Comparative Evaluation of Silibinin Effects on Cell Cycling and Apoptosis in Human Breast Cancer MCF-7 and T47D Cell Lines

Zohreh Jahanafrooz1, Nasrin Motameh1*, Behnaz Bakhshandeh2

Abstract

Silibinin is a natural polyphenol with high antioxidant and anticancer properties. In this study, its influence on two of the most commonly employed human breast cancer cell lines, MCF-7 and T47D, and one non-malignant MCF-10A cell line, were investigated and compared. Cell viability, the cell cycle distribution and apoptosis induction were analyzed by MTT and flow cytometry, respectively. The effect of silibinin on PTEN, Bcl-2, P21, and P27 mRNAs expression was also investigated by real-time RT-PCR. It was found that silibinin caused G1 cell cycle arrest in MCF-7 and MCF-10A cells but had no effect on the T47D cell cycle. Silibinin induced cytotoxic and apoptotic effects in T47D cells more than the MCF-7 cells and had no cytotoxic effect in MCF-10A cells under the same conditions. Silibinin upregulated PTEN in MCF-7 and caused slightly increased P21 mRNA expression in T47D cells and slightly increased PTEN and P21 expression in MCF-10A cells. Bcl-2 expression decreased in all of the examined cells under silibinin treatment. P27 mRNA expression upregulated in T47D and MCF-10A cells under silibinin treatment. PTEN mRNA in T47D and P21 and P27 mRNAs in MCF-7 were not affected by silibinin. These results suggest that silibinin has mostly different inhibitory effects in breast cancer cells and might be an effective anticancer agent for some cells linked to influence on cell cycle progression.

Keywords: Breast cancer cells - silibinin - bcl-2 - cell cycling - PTEN - P21 - P27

Introduction

Breast cancer is the most common malignancy in women worldwide, however there is no efficient method for successful treatment of this cancer like many others. Targeted drugs take care of their intended targets very effectively but, with the continued administration, tumor cells reduce their dependency on the targeted factor/pathways and switch to an alternative survival pathway. It has, therefore, been advocated that multitargeted cancer therapy should result in prolonged disease-free survival (Ahmad A, 2013).

Use of some natural anticancer agents can dissolve this problem because they target multiple signaling pathways. In addition natural anticancer agents have lower/no cytotoxicity for normal cell (Asad et al., 1998). Silibinin is a polyphenolic flavonolignan isolated from milk thistle (Silybum marianum), and is one of the chemopreventive and anticancer agents that has attracted attention for the prevention of or treatment for cancer (Sharma et al., 2003; Deep and Agarwal, 2007). There are many published reports about different anticancer activities of silibinin (Zi and Agarwal, 1999; Sharma et al., 2003; Deep and Agarwal, 2007; Roy et al., 2007; Wang et al., 2010; Duan et al., 2011; Tiwari et al., 2011).

Silibinin induces p21/Cip1 and p27/Kip1, two main cell cycle progression inhibitors, and G1 arrest in prostate cancer cells irrespective of p53 status (Roy et al., 2007). Silibinin inactivates p53 and induces autophagic death in human fibrosarcoma HT1080 cells via reactive oxygen species-p38 and c-Jun N-terminal kinase pathways (Duan et al., 2011). Studies have demonstrated that silibinin induced apoptosis in breast cancer cell lines and caused overexpression of Bax and underexpression of Bcl-2 (; Wang et al., 2010; Tiwari et al., 2011). So far there is no report about cytotoxic effects of silibinin on normal cells, Zi et al. (Zi and Agarwal, 1999) in 1999 showed that silibinin did not show a considerable inhibition of NIH 3T3 and normal human prostate cell growth or Tiwari et al. (Tiwari et al., 2011) in 2011 showed that IC50 concentration of silibinin on MCF-10A was found to be significantly higher than MCF7 and T47D.

The objective of the current study was to investigate and compare the silibinin effect on cell viability, cell cycle and apoptosis in breast cancer MCF-7 and T47D cell lines, both are ER positive and from luminal A subtype but T47D cells express mutant p53 protein, and less well differentiated (Adams et al., 2006; Vojtesek and Lane,
Materials and Methods

Cell culture

MCF7 (human breast adenocarcinoma cell line) and T47D (human ductal breast epithelial tumor cell line) human breast cancer cell lines were obtained from National Cell Bank of Iran (Tehran, Iran). Two cell lines were cultured in RPMI-1640 (GIBCO, UK) medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/streptomycin (pen/strep) antibiotics (Gibco, Scotland) at 37°C in 5% CO₂ under 90-95% humidity. MCF-10A cells were cultured in DMEM/F12 (Invitrogen, USA) supplemented with 15% FBS, 10 μg/ml insulin (Sigma-Aldrich), 100 μg/ml hydrocortisone (Sigma-Aldrich), and 1% penicillin/streptomycin antibiotics (Gibco, Scotland) at 37°C in 5% CO₂ under 90-95% humidity. Silibinin (Sigma, USA) was dissolved in dimethylsulfoxide (DMSO, Sigma, USA) and the amount of DMSO never exceeded 0.1% (v/v) in the whole experiment.

