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Original Article

In vitro and *in vivo* anticancer efficacy of silibinin against human pancreatic cancer BxPC-3 and PANC-1 cells

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ABSTRACT

Silibinin suppresses the growth of many cancers; however, its efficacy against pancreatic cancer has not been evaluated in established preclinical models. Here, we investigated *in vitro* and *in vivo* effects of silibinin against lower and advanced stages of human pancreatic carcinoma cells. Silibinin (25–100 μM) treatment for 24–72 h caused a dose- and time-dependent cell growth inhibition of 27–77% ($P < 0.05$–0.001) in BxPC-3 cells, and 22–45% ($P < 0.01$–0.001) in PANC-1 cells. Silibinin showed a strong dose-dependent G1 arrest in BxPC-3 cells (upto 72% versus 45% in control; $P < 0.001$), but a moderate response in advanced PANC-1 cells. Cell death observed in cell growth assay, was accompanied by up to 3-fold increase ($P < 0.001$) in apoptosis in BxPC-3 cells, and showed only slight effect on PANC-1 cells. Dietary feeding of silibinin (0.5%, w/w in AIN-93M diet for 7 weeks) inhibited BxPC-3 and PANC-1 tumor xenografts growth in nude mice without any apparent change in body weight gain and diet consumption. Tumor volume and weight were decreased by 47% and 34% ($P \leqslant 0.001$) in BxPC-3 xenograft, respectively. PANC-1 xenograft showed slower growth kinetics and silibinin decreased tumor volume by 34% ($P < 0.001$) by 7 weeks. Another 4 weeks of silibinin treatment to PANC-1 xenograft showed 28% and 33% decrease in tumor volume and weight, respectively. Silibinin-fed group of BxPC-3 tumors showed decreased cell proliferation and angiogenesis and an increased apoptosis, however, considerable inhibitory effect was observed only for angiogenesis in PANC-1 tumors. Overall, these findings show both *in vitro* as well as *in vivo* anticancer efficacy of silibinin against pancreatic cancer that could involve inhibition of cell proliferation, cell cycle arrest, apoptosis induction and/or decrease in tumor angiogenesis.

1. Introduction

Pancreatic cancer is one of the most aggressive and lethal cancer, with 1- and 5-year relative survival rates for all stages combined, of 25% and 6%, respectively [1]. In spite of its low incidence, pancreatic cancer accounts for the fourth leading cause of cancer related deaths worldwide [2]. The reason mainly attributed to this is detection at advanced stages of the disease and the fact that, it is one of the most therapeutically resistant cancers [3]. The therapeutic resistance of pancreatic cancer is likely to be due to many factors, which includes the high frequency of *KRAS*-activating mutations (*KRAS*), *EGFR* amplification, IGFR, VEGF and Akt signaling activation [4]. Gemcitabine has been the standard of care in the first line treatment of metastatic pancreatic cancer for more than a decade, after it was shown to moderately improve survival compared to 5-fluorouracil [5]. But in many patients there is development of resistance to gemcitabine therapy. Other targeted approaches used have shown limited benefits and given the fact that multiple growth pathways are activated in pancreatic adenocarcinomas and these tend to work in overlapping fashion [6]; there is a desperate need for better strategies for the management of the disease. Anti-neoplastic phytochemicals which in general have a multi-targeted nature and inhibit one or more neoplastic events could be used to complement current therapies in view of the fact that they reduce or delay the risk of cancer progression.

Silibinin, a major bioactive component of the plant *Silybum marianum* is well studied for its efficacy in prostate cancer and is currently in phase II clinical trial for prostate cancer patients [7]. Additionally, it has shown strong efficacy in cell culture as well as pre-clinical carcinogen-induced or tumor xenograft models of skin,

Abbreviations: EGFR, epidermal growth factor receptor; VEGF, vascular endothelial cell growth factor; CDK, cyclin-dependent kinase; CDKI, cyclin-dependant kinase inhibitor.

