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Polyphyllin I induced-apoptosis is enhanced by inhibition of autophagy in human hepatocellular carcinoma cells

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ABSTRACT

Background: Polyphyllin I (PPI), a bioactive phytochemical isolated from the rhizoma of *Paris polyphyllin*, exerts preclinical anticancer efficacy in various cancer models. However, the effects of PPI on regulatory human hepatocellular carcinoma (HCC) cell proliferation and its underlying mechanisms remain unknown.
Purpose: This study investigated the antiproliferation effect of PPI on HCC cells and its underlying mechanisms.
Methods: Cell viability was measured by MTT assay. Cell death, apoptosis and acidic vesicular organelles (AVOs) formation were determined by flow cytometry. Protein levels were analyzed by Western blot analysis.
Results: PPI induced apoptosis through the caspase-dependent pathway and activated autophagy through the PI3K/AKT/mTOR pathway. Blockade of autophagy by pharmacological inhibitors or RNA interference enhanced the cytotoxicity and antiproliferation effects of PPI. Moreover, chloroquine (CQ) enhanced the antiproliferation effect of PPI on HCC cells via the caspase-dependent apoptosis pathway by inhibiting protective autophagy. Therefore, the combination therapy of CQ and PPI exhibited synergistic effects on HCC cells compared with CQ or PPI alone.
Conclusion: The current findings strongly indicate that PPI can induce protective autophagy in HCC cells, thereby providing a novel target in potentiating the anticancer effects of PPI and other chemotherapeutic drugs in liver cancer treatment. Moreover, the combination therapy of CQ and PPI is an effective and promising candidate to be further developed as therapeutic agents in the treatment of liver cancer.

Introduction

HCC is the sixth most common malignancy worldwide and the third leading cause of cancer death. Currently, HCC is highly resistant to conventional systemic therapies, and the prognosis of advanced HCC patients remains poor (Wysocki, 2010). Given its high morbidity and mortality rates, HCC has become a global problem (Forner et al., 2012). Thus, further exploration and validation of therapeutic regimens for HCC are urgently required.

Macroautophagy (autophagy) is a lysosomal degradation pathway for the breakdown of intracellular proteins and organelles. Emerging evidence has shown that autophagy can be a novel target for cancer therapy, but whether autophagy promotes the survival or death of cancer cells is controversial (Gozuacik and Kimchi, 2004). On one hand, autophagy suppresses tumorigenesis by isolating damaged organelles or proteins and by limiting cell growth and genomic instability (Mathew et al., 2009; Yang et al., 2011). On the other hand, autophagy enables tumor cells to survive in the environmental stressors and injuries caused by chemotherapy, radiation therapy, and targeted therapies (Hu et al., 2012). Therefore, inhibition of autophagy may increase cytotoxicity when applied in combination with anticancer drugs. Understanding and modulating autophagy are important to develop new approaches for cancer therapy and prevention.

PPI, also known as Polyphyllin D, is a steroidal saponin extracted from the rhizoma of *Paris polyphyllin* (Fig. 1A). PPI inhibits the growth of tumor cells, including human breast tumor cells (MCF-7, MDA-MB-231) (Lee et al., 2005), lung cancer cells (A549) (Kong et al., 2010), HCC cells (HepG2) and their multidrug resistant cells (R-HepG2) (Cheung et al., 2005). PPI also directly triggers mitochondrial membrane permeabilization and mitochondrial damages, which induce apoptosis in various tumor cell lines (Gao et al., 2012; Ong et al., 2008). *In vivo* studies indicate that PPI effectively inhibits tumor growth in nude mice without exerting significant toxicity to the host

Abbreviations: DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; 3-MA, 3-methyladenine; Rap, rapamycin; Baf A1, bafilomycin A1; EdU, 5-ethynyl-2-0-deoxyuridine; PI, propidium iodide; AO, acridine orange; siRNA, small-interfering RNA; CI, combination index.

