoblotting. The immunoblots were visualized using enhanced chemiluminescence detection reagent (Amersham; GE Healthcare, Chicago, IL, USA). A LAS-3000 CCD system was used for imaging and a Multi Gauge V2.02 both from (Fujifilm Holdings Corporation, Tokyo, Japan) was used for analysis.

Statistical analysis. Data were expressed as the mean ± SD of three independent experiments. Statistical significance was determined by performing independent Student's t-test with SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.
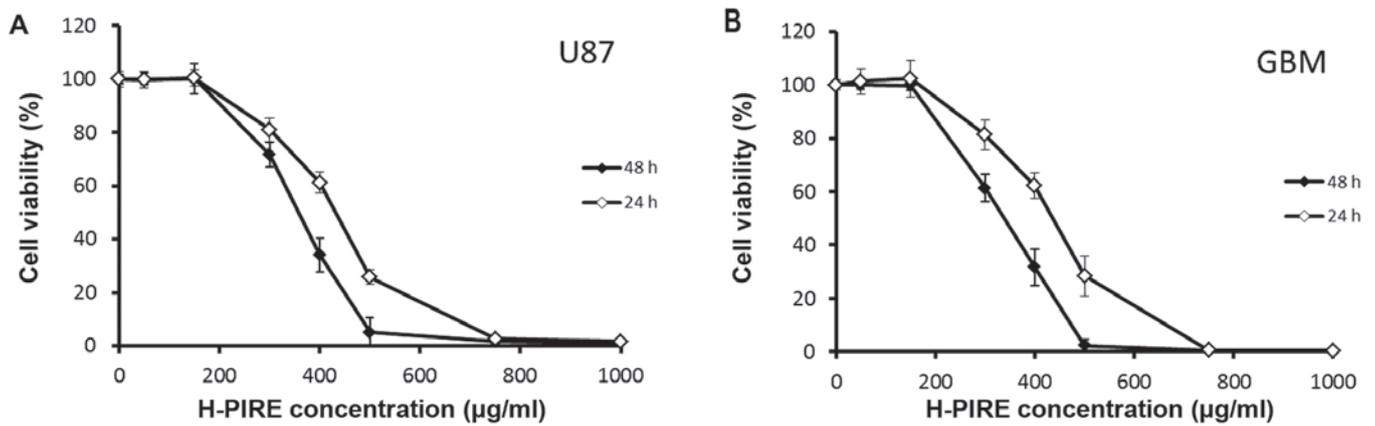
Figure 1. Dose-dependent effect of H-PIRE treatment (0-1,000 μg/ml) on viability of (A) U87 cells and (B) GBM cells. Cell viability was determined by WST-1 assay at 24 and 48 h. H-PIRE, hexane fraction of *Pluchea indica* root extract; GBM, glioblastoma multiforme.
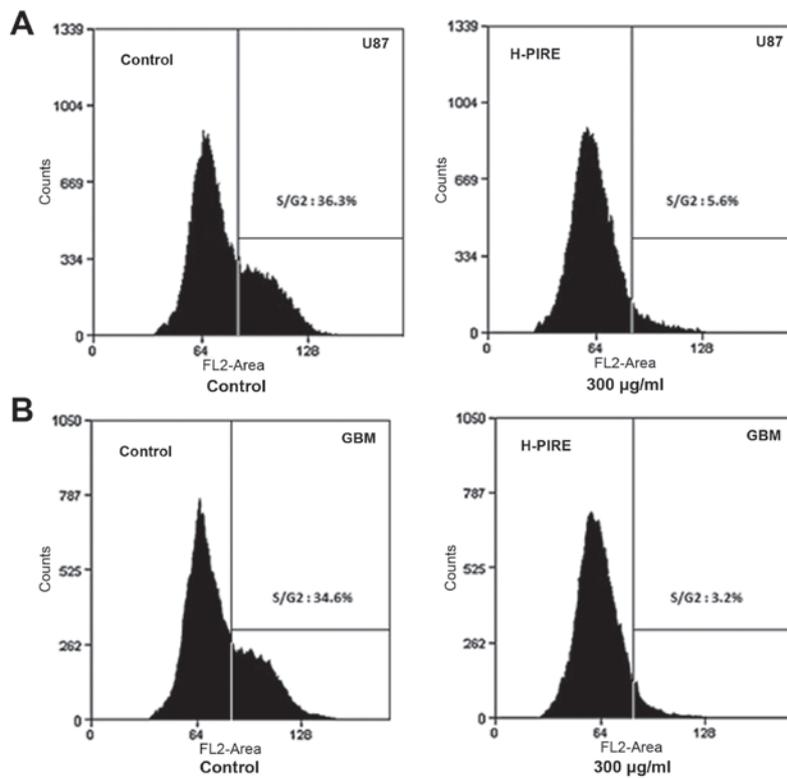

