International Journal of Biotechnology and Biomedical Sciences
p-ISSN 2454-4582, e-ISSN 2454-7808, Volume 3, Issue 2; July-December, 2017 pp. 155-159

http://www.krishisanskriti.org/Publication.html

Berberine Inhibits the Expression of c-Src and Growth of MDA-MB-213 Breast Carcinoma Cells

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Abstract—*Breast cancer is a major health problem among females around the globe. Presently available treatments are not effective in the advanced stages of breast cancer. Phytochemicals provide new insights into cancer therapy and prevention. In the present study, we have shown anticancer potential of berberine, a quaternary ammonium salt from the protoberberine group of benzylisoquinoline alkaloids found in plants. Berberine at 20-80 µM concentrations inhibited the growth of MD-MBA-231 breast cancer cells in a dose- and time-dependent fashion. Berberine also down-regulated the expression of many proteins involved in the regulation of cell division. Berberine inhibited the expression of CDK2, 6 and Cyclin D1. It has also inhibited the expression of c-Src, which is an important molecule and has role in cell proliferation, survival, metastasis and angiogenesis. p53 and p21 play important roles in the regulation of cell cycle, thus effect of berberine on the expression of p53 and p21/cip1 was also studied and observed to be up-regulated by the berberine treatment. Overall, berberine may inhibit proliferation and survival of breast cancer cells involving c-Src, CDKs, p53 and p21/cip1.*

Keywords*: Berberine, Breast cancer, Cell viability, c-Src, Cell cycle.*

Abbreviations: SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CDKI, Cyclin dependent kinase inhibitor; HER-2, human epidermal growth factor receptor, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

1. INTRODUCTION

Cancer is a leading cause of death globally with 8.2 million deaths in 2012 (1). Global comparison of cancer incidences between 2005 and 2015, indicates 33% of overall increase globally (2). In men, prostate cancer steps first for cancer incidences while lung cancer is leading cause of cancer related death. Breast cancer accounts for highest incidence and death of women worldwide. In India, total incidence and mortality rates due to top ten cancers are highest among females. Molecular subtypes of breast cancer are luminal-A, luminal-B, HER-2 enriched, normal like, basal like/triple negative (3). Out of known molecular subtypes, triple negative type of breast cancer (TNBC) has more prevalence in India, which accounts one from every three women diagnosed with carcinoma of breast. As triple negative breast cancer is highly advanced disease type with high prevalence there is a huge possibility for TNBC to be one of the important factor for higher fatality of Indian women due to breast cancer (4). Major risk factors to develop carcinoma of breast include age, family history, diet, consumption of alcohol and oral contraceptive etc. (5).

Regulation of mammalian cell cycle involves activation and inactivation of many cell cycle regulatory molecules which include group of well conserved family of serine/ threonine kinase (CDKs) along with regulatory molecules cyclins and an inhibitory molecule which controls the activity of CDKs. Cyclin-dependent kinase is a serine/threonine protein kinase which signals cells to advance next stage of the cell cycle. According to different phase of the cell cycle, specific types of CDK-Cyclin complexes are activated. Cyclin D1 has key role in cell cycle as all growth factor signaling regulates its expression. Upon activation it binds to CDK4/6 to phosphor late the RB and activates the E2F family proteins, responsible for transcription of regulators of next phase of cell cycle (6,7).

c-Src is a non-receptor tyrosine kinase proto-oncogene that is found to be mutated in several cancer types including breast cancer. Additionally, it is involved in several important regulatory mechanisms including cell proliferation, survival, metastasis, and angiogenesis. Thus, inhibiting Cyclin-CDK and c-Src can inhibit the growth of tumors (8,9).

Historically till date, plants are the common source for medication, in the form of traditional way or as an active compound in drug. As per the WHO, 80% people are been treated with plant-based medicine as a first line of treatment (10). Phytochemicals comprise some wonderful properties which enable them to be a potential compound for human health care, specifically cancer. Berberine is an alkaloid

anciently used in Ayurvedic medicines, and apart from its traditional use, vigorous research of past decade proves potential of berberine against cancer, diabetes and many infectious diseases (11-14). In the present study we have shown growth inhibitory activities of berberine against MD-MBA-231 cells, we have also shown that berberine decreased the level of Cyclin D1, CDK6 and CDK2, which show that it has anti-mitogenic properties. Additionally, berberine upregulated the expression of p53 and p21 moderately. Berberine also down regulated the expression of c-Src, which is an important regulator of cell proliferation and survival.