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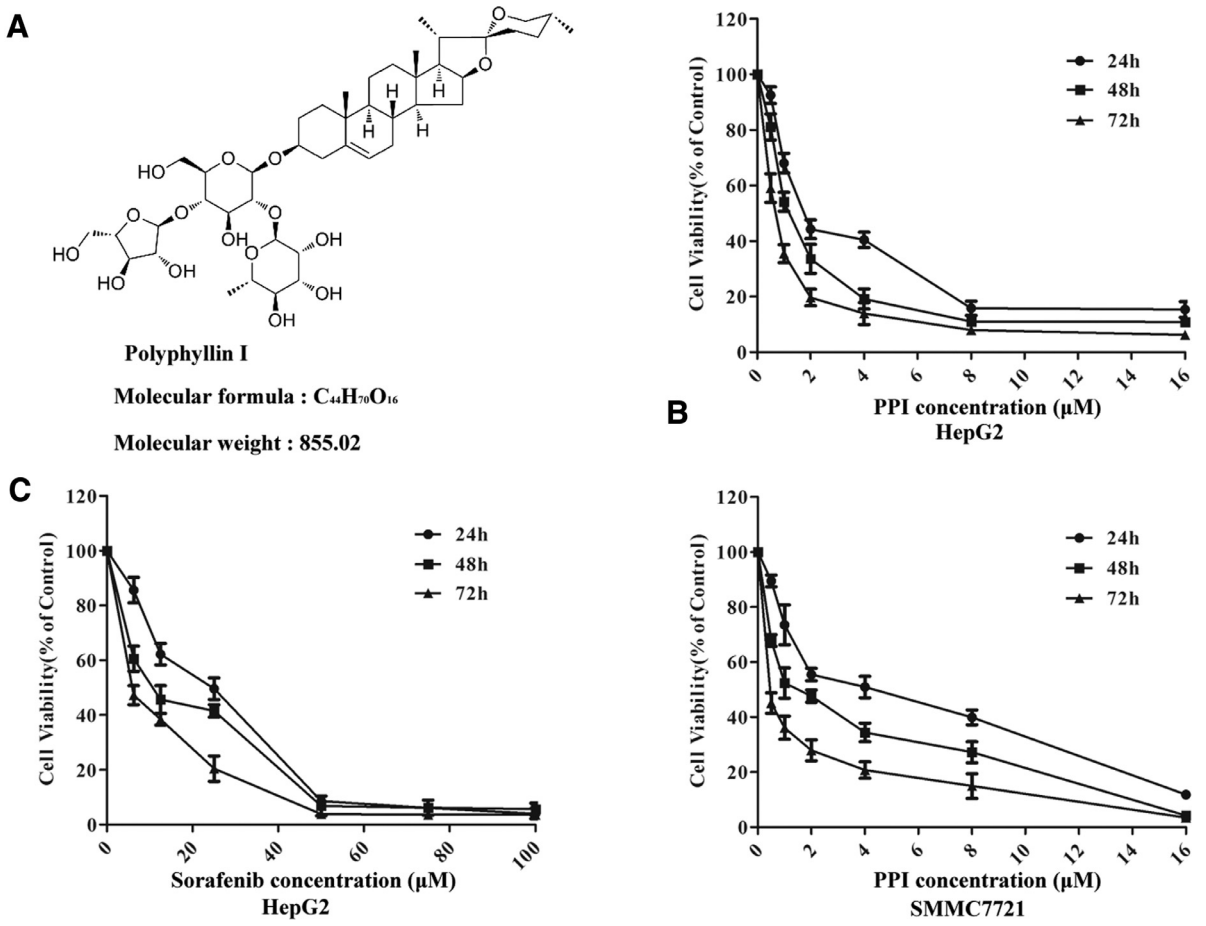

Fig. 1. PPI inhibited the cell viability of HCC cells. (A) Chemical structure and molecular weight of PPI. (B) HepG2 and SMMC7721 cells were treated with different concentrations (0–16 μM) of PPI for 24 h, 48 h, 72 h. Cell viability was determined by MTT assay. (C) Sorafenib was used as a positive control in cell viability assay in HepG2 cells.

(Man et al., 2011). However, whether PPI can induce autophagy in HCC, including its detailed molecular mechanism, remains unclear.

This study evaluated the capacity of PPI to inhibit cell proliferation and induce autophagy in HCC cells. PPI-induced apoptosis and cell death were also investigated after employing genetic and pharmacologic agents that suppressed autophagy. Finally, the efficacy of the combination therapy of PPI and CQ against HCC cells was assessed to develop a promising strategy for HCC therapy.

Materials and methods

Reagents and chemicals

PPI (purity ≥ 98%) was purchased from Goren Biotechnology Company (Nanjing, China). The compound was dissolved in DMSO (Sigma–Aldrich, St. Louis, MO) as a stock solution of 50 mM and then added to extracellular solutions to obtain the desired concentration. The final concentration of DMSO was lower than 0.1%, which did not affect the test. DMEM, RPMI 1640 medium and FBS were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). The pan-caspase inhibitor Z-VAD-FMK, 3-MA, Rap, Baf A1 and CQ were purchased from Tocris Bioscience (Tocris Cookson Limited, Bristol, UK). Sorafenib was purchased from AbMole (Shanghai, China), and EGF was purchased from Biovision (San Francisco, USA). Primary antibodies of Beclin-1, Atg-5, LC3B, procaspase-3, procaspase-9, PARP, cleaved Caspase-3, cleaved Caspase-9, cleaved PARP, Phospho-mTOR (S2448), mTOR, Phospho-AKT (S473), AKT (pan), Phospho-p70S6K (T389), p70S6K, Phospho-4EBP1 (T37/46), 4EBP1, GAPDH, and secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

HCC HepG2 and SMCC7721 cells were purchased from the Typical Culture Preservation Commission Cell Bank (Shanghai, China). HepG2 and SMCC7721 cells were cultured in DMEM and RPMI 1640 media, respectively. The media were supplemented with 10% (*v*/*v*) FBS, 50 μg/ml penicillin and 50 μg/ml streptomycin at 37 °C and 5% CO_2 in a humidified environment.

Cell viability assay and colony formation assay

Cell viability assay and colony formation assay were performed as previously described (Zhang et al., 2013).

CFDA-SE cell tracer kit

Cell proliferation was further determined using the CFDA-SE cell tracer kit (Beyotime, Haimen, China) in accordance with the manufacturer's instructions. HepG2 and SMCC7721 cells were labeled with CFDA-SE and then seeded on six-well plates. After 24 h incubation, the medium was replaced with fresh medium containing different concentrations (0–4.0 μM) of PPI. The cells were harvested after 24 h and then washed twice with PBS. The fluorescence intensity was measured by flow cytometry (BD Biosciences, San Jose, CA).

EdU incorporation assay

HepG2 and SMCC7721 cells were incubated at 1×10^5 cells per well on six-well plates by using the keyFluor488 Click-iT EdU Flow Cytometry Kit (Keygen Biotech, Nanjing, China) in accordance