Figure 2. Results of cell cycle analysis of (A) U87 cells and (B) GBM cells after 24 h treatment with 0.1% DMSO (control) or 300 μg/ml H-PIRE. Cell cycle analysis was performed by flow cytometry in cells stained with PI. GBM, glioblastoma multiforme; DMSO, dimethyl sulfoxide; H-PIRE, hexane fraction of *Pluchea indica* root extract; PI, propidium iodide.

Results

Phytochemical screening. Table I shows that the preliminary phytochemical analysis of H-PIRE revealed a high content of phenols, flavonoids and proanthocyanidins, which are biologically active phytochemical compounds found in many medicinal herbal extracts. Secondary metabolites such as polyphenols and flavonoids interact with proteins and are useful for inhibiting multidrug resistance (25), suppressing tumor angiogenesis and preventing inflammation (26). Thus, the phytochemical composition of H-PIRE was consistent with the reported medicinal properties of *P. indica*.

Suppression of GBM cell proliferation by H-PIRE. The cytotoxic effects of H-PIRE on GBM cells were examined by assays of WST-1 cell proliferation. Two GBM cell lines were treated with varying concentrations of H-PIRE (range, 0-1,000 μg/ml) at 24 and 48 h. Fig. 1 shows the experimental results. In both cell lines, H-PIRE reduced cell viability in a dose-dependent manner. At 24 and 48 h, the IC_{50} values (mean ± SD, μg/ml) for H-PIRE were 422.6±38.3 and 353.3±18.7 in U87 cells and 416.4±35.5 and 334.1±29.6 in GBM cells, respectively. That is, the IC_{50} values indicated that H-PIRE suppressed proliferation in both human GBM cell lines.
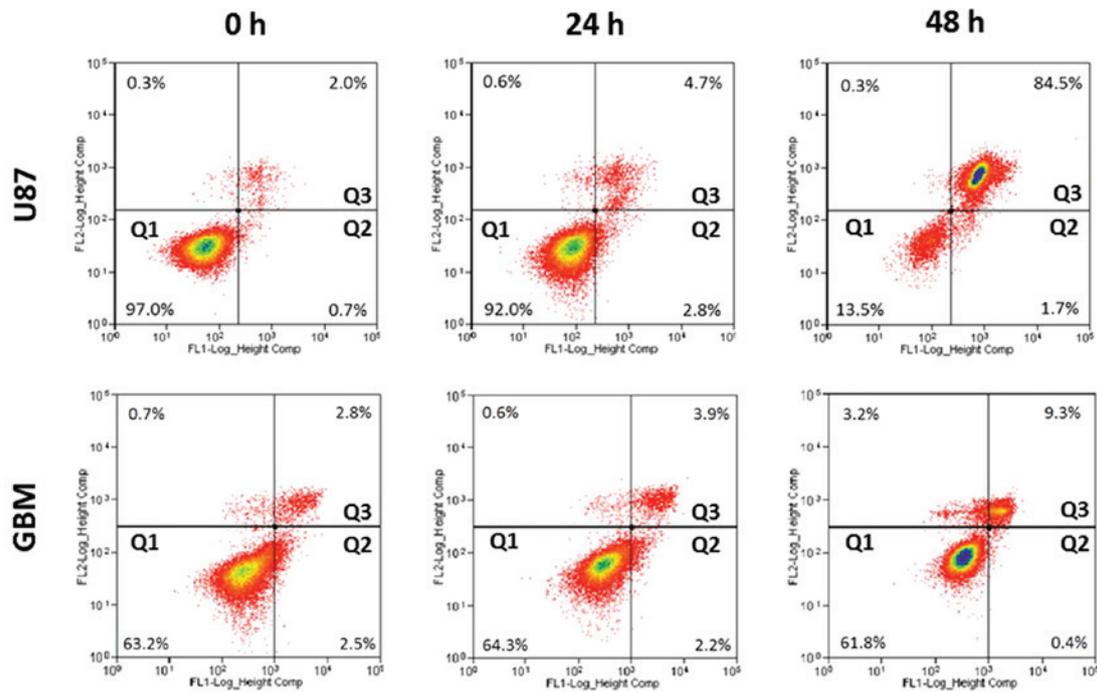
Figure 3. Results of flow cytometric analysis of apoptosis in U87 and GBM cells treated with 300 μg/ml H-PIRE for 0, 24 and 48 h then stained with FITC-Annexin V and PI. Q1, the lower-left quadrant shows living cells; Q2, the lower-right quadrant shows early apoptotic cells; and Q3, the upper-right quadrant shows necrotic or late-apoptotic cells. GBM, glioblastoma multiforme H-PIRE, hexane fraction of *Pluchea indica* root extract; FITC, fluorescein isothiocyanate; PI, propidium iodide.
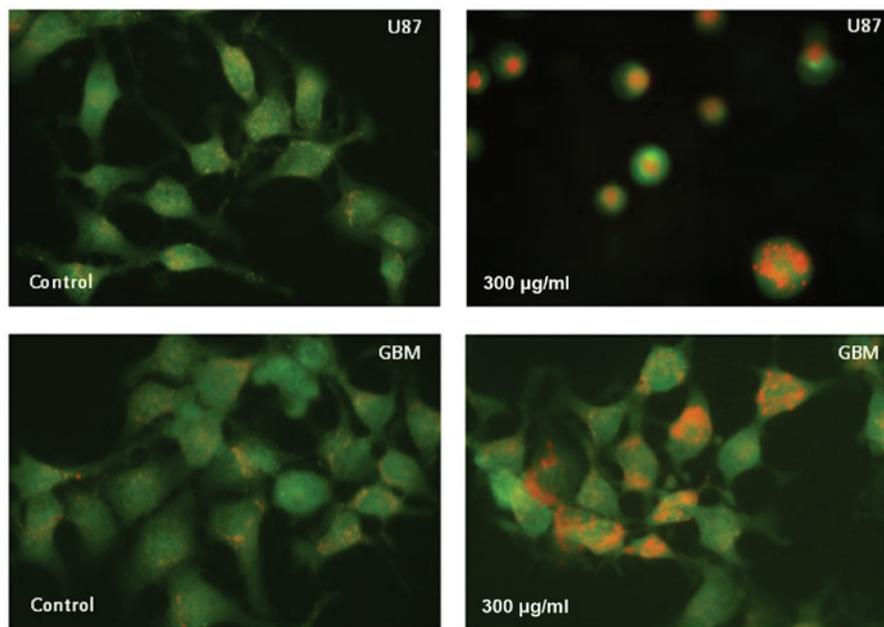