2. MATERIALS AND METHODS

2.1. Reagents and Antibodies

Molecular and analytical grade chemicals were used to prepare reagents and buffers. Acrylamide, ammonium persulphate, bis-acrylamide, β-mercapto ethanol, ethylene diaminetetra-acetate (EDTA), milk-powder, methanol, NP-40, sodium dodecyl sulphate (SDS), sodium orthovanadate, sodium carbonate anhydrous salt, sodium chloride, TEMED, MTT dye, pifithrin-α was purchased from Sigma Aldrich, USA. Tissue culture treated plastic plates were obtained from BD Biosciences, USA.

Fetal bovine serum was from Gibco-BRL, USA while protein ladder was procured from Bio-Rad, USA. Complete cocktail of protease inhibitor and phosphatase inhibitor were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA).Nitro-cellulose membrane and enhanced chemiluminescence (ECL) detection system was procured from Amersham Biosciences USA, Millipore. Antibodies against CDK-2, CDK-6, Cyclin D1, c-Src, p53, p21, secondary antibodies of rabbit & mouse and β-actin were purchased from Santa Cruz.

2.2 Cell culture maintenance and treatments

MDA-MB-231 are cell line of human adenocarcinoma of breast and was purchased from National Centre for Cell Science (NCCS), Pune, India. MDA-MB-231 cells were cultured in DMEM/F12 (GIBCO, USA), which was supplemented with 10% fetal bovine serum and 1% of PSA (Penicillin (100units/ml), Streptomycin (100µg/ml) and Antimycotic amphotericin B (250ng/ml)) in 5% CO_2 humidified atmosphere at 37°C. At 80% confluency, cells were sub-cultured in 1:3 ratios. Berberine was obtained from Sigma, in chloride form and the stock was prepared by dissolving powder of berberine in DMSO and stored at 4°C. The stock solution was prepared in such a way that final concentration of DMSO was achieved less than 0.2% (v/v) in all treatment groups. Cells were treated with 20, 40 and 80 µM of berberine for experiments.

2.3 MTT Assay

MTT assay was performed using the procedure described by Mosmann et al, 1983 with a slight change as using DMSO as solvent (15,16). 8000 cells were seeded in 96 well plates. Cells were allowed to attached and grow for 24 h at 37°C in a CO_2 incubator. After 24 h of seeding, at 75% confluency, cells were treated with three different (20, 40, 80 µM) concentrations of berberine for two different time points (24 h and 48 h). After the treatment time, media were removed gently and 100 µl of MTT (5 mg/ml in 1X PBS) was added and incubated for 4 h at 37°C. After 4 h of incubation time, MTT reagent was gently removed from all the wells and incubated with 100 µl DMSO for 15-20 min at 37°C. DMSO dissolves formazan crystals resulted into formation of blue color. The optical density of the blue color was measured at 570 nm by multimode plate reader (Synergy H1 BioTek). The experiment was done in triplicates for each time points.

2.4 Immunoblotting

Cells were seeded and treated with the indicated doses of berberine for 24 hours, after treatment time, cells were harvested and lysed with lysis buffer. Protein was collected by high speed centrifugation. Protein concentration was measured by Bradford method and separated by SDS-PAGE and transferred onto a membrane by wet transfer and probed with specific antibodies and detected using enhanced chemiluminescent (ECL). Fold difference of the expression of targeted proteins was calculated using the ImagJ software (17).

2.5 Statistical Analysis

Statistical analysis was done using Graph Pad Prism software version 6 and quantitative data were presented as mean $\pm$ SEM. Student's *t*-test was performed to determine the difference between groups. All experiments were done twice or thrice. *p*-value lower than 0.05 was considered statistically significant. Densitometry was done using ImagJ (NIH, USA) software to analyze fold difference in expression of the gene (PCR) and protein (western blotting) of control and treated samples.

3. RESULTS

3.1 Effect of Berberine on the Viability of Human Breast Cancer Cell Line MDA-MB-231

The effective dosage of berberine and its effect on the viability of breast cancer cell line were determined by using MTT assay. Metabolically active cells (viable cells) are capable for reduction of yellow tetrazolium salt of MTT into formazan crystals. Metabolic reduction of MTT was done by dehydrogenase enzyme to generate the reducing equivalents like NADH and NADPH. Resulted intracellular purple colored formazan was dissolved and measured as described in materials and methods. This experiment was performed to

determine the effective dose of berberine on MDA-MB-231 cells. Berberine significantly (*P<0.0001*) inhibited the viability of MDA-MB-231 cells at three different concentration (20, 40, 80μM) and two time points 24 and 48 h. The viability of cells was inhibited by berberine in time- and dose-dependent fashion (Figure 1). Berberine strongly inhibited the viability of the breast cancer cell line at all the three concentratins, 20, 40 and 80 μM.