Cell viability investigation

To evaluate the cell proliferation and cell viability under different concentrations of silibinin, MTT (Sigma, USA) assay was applied. Cells of mentioned three lines were cultured in 96-well plates with 8000 cell/well density. Next day, different concentrations of silibinin (0, 100, 150 and 200 μM) were supplemented to the media for 48h. Four hours before the end of incubation time, 10 μl MTT solution (5mg/ml) was added to each well, followed by incubation at 37°C for 4h. The supernatant was removed and insoluble formazan crystals (formed in living cell by mitochondrial dehydrogenases) were solubilized by adding 100μl DMSO to each well. Optical density (OD) of each well was measured at 570 nm with Elisa reader (Awarnesse, USA). Individual samples were washed with PBS and fixed with 70% ethanol at 4°C. After 2h, fixed cells were pelleted and stained with 10μl PI (2 μg/ml) in the presence of 5μl Ribonuclease (Sigma-Aldrich, 100μg/ml) for 30 min at 37°C. Data were analyzed using win MDI2.9 software (Beckman Coulter, CA, USA).

RNA extraction and Quantitative RT-PCR analysis

20*10⁴ cells/well were treated with 0 and 150 μM silibinin doses at 48h. Total RNA was isolated from the treated cells, using RNX Plus™ kit (CinnaColon, Tehran, Iran), according to manufacturer instruction. The 260/280 and 260/230 absorbance ratios and RNA concentration were determined by Nanodrop (Thermo scientific, Wilmington, USA). For cDNA synthesis, 2μg of extracted RNA was reverse transcribed into cDNA according to the manufacturer’s protocol, using DNase I (TAKARA, Japan), EDTA (Cat. #PR991627), dNTP (Cat. #DN7604C, CinnaClon), and random hexamer primer (Cat. #S0142), Reverse Transcriptase 10000 u (Cat. #EP0441), RiboLock RNase Inhibitor 2500 u (Cat. #E00381) (all from Fermentas), DEPC Water (Cat. #MR8244C, CinnaClon). To assess the alterations of PTEN, Bcl-2, P21, and P27 mRNAs in the MCF-7, T47D, and MCF-10A breast cell lines.

Results

Effect of silibinin on cell viability

We first determined the cytotoxic effect of silibinin by treating the MCF-7, T47D, and MCF-10 cells with different concentrations of silibinin (0,100, 150 and 200 μM) for 48h. MTT assay showed no effect of silibinin on cell cycle distribution, 20 *10⁴ cells/well were seeded in six-well plate and incubated for 24h. Silibinin in 0 and 150 μM concentrations was supplemented to the wells after 24h serum-starvation. After 48h incubation, the cells were washed with PBS and fixed with 70% ethanol at 4°C. After 2h, fixed cells were pelleted and stained with 10μl PI (2 μg/ml) in the presence of 5μl Ribonuclease (Sigma-Aldrich, 100μg/ml) for 30 min at 37°C. Data were analyzed using win MDI2.9 software (Beckman Coulter, CA, USA).

Apoptotic analysis

To investigate the apoptotic effect of silibinin in 0 and 150 μM concentrations at 48h in cancerous cell lines (MCF7, T47D), 10⁴ cells of each treatment was washed twice with PBS and analyzed using Annexin V-FITC Apoptosis Detection Kit (EXBIO, Czech Republic). The cells were double stained with Annexin V-FITC and PI, following manufacturers’ protocol and analyzed by flow cytometry.

Statistical analysis

All experiments were performed at least three times. Student’s two-tailed t-test was used to compare data between two groups. A p-value <0.05 was considered statistically significant. The results were expressed as means ± SD.
MCF-10A proliferation. Analysis of T47D proliferation under silibinin induction represented susceptibility of this cell line and significant lesser in T47D proliferation. The viability of MCF-7 was decreased gradually by silibinin treatment (Figure 1). According to the results, the 150 μM silibinin supplementation because of moderate effect on both cancer cell lines was chosen as an optimum concentration for the subsequent experiments.

Cell cycle alteration by silibinin induction

For cell cycle evaluation, all treatments were stained with Ribonuclease /PI followed by flow cytometry analysis. MCF-7 population was found 2.13±0.021% in subG1 phase, 66.95±1.65% in G1 phase, 16.33±0.134% in S phase, and 14.00±1.52% in G2/M phase in control group. After treatment by 150 μM silibinin for 48 h, the percentage of cells in subG1 and G1 phases were increased to 5.65±0.29% (p=0.04) and 83.14±2.71% (p=0.02), respectively with decrease in S phase to 4.13±1.55% (p=0.01), and no significant change in G2/M phase (Figure 2A).