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colon, bladder and lung cancer [8–11]. A pilot study has also been done in human colon cancer patients with silibinin showing its clinical potential in controlling colon cancer [12]. Studies conducted on various models *in vitro* and *in vivo* show that silibinin works by inhibition of epidermal growth factor receptor (EGFR), insulin-like growth factor receptor-1 (IGFR-1), vascular endothelial growth factor (VEGF) and nuclear factor-kappa B (NF-κB) pathways [13–15]. Studies from our group have also shown that silibinin could also help in chemosensitizing the cancer cells to chemotherapeutic agent, doxorubicin [16]. Another preliminary study demonstrated that silibinin could inhibit pancreatic cancer cell growth *in vitro* [17]. But, the *in vivo* efficacy and the associated molecular alterations due to silibinin against pancreatic cancer have not been evaluated yet. Therefore, in the present study we evaluated the efficacy of silibinin using two pancreatic cancer cell lines representing an early (BxPC-3) and advanced (PANC-1) stages of the disease both *in vitro* in culture and *in vivo* in athymic nude mice. Additionally, we investigated the potential *in vivo* biomarkers of silibinin efficacy and associated molecular alterations in pancreatic tumor xenografts and found that silibinin showed prominent anti-proliferative, anti-angiogenic and pro-apoptotic activity in early stage of pancreatic tumor cells, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell lines and reagents

BxPC-3 and PANC-1 cells were from ATCC (Manassas, VA, USA). Cells were cultured in DMEM with 10% fetal bovine serum and 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. Silibinin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cells were treated with different doses of silibinin (25, 50 and 100 μM final concentrations in medium) dissolved in DMSO for the desired time periods in cell culture. An equal amount (0.1% v/v) of DMSO (vehicle) was present in all the treatment groups including control.

2.2. Cell growth and death assays

Cells were plated at 5000 cells/cm^2, and after 24 h, fed with fresh medium and treated with different doses of silibinin (25, 50 and 100 μM) in complete medium. After 24, 48 and 72 h of these treatments, total cells were collected by brief trypsinization, and washed with PBS. Total cell number was determined by counting each sample in duplicate using a hemocytometer under an inverted phase contrast microscope (Olympus) using trypan blue dye. Dead cells could not exclude the dye and showed blue staining of the cell. Each treatment for each time point was done in triplicates.

2.3. Cell cycle analysis

BxPC-3 and PANC-1 cells were grown at similar confluency as in cell growth assay, and treated with desired doses of silibinin (25, 50 and 100 μM) in complete medium for 24 and 48 h. At the end of each treatment time, cells were collected after a brief incubation with trypsin–EDTA followed by processing for cell cycle analysis as reported earlier [18]. Briefly, 0.5×10^5 cells were suspended in 0.5 ml of saponin/propidium iodide (PI) solution (0.3% saponin (w/v), 25 μg/ml PI (w/v), 0.1 mM EDTA and 10 μg/ml RNase (w/v) in PBS), and incubated over night at 4 °C in dark. Cell cycle distribution was then analyzed by flow cytometry using FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.4. Apoptosis assay

To quantify silibinin-induced apoptotic death of human pancreatic carcinoma BxPC-3 and PANC-1 cells, annexin V/PI staining was performed followed by flow cytometry, as described earlier [19]. Briefly, after treatment, both floating and attached cells were pooled and subjected to annexin V/PI staining using the Vybrant Apoptosis Assay Kit2 (Molecular Probes, Inc., Eugene, OR, USA) and following the step-by-step protocol provided by the manufacturer.

2.5. Animals and diet

Athymic (*nu*/*nu*) male nude mice were obtained from the National Cancer Institute (Bethesda, MD) and housed under standard laboratory conditions (pathogen-free conditions with a 12 h light/12 h dark schedule). For all of the animal studies, γ-irradiated sterile AIN-93M (Dyets Inc., Bethlehem, PA) purified rodent pellets containing no silibinin (control diet) or 0.5% (w/w) silibinin (test diet) were used. Animal care and experiments were conducted in accordance with an approved protocol by the Institutional Animal Ethics Committee.