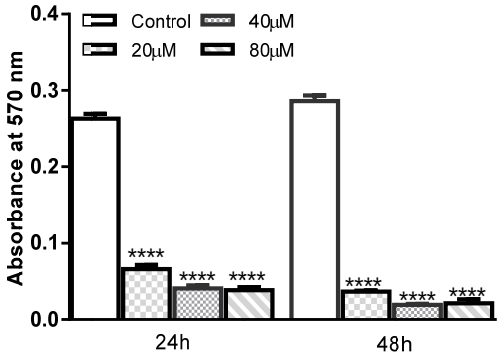

Figure 1. Inhibitory effect of berberine on cell viability of human breast cancer cells. MDA-MB-231 cells were treated with berberine (20-80 μmol/L) for 24 and 48 h, and assessed by MTT assay. Each column represents mean of three independent treatment samples and were reproducible in two to three independent experiments; Data presented as mean ± SEM. ****, $P < 0.0001$, berberine-treated groups compared with DMSO control.

3.2 Effect of Berberine on the Protein Levels of CDK Inhibitors, Cyclins, and CDKs

Effect of berberine on cell cycle regulatory proteins was analyzed by western blot analysis. CDK6 and CDK2 are protein kinases that cause the phosphorylation of Rb at different sites which is needed for the advance of the cell cycle. Cyclin D1 is an important regulatory molecule which acts in G1 phase of the cell cycle and quickly degrades in the S-phase. Berberine down regulated the expression of CDK6 and CDK2 at 20 and 40μM concentrations after 24 h. Also, berberine completely inhibited the expression of Cyclin D1 at same concentrations and treatment time point (Figure 2).

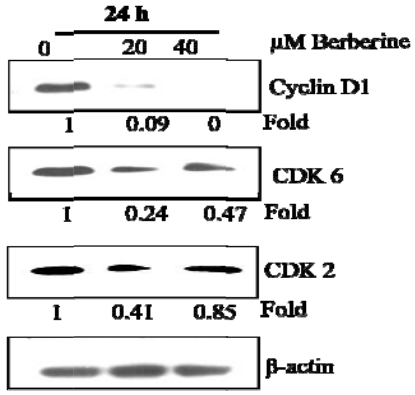

Figure 2: Inhibitory effect of berberine on G1 phase cell cycle regulatory molecules.

Cells were treated with berberine for 24 and 48 h and western blotting was done for cyclin D1, CDK6 and CDK2. Membranes were stripped and reprobed for β-actin as loading control. Data represented were reproducible in two independent experiments.

3.3 Berberine Downregulated the Expression of c-Src in MD-MBA-213 Breast Cancer Cells

c-Src is an important regulator of several important events inside the cells, it has role in the regulation of cell proliferation, survival, metastasis and angiogenesis. c-Src directly regulates the subcellular localization and post-translational protein stability of CDK inhibitors and is also able to regulate upstream regulators of p21/cip1. p21/cip1is an important regulator of cell cycle, and found to be down-regulated and mis-localized in several cancer types including breast cancer. Thus, we studied the expression of c-Src by western blot analysis and found that berberine down regulates the expression of c-Src at 20 and 40 μM concentrations at 48 h of treatmnet. Result of the study revealed that berberine strongly decreased expression of c-Src in MDA-MB-231.

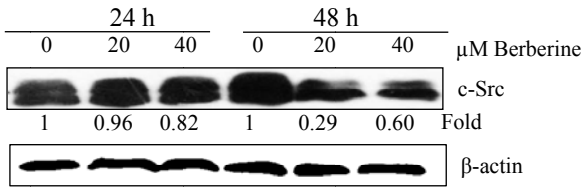

Figure 3. Berberine modulates c-Src in MDA-MB 231 cells. Cells were treated with 20-40 μmol/L berberine or DMSO for 24 and 48 hours. Whole cell lysate was collected and western blotting was done for c-Src. Beta-actin was used as loading control. Data represented were reproducible in two/three independent experiments.