Figure 4. Fluorescence microscopic images of U87 and GBM cells stained with AO. Cells were treated with 0.1% DMSO (control) or 300 μg/ml of H-PIRE for 24 h. Merging green and red channels are visible. AO, acridine orange; GBM, glioblastoma multiforme; H-PIRE, hexane fraction of *Pluchea indica* root extract; DMSO, dimethyl sulfoxide.

Suppression of cell cycle progression by H-PIRE. The effect of H-PIRE on the cell cycle was investigated by flow cytometry. The U87 and GBM cells were incubated with 300 μg/ml H-PIRE for 24 h. The incubated cells were then collected, fixed and stained with PI for flow cytometric analysis of DNA content. Fig. 2 shows that the H-PIRE treatment significantly decreased the cell populations in the S and G2/M phases.

However, the sub-G1 population showed no increase, which indicated that the treatment did not induce apoptosis. Thus, H-PIRE suppressed progression of the human GBM cell cycle through the G0/G1 phase.

Induction of apoptosis by H-PIRE. Apoptotic effects of H-PIRE were investigated by flow cytometry of unfixed cells
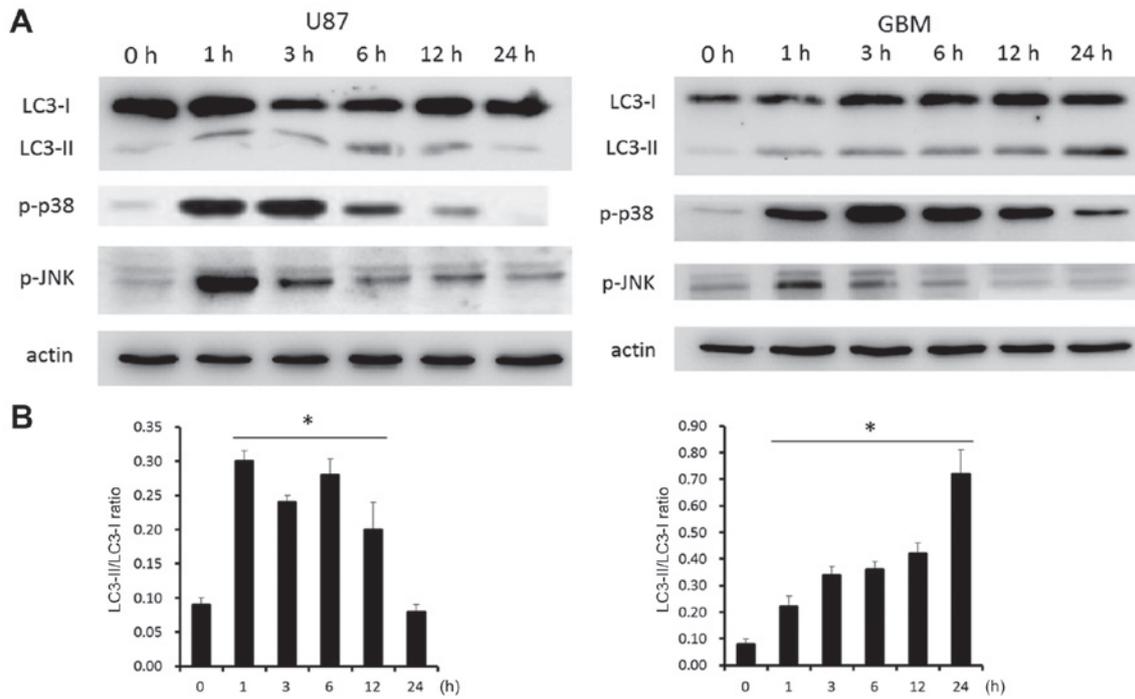
Figure 5. Expression of LC3, p-p38 and p-JNK proteins in GBM cells. (A) Results of western blot analysis of LC3, p-p38 and p-JNK proteins in U87 and GBM cells after varying durations of treatment with 300 µg/ml H-PIRE. (B) The LC3-II/LC3-I ratio changed in a time-dependent manner. Results are presented as means ± SD in three experiments. *P<0.05 in comparison with baseline. SD, standard deviation; H-PIRE, hexane fraction of *Pluchea indica* root extract.
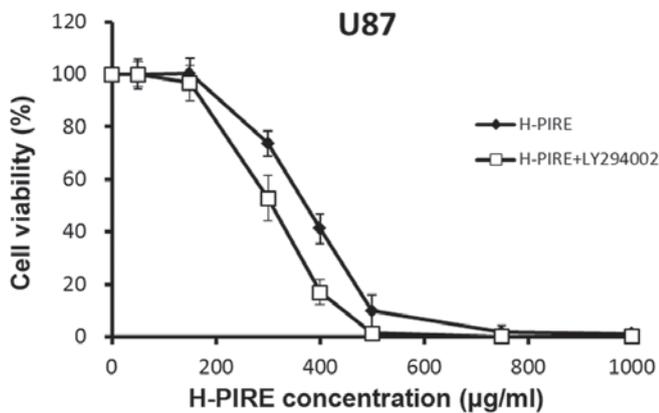

Figure 6. Combined treatment with H-PIRE and LY294002 had additive effects on human GBM cell survival. The U87 cells were treated with 0-1,000 µg/ml H-PIRE, with or without 10 µM LY294002, for 48 h. Cell viability was determined by WST-1 assay. H-PIRE, hexane fraction of *Pluchea indica* root extract.

stained with Annexin V and PI. Fig. 3 shows that, at 24 and 48 h after treatment with 300 µg/ml H-PIRE, the U87 and GBM cells showed significantly increased percentages of dead cells in quadrant 3 but no increase in early apoptosis in quadrant 2. These experimental results suggest that cell death induced by H-PIRE in glioblastoma cells is probably not associated with apoptosis.

Detection of AVOs. Formation of AVOs, which is a morphological characteristic of cells undergoing autophagy, can be detected by fluorescence microscopy of living cells stained with AO, which emits red fluorescence at low pH. The staining

results in Fig. 4 show that AVO expression was increased in U87 and GBM cells at 24 h after treatment with 300 µg/ml H-PIRE. These results clearly indicate that H-PIRE induces autophagy in human glioblastoma cells.

