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Silibinin and Paclitaxel Cotreatment Significantly Suppress the Activity and Lung Metastasis of Triple Negative 4T1 Mammary Tumor Cell in Mice

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Abstract

The *in vitro* and *in vivo* bioactivities of silibinin (SB), paclitaxel (PTX) and SB and PTX in combination (SB+PTX) against murine metastatic mammary 4T1 cancer cell line were investigated. Isobologram and combination index (CI) analyses showed that SB and PTX can function synergistically in the inhibition of 4T1 cell proliferation with a CI value < 1. Both SB and PTX alone or SB+PTX treatment inhibited 4T1 cell migration and motility possibly through downregulation of the serpin protease nexin-1 (PN-1) and N-cadherin expression, inhibition of matrix metalloprotease (MMP)-9 activity, and upregulation of E-cadherin. Flow cytometry and Western blot analyses demonstrated that both drugs deregulated cell-cycle mediators and induced apoptosis in 4T1 cells. A real-time *in vivo* bioluminescence imaging system to monitor the breast cancer cell metastasis in syngeneic BALB/c mice was established using a stable $4T1^{pGL\text{-}COX\text{-}2/Luc}$ cell clone carrying a COX-2 promoter driven-luciferase reporter gene. *In vivo* study using the allograft $4T1^{pGL\text{-}COX\text{-}2/Luc}$ metastatic mouse model indicated that SB co-treated with PTX can significantly suppress lung metastasis of 4T1 cells likely through inhibiting cell proliferation and angiogenesis. Together, this study demonstrates that SB could act synergistically with PTX in 4T1 cells, providing a therapeutic option for highly metastatic triple negative breast cancer.

Keywords: Silibinin, Paclitaxel, Triple negative breast cancer, Lung metastasis, Synergistic effect

Introduction

Breast cancer is the most common cancer in women, and the incidence, morbidity and mortality of the disease remain a major concern globally (Maxmen 2012). Immunohistochemical markers such as the expression of hormone receptors, *e.g.*, estrogen receptor (ER) and progesterone receptors (PR) and the overexpression of HER2, provide therapeutic predictive value and clinical guidance in selection of breast cancer treatments. While effective targeted therapeutic modalities exist for women with hormone receptor positive and HER2-positive disease, chemotherapy is the only systemic therapy available for women with triple negative breast cancer (TNBC) (Lin *et al*., 2012).

Chemotherapy is used extensively in treating cancers, but its effectiveness, drug resistance and adverse side

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effects in patients are common concerns. For instance, the side effects of a commonly used anticancer drug paclitaxel (PTX) include pain, hair loss, diarrhea, nausea, vomiting and lowered white blood and red blood cell counts with increased risk of infection and anemia in patients (Salvinelli *et al*., 2003; Lee *et al*., 2012). This has led to the search for adjuvant therapy combining cytotoxic chemotherapeutic drugs with less toxic agent(s) with the potential to boost antitumor activity and reduce side effects that are considered to be an alternative approach for cancer treatment (Lin *et al*., 2012; Sarkar & Li 2006).

Silibinin (SB), the most active flavonolignan constituent present in the milk thistle (*Silybum marianum* L.) extract silymarin has demonstrated pleiotropic effects against a variety of cancers (Deep & Agarwal 2010). Silymarin was reported previously for its clinical properties in the management of hepatic disorders (Wellington & Jarvis 2001). Preclinical studies indicate that SB or silymarin are also effective against epithelial-type cancers, such as prostate, lung, ovarian, colon, and skin cancers (Colombo *et al*., 2011; Kaur & Agarwal 2007; Zhou *et al*., 2008). SB or silymarin has thus been considered a novel candidate for cancer chemotherapy and chemoprevention as it is well tolerated and is considered safe (Hoh *et al*., 2006), and can enhance chemosensitivity to select anticancer drugs, *e.g.*, 5-fluorouracil, doxorubicin or PTX (Colombo *et al*., 2011; Zhou *et al*., 2008).

In the present study, we investigated the therapeutic effect and underlying mechanisms of SB, PTX and SB PTX cotreatment on triple negative breast cancer cells 4T1 *in vitro* and in an experimental lung metastatic mouse model. Our results demonstrate that SB and PTX can act synergistically by suppressing 4T1 cell activity through inhibiting migration, modulating cell-cycle machinery and inducing apoptosis. Both drugs also suppressed lung metastasis of 4T1 cells in syngeneic BALB/c mice.

Materials and Methods

Cell lines and culture conditions

The 4T1 murine metastatic breast cancer cell line was obtained from the American Type Culture Collection (ATCC, CRL-2539; Manassas, VA, USA). The TS/A murine adenocarcinoma cell line was a gift from Dr. Ning-Sun Yang of the Agriculture Biotechnology Research Center, Academia Sinica, Taiwan. The human normal breast epithelial cell line H184B5F5/M10 (BCRC 60197) was purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). All cell lines were cultured in indicated media supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified 5% CO_2 incubator at 37°C.

Animals

All animal care and experimental procedures adhered to the guidelines of Academia Sinica Institutional Animal Care and Utilization Committee. Female BALB/cByJNar1 mice (six-week-old) obtained from National Laboratory Animal Center, Taipei, Taiwan) were given a standard laboratory diet and distilled water ad libitum and kept on a 12 h light/dark cycle at 22 ± 2°C in the Animal Facility of Agricultural Biotechnology Research Center, Academia Sinica.

Cell proliferation and cell cycle analyses

In cell proliferation assays, 4T1 cells were cultured in 96-well plates at 4×10^3 cells/well and allowed to adhere overnight. The cells were then treated for 24 h with 0.2% vehicle of dimethyl sulphoxide (DMSO) or indicated concentrations of silibinin (SB) and paclitaxel (PTX) (St. Louis, Mo, USA). Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based colorimetric assay.

Analysis of cell cycle was carried out as described previously (Shyur *et al*., 2004). 4T1 cells (2×10^5 cells/mL) were synchronized by incubation in medium containing 1% FBS for 12 h. The low-serum (1% FBS) medium was then replaced by medium containing 10% FBS, and the 4T1 cells were treated with vehicle, SB, PTX, or SB+PTX for 6, 12, 24, and 48 h, respectively. Both adherent and floating cells were collected, washed with phosphate-buffered saline (PBS) and fixed with 1 mL of ice-cold 70% ethanol overnight at 4°C. Cells were stained with 0.2 mg/mL propidium iodide in darkness for 30 min at room temperature and analyzed by flow cytometry (FACS Calibular, BD Biosciences, Bedford, MA, USA).

Western blotting

Western blot analysis was performed following procedures described previously (Chiang *et al*., 2005). Protein content was measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were resolved by 5–20% gradient SDS-PAGE and then electrotransferred to polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA, USA). The blot was incubated in blocking buffer (3% skim