The population of control group of T47D cells was divided to 1.41±0.64% in subG1 phase, 67.43±1.58% in G1 phase, 17.60±0.79% in S phase, and 13.60±1.26% in G2/M phase. Under 150 μM silibinin treatment for 48 h, the percentage of cells in subG1 and partly G2/M phases were increased to 4.81±2.93% (p=0.25) and 14.1±1.51% (p=0.75), respectively with decreased percentage in S and partly G1 phase to 14.02±0.98% (p=0.06) and 66.01±2.84% (p=0.60), respectively (Figure 2B).

Investigation of control group of MCF-10A cells showed allocation of 0±0.1% to subG1 phase, 69.1±0.1% to G1 phase, 20.1±0.08% to S phase, and 10.4±0.5% to G2/M phase. Under treatment by 150μM silibinin for 48 h, the percentage of cells in subG1 phases was remained 0±0.1%, the percentage of cells in G1 phases were increased to 84.3±0.4% (p=0.01), and the percentage of cells in S and G2/M phases were decreased to 8.1±0.16% (p=0.006) and 71±0.08 (p=0.07), respectively (Figure 2C).

The impact of silibinin in apoptotic induction

As illustrated in Figure 3, silibinin induced significant apoptosis cell death in MCF-7 and T47D cells with no effect on MCF-10A. Flow cytometry experiments indicated 25.2 ± 0.4% and 41.6 ± 2.2% apoptotic cell death by both Annexin V+ and Annexin V+PI+ evaluations in MCF-7 and T47D cells under 150μM silibinin supplementation, respectively. Silibinin also induced necrotic cell death in the both cell lines, 11.9±0.16% in MCF-7 and 16.5±0.27 in T47D cells (investigated by PI assay).

Implication of PTEN, Bcl-2, P21, and P27 transcriptions under silibinin induction

We measured PTEN (as a tumor suppressor protein), Bcl-2 (as an anti-apoptotic factor) transcriptions, and P21 and P27 (cyclin-dependent kinase inhibitors) in the MCF-7, T47D, and MCF-10A cells by the real-time RT-PCR analysis to determine their possible role

Table 1. The Primers and the Size of PCR Products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Access Number</th>
<th>Primers’ Sequences</th>
<th>Amplicon Size</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>NM_002046.5</td>
<td>F: 5’TGTGAACCATGAGAAGTATGAC3' R: 5’ATGAGTCTCCTTCCACGATACC3'</td>
<td>123bp</td>
</tr>
<tr>
<td></td>
<td>NM_001256799.2</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>NM_001289745.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NM_001289746.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN</td>
<td>NM_000314.4</td>
<td>F: 5’TGGCAGAGTTGGCAACAATATCC3’a R: 5’TTCCTCCAGCTTTACAGTGAATTG3'</td>
<td>138bp</td>
</tr>
<tr>
<td>BCL2</td>
<td>NM_000633.2, NM_000657.2</td>
<td>F: 5’AACGTGCTCCTCATGAAGTAAAG3’ R: 5’TIAATTGCTGTCTGGTGCATTC3’</td>
<td>142bp</td>
</tr>
<tr>
<td>P21</td>
<td>NM_00120777.1</td>
<td>F: 5’TGTCTCTCCTTTCCTCCTCCTC3’ R: 5’TCCAGAGGGTAACCACCCA3’</td>
<td>95bp</td>
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<td>NM_001291549.1</td>
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<td></td>
<td>NM_000389.4</td>
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<td>P27</td>
<td>NM_004064.4</td>
<td>5’AACCGACGATTCCTTCACCTC3’ 5’GTTTACGTTTCAGCTTCTG3’</td>
<td>132bp</td>
</tr>
</tbody>
</table>

*a The primer is located on exon junction
in the induction of cell cycle arrest and apoptosis after silibinin administration. The specificity of all primers was determined by examining the melting curves. Statistical analysis of the primers efficiencies was done by LinReg PCR software (Amsterdam, The Netherlands, version 2012.0). By 2-ΔΔCt method, real-time PCR results showed a significant increase in the PTEN gene expression in treated MCF-7 cells, compared to the control cells (3.24±0.170 times, p=0.034). Silibinin also augmented PTEN transcription in MCF-10A cells (1.32±0.12 times, p=0.17). Interestingly, silibinin showed no effect on PTEN transcription in treated T47D cells in comparison to control group (0.92 ±0.190 times, p=0.641). Silibinin decreased Bcl-2 expression in all of examined cells, 0.43±0.04 times with p=0.04 in MCF-7, 0.63±0.11 times with p=0.12 in T47D, and 0.68±0.09 times with p=0.12 in MCF-10A cells. Silibinin slightly increased P21 mRNA expression in T47D (1.5±0.14 times, p=0.12) and MCF-10A (1.2±0.12 times, p=0.24) cells. P27 mRNA expression upregulated by silibinin in T47D (3.44±0.27 times, p=0.047) and MCF-10A (3.35±0.5 times, p=0.09), however P21 and P27 mRNA in MCF-7 cells were not affected by silibinin (Figure 4).