Expression of autophagy-related proteins. The mechanism of cell death induced by H-PIRE was investigated by examining the expression of autophagy-related proteins in U87 and GBM cells. The cells were treated with 300 µg/ml of H-PIRE for 0 to 24 h. Western blot analysis was then used to examine the expression of microtubule-associated protein light chain 3 (LC3)-I, LC3-II, phosphorylated-p38 and phosphorylated-JNK. Fig. 5A shows that, at 1 h after the H-PIRE treatment, LC3-II was significantly upregulated relative to LC3-I, which significantly increased autophagosome formation (27). In both cell lines, expression of phosphorylated-p38 protein was significantly increased at 1 h after H-PIRE treatment and peaked at 3 h after treatment. Phosphorylated-p38 protein expression remained at the peak level for >12 h in U87 cells and for 24 h in GBM cells before dropping back to the base level. Expression of phosphorylated-JNK protein was substantially increased at 1 h after H-PIRE treatment and then gradually decreased to the base level in the two cell lines. The results indicated that, in both cell lines, the stress induced by H-PIRE activated the p38 and JNK signaling pathways, which are known to play key roles in the induction of autophagy (27).

Fig. 5B shows that the LC3-II/LC3-I ratio increased in a time-dependent manner in both cell lines. The ratio peaked at 0.30 at 1 h and started to decline after 12 h in U87 cells. By contrast, the ratio increased steadily and peaked at 0.73 at 24 h in GBM cells. In addition, H-PIRE treatment resulted in different FITC-AV and PI staining pattern at 48 h (Fig. 3)

and cell morphology at 24 h (Fig. 4) in U87 and GBM cells. These data may reflect the intrinsic difference in the manner the two types of cells responded to autophagic stress. The overall results indicate that H-PIRE activates p38 and JNK and induces autophagy in human GBM cells.

Additive cytotoxic effects of combined treatment with H-PIRE with PI3K inhibitor. Rapamycin and LY294002 (a pan-PI3K inhibitor) reportedly exert synergistic effects on the induction of autophagy and reduction of cell survival in malignant glioma cell lines, including U87 (28). Specifically, rapamycin induces autophagy by deactivating mTOR (29). To determine whether inhibition of the PI3K pathway and H-PIRE treatment synergistically affect cell viability, U87 cells were treated with H-PIRE (0-1,000 μg/ml) with or without 10 μM LY274002. Fig. 6 shows the cell viability assay results, which indicated that the addition of LY294002 enhanced the reduction of cell viability induced by H-PIRE treatment. The addition of LY294002 reduced IC_{50} of H-PIRE from 353.3±18.7 to 298.6±20.3 μg/ml. These results indicate that the combined treatment of H-PIRE and LY294002 have additive effects on the reduction of cell survival in human GBM cells.

Discussion

Previous findings have demonstrated the anticancer properties of crude aqueous fraction and ethanol fraction of the *P. indica* root extract (20). The present study further investigated the suppressive effects of H-PIRE on GBM cells. The hydrophobic nature of H-PIRE enables it to penetrate the blood-brain barrier and effectively target GBM cells in the brain.

Our experimental results indicate that H-PIRE has a potent suppressive effect on human GBM cell growth, apparently by inducing cell cycle arrest in the G0/G1 phase but without inducing apoptosis (the sub-G1 fraction). However, due to the complex nature of H-PIRE, we cannot exclude that apoptosis may be induced by H-PIRE at much higher concentrations. Another possible mechanism of the suppressive effects of H-PIRE is the induction of autophagy through the increased formation of AVOs, increased expression of LC3-II, and increased phosphorylation of JNK and p38 proteins.

The activation of p38 and JNK is usually associated with antiproliferative activity (30) and induction of autophagy (31,32). Recent studies suggest that p38 and JNK have important roles in the crosstalk between apoptosis and autophagy under cellular stress conditions (33). Although autophagy may be associated with cell survival or death (34), significantly higher than normal autophagy is usually associated with cell death (35). Although inducing autophagy is considered an effective way to induce GBM cell death (36,37), our experiments showed that GBM cells are insensitive to most apoptotic stimuli.

The PI3K, the protein kinase B (Akt), and the mammalian target of rapamycin (mTOR) form a cell survival pathway important for cell growth and proliferation. This pathway is also critical for tumorigenesis (38) and is now recognized as an important target in cancer treatment (39). Our experiments showed that pre-treating U87 cells with LY294002 inhibited PI3K activity, which then increased their sensitivity to H-PIRE

treatment. Thus, since H-PIRE treatment substantially reduces GBM cell survival through an autophagy-related pathway, combined treatments with H-PIRE and other growth inhibitors may have synergistic effects in conventional cancer treatment.

In conclusion, findings of this study show that H-PIRE suppresses proliferation and reduces viability in GBM cells. As it activates the p38 and JNK pathways, H-PIRE can also be used to induce autophagy in GBM cells. However, further studies are needed to elucidate the molecular pathway of anticancer activity induced by H-PIRE.

Acknowledgements

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