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Herbal product silibinin-induced programmed cell death is enhanced by metformin in cervical cancer cells at the dose without influence on nonmalignant cells

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ABSTRACT

Silibinin is known to display high efficacy against cancer cells and for hepatic protection. Metformin, a well-known antidiabetic agent, has recently been reported to inhibit cancer. In the present study, we investigated the effect of metformin on silibinin-induced programmed cell death in cervical cancer cells (C-33A). MTT assay and Western blot assays were performed to quantify cell viability and the expression of signaling proteins, respectively. Combined treatment with metformin and silibinin decreased cell survival in synergistic manner in C-33A cells at a dose that did not affect nonmalignant cells (HUVECs). Silibinin and metformin increased PTEN and AMPK expression in C-33A cells, respectively. Combined treatment caused a greater increase in the expression of activated caspase-3 or AIF, indicating apoptosis. Combined treatment with silibinin and metformin may induce programmed cell death of human cervical cancer cells at a dose that does not affect HUVECs. This finding reveals a potential therapeutic strategy of cervical cancer.

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Introduction

Cervical carcinoma, the popular female cancer around the world, is the seventh leading cause of cancer death in women (Siegel et al., 2014). Nowadays, cervical cytology screening is useful to reduce the mortality of this cancer. Various strategies for reducing cervical cancer have been reported, including immune therapy of cytokines, polyamine synthesis inhibitors, individual micronutrient supplementation and pharmaceutical agents, and all have indicated a limited success (Follen et al., 2003; Bell and Alvarez, 2005). Moreover, Human papillomavirus (HPV) vaccine strategies have also been applied to reduce cervical cancer risk. However, the actual impact of cervical cancer initiation remains controversial and managing lesions remains necessary to be investigated.

Milk thistle (*Silybum marianum*) has widely been utilized in liver diseases as a popular dietary supplement in the United States and Europe (Kroll et al., 2007). Silibinin, a polyphenolic flavonoid, is the major active compound in milk thistle (Singh and Agarwal, 2004; Gazak et al., 2007). Milk thistle is known to be safely and tolerably protecting the liver against chemical or alcohol-related injury (Ball and Kowdley, 2005; Hackett et al., 2013). The inhibitory effect of silibinin has been demonstrated in multiple cancer cell lines, including lung (Chu et al., 2004), liver (Lah et al., 2007; Cui et al., 2009), skin (Mallikarjuna et al., 2004; Mohan et al., 2004), colon (Yang et al., 2003) and prostate cancers (Singh et al., 2002; Tyagi et al., 2002).

Metformin is an antidiabetic agent widely used to treat diabetic patients. Its action is mainly mediated by the activation of AMP-activated protein kinase (AMPK), which inhibits hepatic gluconeogenesis and enhances glucose uptake in skeletal muscle and adipose tissue (Zhou et al., 2001). In addition, metformin has been reported to reduce cell proliferation in several human tumors including breast cancer (Zhuang and Miskimins, 2011; Lee et al., 2014), pancreatic cancer (Bao et al., 2012) and gastric cancer (Kato et al., 2012). Metformin also inhibited tumor growth in xenograft mouse models of breast cancer (Anisimov et al., 2005), prostate cancer (Ben Sahra et al., 2008), ovarian cancer (Rattan et al., 2011) and melanoma (Janjetovic et al., 2011). The administration of metformin to diabetic patients was associated with lower risks of cancer incidence and mortality (Noto et al., 2012). Colorectal cancer patients with diabetes who were treated with metformin as part of their diabetic therapy appeared to exhibit a superior overall survival rate (Luo et al., 2012; Smiechowski et al., 2013).

Both silibinin and metformin showed anticancer activities. Thus, we administered metformin with silibinin in combination to investigate the anticancer efficacy in C-33A cells. In the present study, an effective dose of this combination of drugs that did not affect nonmalignant cells was found.

Materials and methods

Materials

Antibodies against activated caspase-3 and apoptosis induce factor (AIF) were purchased from Millipore (Millipore, Bedford, MA, USA). Antibodies against the phosphatase and tensin homolog (PTEN), signal transducer and phosphorylated (p-) protein kinase B (Akt), phosphorylated 5′-adenosine monophosphate (AMP)-activated protein kinase (p-AMPK) and β-actin (actin) were the products of Abcam (Cambridge, MA, USA). Metformin and silibinin were purchased form Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Human cervical cancer cells (C-33A) and human umbilical vein endothelial cells (HUVECs) were purchased from the Culture Collection and Research Center of the Food Industry Institute (Hsin-Chiu City, Taiwan). C-33A cells were cultured in α-MEM (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin sulfate (10 μg/ml) (GIBCO). HUVECs were nonmalignant epithelial cells which are widely used as normal cells in cancer studies (Kamat et al., 2011; Dil and Banerjee, 2012; Hoang et al., 2012). HUVECs were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air. After approximately 60% confluence, the medium was replaced with serum-free cell medium, to prevent the cell growth in the period of treatment, containing various concentrations of silibinin (0, 50, 100, 150 or 200 μM) (Lah et al., 2007; Cui et al., 2009) and metformin (0, 5, 10, 15 or 20 mM) (Janjetovic et al., 2011; Luo et al., 2012) and the cells were cultured for 24 h as performed in previous studies (Yu et al., 2012; Su et al., 2013a,b). The cells were harvested via treatment with 0.25% trypsin and 0.2 g/l EDTA for further studies.

Cytotoxicity assay

Cell viability and survival was determined via the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Scudiero et al., 1988). Briefly, 10^4 cells were seeded on 96-well plates in triplicate and treated with different combinations of concentrations of metformin and silibinin for 24 h. After treatment, the medium was replaced with 100 μl/well of fresh medium and 10 μl of MTT (final concentration of 0.5 mg/ml) was added to each well. The plates were incubated at 37 °C for 4 h, allowing the viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. The formazan crystals were dissolved using a solution of 0.01 M HCl/10% SDS. Finally, the absorbance of each individual well was determined at 595 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek, U.S.). The results of the assays were expressed as the means ± SEM. The data were collected from at least three independent experiments.

Analysis of synergy by combination index (CI)

The Loewe additivity model was used as a second method of analyzing the interaction between silibinin and metformin (Lee et al., 2007). The interaction between the compounds is reported as the combination index in the following equation:

$$CI = \left(\frac{d1}{D_{x,1}}\right) + \left(\frac{d2}{D_{x,2}}\right)$$

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