Discussion

Silibinin as a natural flavonoid, induces various cell functions including growth inhibition, cell cycle arrest, anti-proliferative effect and apoptotic induction which could be applied as anticancer agent (Sharma et al., 2003; Agarwal et al., 2003; Roy et al., 2007; Li et al., 2008; Mokhtari et al., 2008). Consistent with our viability assessment, Tiwari et al. (2011) reported a dose-dependent reduction in the proliferation of MCF7 and T47D cells after silibinin administration and interestingly viability of non-cancerous MCF-10A cells were not affected by silibinin supplementation.

Cell cycle progression is an important biological event having controlled regulation in normal cells, which almost universally becomes aberrant or deregulated in transformed and neoplastic cells. In this regard, the potential prognostic role of cell cycle regulators and natural agent’s effects in cancer therapy has been under focus. Some investigations have shown the inhibitory effects of silibinin on the cell cycle progression in various cancers (Zi et al., 1998; Roy et al., 2007). So in this study, the influence of silibinin on the cell cycle of MCF7, T47D and MCF-10A was evaluated. Data obtained from the cell cycle distribution in the cells treated with silibinin revealed G1 arrest in MCF-7 and MCF-10A cells, significantly decreased cell percentage in S phase and decreased percentage of cells in G2/M phases in MCF-7 and MCF-10A cells, while increased percentage of cells in subG1, slightly decreased percentage of cells in S phase with no G1 arrest in T47D cells.

Studies have demonstrated that silibinin induces apoptosis in breast cancer cell lines (Wang et al., 2010). Our data reinforced this point by showing that the ability of silibinin to inhibit both MCF-7 and T47D cells growth by at least partially inducing apoptosis and necrosis. Apoptotic and necrotic responses of T47D cells were more sensitive to silibinin than MCF7 cells. One of the suggested reasons for this phenomenon is that the deficiency of p53 renders cancer cells more sensitive to the induction of apoptosis by chemotherapeutic agent due to the direct involvement of p53 in DNA repair and survival signaling, and this feature favors silibinin for the therapy of tumors with dysregulated p53 function (Kaina, 2003).
Previous studies have shown that enforced expression of PTEN induces a predominant G1 arrest, which is consistent with the capacity of PTEN to evoke increased expression of p27/Kip1 cyclin dependent kinase inhibitor via its lipid phosphatase activity and its capability to decrease cyclin D via its protein phosphatase function in breast cancer (Lu et al., 1999; Weng et al., 2001), and PTEN indirectly leads to activation of p21/Cip1 and p27/Kip1 along with dephosphorylation of pBad in PI3 kinase/Akt pathway (Li et al., 2012), so in this research, we evaluated silibinin effect on PTEN mRNA expression in MCF-7, T47D, and MCF-10A cell lines. According to our study, silibinin increased PTEN mRNA level in MCF-7 and MCF-10A cells but had no effect on T47D cells, and this increase was accompanied by the G0 stage or G1 arrest in MCF-7 and MCF-10A cells, treated with silibinin. Silibinin caused increase in P21 and P27 mRNA in T47D and MCF-10A cells with no changes in MCF-7. P21 and P27 upregulation by silibinin was in accordance with both cell cycle arrest and PTEN upregulation in MCF-10A, but in cancer cells (T47D and MCF-7) there was not a combination between these genes and cell cycle arrest and PTEN mRNA changes under silibinin treatment. As mentioned above, silibinin had significant apoptotic effects both in MCF-7 and T47D cells but not in MCF-10A cells and in this view, PTEN upregulation was along with apoptotic induction by silibinin only in MCF-7 cells. Bcl-2 mRNA expression as a direct anti-apoptotic factor, was also evaluated under silibinin treatment. Our results showed downregulation of Bcl-2 in all of our cell lines, and apoptotic induction by silibinin in breast cancer cell lines was accompanied by Bcl-2 downregulation.

In conclusion, silibinin had both different (in cell cycle and PTEN mRNA regulation) and similar (in apoptosis and Bcl-2 mRNA regulation) inhibitory effects in breast cancer cells.

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References