2.6. Tumor xenograft study

To establish BxPC-3 and PANC-1 tumors in mice, these cells were grown in culture, then detached by trypsinization, washed, and resuspended in serum-free DMEM. Six-week-old athymic nu/nu male mice were s.c. injected with 3×10^6 cells of each type were mixed with matrigel (1:1) in the right flank of each mouse to initiate tumor growth. Mice were randomly divided into two groups for each cell type, each having 10 mice. After 24 h, mice were transferred from regular diet to AIN-93M purified diet (group I, control diet; group II, 0.5% w/w silibinin diet) for 7 weeks for BxPC-3 groups and 11 weeks for PANC-1 groups (because of its relatively slower growth rate). Food consumption and animal body weight were monitored twice weekly throughout the study. Once the tumor xenografts started growing, their sizes were measured twice weekly in two dimensions. The tumor volume was calculated by the formula: $0.5236\, L_1(L_2)^2$, where L_1 is long diameter, and L_2 is short diameter [20]. At the end of experiment, tumors were excised and weighed to record wet tumor weight and one part was fixed in buffered formalin and processed for immunohistological analysis.

2.7. Immunohistochemical staining for PCNA and CD31

The paraffin-embedded tumor sections (5-μm thick) were processed as described recently [21] and incubated with specific primary antibodies, including mouse monoclonal anti-proliferating cell nuclear antigen (PCNA; Dako) and goat polyclonal anti-CD31antibody (Santa Cruz Biotechnology, Inc.) for 1 h at 37 °C followed by overnight incubation at 4 °C in humidity chamber. Negative controls were incubated only with universal negative control antibodies under identical conditions. The sections were then incubated with appropriate biotinylated secondary antibody (1:200–400 dilutions) for 60 min at room temperature. Thereafter, sections were incubated with conjugated horseradish peroxidase streptavidin (Dako) for 45 min, followed with 3,3′-diaminobenzidine (Sigma Chemical Co.) working solution, and counterstained with hematoxylin.

The proliferation index (per 400× microscopic field) was determined as number of PCNA-positive (brown) cells × 100/total number of cells. Tumor microvessel density was quantified by counting the CD31-positive cells and the total number of cells at 10 randomly selected fields at 400× magnification [21]. Zeiss Axioscop 2 microscope (Carl Zeiss, Inc.) was used for microscopic immunohistochemical analyses.

2.8. TUNEL staining of tumor section for apoptotic cells

The 5-μm-thick sections of tumor samples were analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining using Tumor TACS *In situ* Apoptosis kit (R&D Systems, Inc., Minneapolis, MN, USA) as published [21]. Apoptotic cells were counted as DAB-positive cells (brown stained) at five arbitrarily selected microscopic fields at 400× magnification, together with total number of cells. Apoptotic index was calculated as number of apoptotic cells × 100/total number of cells [19].

2.9. Statistical analysis

All statistical analyses for cell culture and *in vivo* data were carried out with Sigma Stat software version 2.03 (Jandel Scientific, San Jose, CA, USA). Student's *t*-test was used for comparing the control group with treatment groups for statistical significance. Paired student's *t*-test was used for tumor volumes. $P < 0.05$ was considered significant.

3. Results

3.1. Silibinin inhibits growth and induces cell death of human pancreatic cancer cells

Silibinin caused a strong dose- and time-dependent inhibition of cell growth of BxPC-3 cells, which were 27–51% ($P < 0.05$–0.001), 32–62% ($P < 0.001$) and 30–77% ($P < 0.001$) after 24, 48 and 72 h of treatment, respectively (Fig. 1A). In similar silibinin treatment of PANC-1 cells, a moderate dose-dependent decrease in cell number was observed as compared to BxPC-3 cells. Silibinin caused 24–45% ($P < 0.01$–0.001), 23–40% ($P < 0.001$) and 22–43% ($P < 0.01$–0.001) cell growth inhibition in PANC-1 cells after 24, 48 and 72 h of treatment, respectively (Fig. 1B). We observed that consistent with the pattern of growth inhibition, silibinin showed