3.4 Berberine Caused Moderate Increase in the Level of p53 and p21

In normal cells, p53 was activated upon DNA damage; induces the transcription of p21/cip1 to halt the cell cycle and allows the repair of the damaged DNA by DNA repair enzymes, but in oncogenic cells, p53 was majorly mutated or inhibited as a result, expression of p21/cip1 is down-regulated and cells can easily escape the cell cycle arrest. We analyzed the expression of p53 and p21/cip1 in berberine treated cells, and found that berberine caused moderate increase in the expression of these two proteins. However, when p53 was moderately inhibited by its chemical inhibitor pifithrin-α (20 μM), the expression levels of p21/cip1 as well as p53 caused by berberine were also decreased (Figure 4).

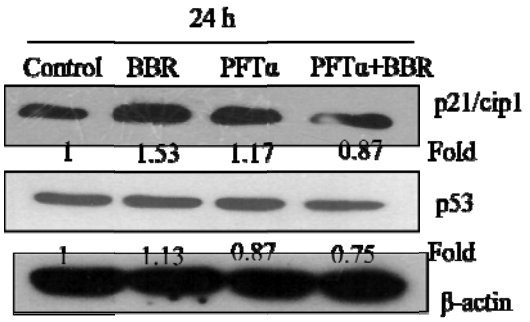

Figure 4. Effect of berberine and pifithrin-α on MDA-MB-231 cells. Cells were treated with berberine (BBR, 20 μM) and pifithrin-α (PFT, 20 μM) alone and in combinations for 24 hours. Whole cell lysate was collected and western blotting was done for p21/cip1 and p53. Beta-actin was used as loading control.

4. DISCUSSION

Even after more than hundred years of cancer research, still, deaths related to cancer and its incidences are high in numbers globally. In India, the consistent increase is seen in both incidence and mortality related to cancer. Women from all over the globe suffering from breast cancer, and moreover, the incidence and mortality are rising year after year in India. The available diagnostics, prognostics, and treatment options are inadequate to manage the alarming scenario of breast cancer in Indian women. Altogether, it is adistressing sign and therefore, a vigorous research is needed in areas like accurate diagnostic and prognostic tools, and more treatment options. In the context of breast cancer chemotherapeutics, the available options are very few with having severe high side effects. Some phytochemicals are known for beneficial effect against cancer with lesser side effects, specifically for breast cancer. Berberine is a potential phytochemical which has continuously growing popularity for its efficacy against diabetes, cardiovascular diseases, and cancer. Hence with this study, we have explored berberine for its mode of action against important cell cycle regulatory molecules in breast cancer cells.

The findings of the study confirmed the inhibitory effect of berberine on cell viability and cell cycle progression of advanced human breast cancer cell line which represents TNBC. Results suggested that berberine mediated inhibition of cell cycle regulatory proteins of G1 phase molecules such as cyclin D1, CDK2 and 6 can play critical roles in halting the cell cycle in these cancer cells.

These proteins are known to have key role in the progression of cell cycle from G1 phase to S phase (18,19). Apart from these proteins, berberine also down-regulated c-Src. After knowing that berberine affects Cyclin and CDKs expression, we investigated the effect of berberine on the expression of p21/cip1 and p53. The findings indicated that berberine also targets p21/cip1, which is essential to arrest the cell cycle advance. Berberine caused increase in the levels of p53 and p21/cip1, however, when p53 was inhibited by pifithrin-α, the berberine caused expression of both p53 and p21 further decreased. This indicated that p21/cip1 may not only regulated by p53 but also by some other pathway. p21/cip1 is a well know inhibitor of the cell cycle and its increased expression by berberine explains the decrease in cell proliferation(20). Further studies are needed to decipher the molecular cross-talk in the presence of berberine and pifithrin-α. Most chemo preventive or therapeutic agent targets wild-type p53 to induce expression of p21/cip1, as majority of oncogenic cells have faulty genes, some of the agents also cause DNA damage to induce cell cycle arrest and apoptosis (21,22).

In conclusion, berberine has shown growth inhibitory properties against human breast cancer MD-MBA-231 cells by down regulating the expression of Cyclin and CDKs, c-Src and caused up regulation of p53 and p21. However, further studies are needed to reveal the nexus of molecular changes caused by berberine.

5. ACKNOWLEDGEMENTS

The work was supported by the funds from the Central University of Gujarat and UPE-2, DST-PURSE, UGC-RN, JNU, India. J. Tak is supported by a fellowship from University Grant Commission, New Delhi, India.

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