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IMPROVING THE EFFICACY OF CYCLOPHOSPHAMIDE BY USING VERAPAMIL AS A P-GLYCOPROTEIN INHIBITOR IN BREAST CARCINOMA

Balakrishnan Babu*, Dhandapani Muthu*, Pitchaikutti Sasikala, Sivamani Ganesan[†]

PG & Research Department of Zoology and Biotechnology, A.V.V.M. Sri Pushpam College (Autonomous), Poondi, Thanjavur-613 503, Tamil Nadu.

*† Corresponding author, * Authors contributed equally.*

ABSTRACT

In women, breast carcinoma is the leading cause of cancer death globally. By the combination of better screening and treatments that moderately improved the survival however much to be done for the women who are refractory to the current therapies. Among Indian females, breast cancer has ranked number one cancer with mortality 12.7 per 100,000 women and age adjusted rate as high as 25.8 per 100,000 women. Statistics reports from various recent national cancer registries were compared for incidence, mortality rates. The age adjusted incidence rate of carcinoma of the breast was found as high as 41 per 100,000 women for Delhi, followed by Chennai (37.9), Bangalore (34.4) and Thiruvananthapuram District (33.7). One of the most common factor for the drug resistance in breast cancer is high expression of P-glycoprotein (P-gp) which is associated with a poor prognosis in patients. Consequently, P-gp represents a potential biomarker of drug resistance. However, a direct role of P-gp as a cause of clinical drug resistance has not been adequately tested in breast cancer. The objective of this study was to evaluate the effect of cyclophosphamide with and without verapamil on P-gp expression. Verapamil was found specific and effective against P-gp expression in breast cancer. In conclusion, treatment efficacy of cyclophosphamide is increased in combination with verapamil against most advanced breast cancer.

Keywords: P-Glycoprotein; Breast Carcinoma; Verapamil; Cyclophosphamide

INTRODUCTION

In both developing and developed countries, breast carcinoma is the most common cancer in the world [1]. The top incidence rate noticed in the high-income countries and highest mortality rate noticed in middle-income countries [2]. One of the study estimated that in 2020, the incidence rate of breast cancer will be 1.7 million and 70% of the breast cancer mortality will occur in the developing countries. Hence, breast cancer has a incredible public health consequence and the reason for that is unavailability of primary prevention, efforts to promote early detection should be highlighted

[3]. Worldwide, in female, breast cancer is the most common cancer representing closely 25% of all cancers with an estimated 1.67 million new cancer cases diagnosed in 2012. Women from rural areas have somewhat a greater number of cases (883,000) compared to developed (794,000) regions [4]. Compared to United Kingdom (95 / 100,000) the age adjusted incidence rate of breast cancer is lower (25.8 / 100,000) in India but mortality is equivalence (17.1 vs 12.7 per 100,000) with United Kingdom [5]. As described in global and Indian studies, there is a significant increase in the incidence and cancer associated morbidity and mortality in Indian subcontinent [6-10]. In most countries, the

prevalence of breast cancer is increasing, and it is highlighted that the incidence rate will escalate further in the next 20 years in spite of current efforts to prevent the disease [11-13]. Risk factors involves in numbers of women with major breast cancer, including late age of first pregnancy, shorter or no periods of breastfeeding, later menopause, increase in obesity, alcohol consumption, inactivity, and hormone replacement therapy [14]. There are many types of chemotherapy used to treat breast cancer. Common drugs include: Capecitabine, Carboplatin, Cisplatin, Cyclophosphamide, Docetaxel, Doxorubicin, etc.

MDR1 protein (P-gp) is a transmembrane glycoprotein with the molecular weight of 170-kDa transmembrane glycoprotein. This protein is encoded by the MDR1 (ABCB1) gene on the human chromosome 7p21. MDR1 protein overexpression has been associated with multidrug resistance in cancer cells including breast [15]. The overexpression of this protein is reason for intrinsic and acquired drug resistance in different cancers [16]. This overexpression can reduce intracellular anticancer-drug concentration as is frequently associated MDR in human cancer cells [17]. Our knowledge is restricted on how these drugs interact with the MDR1 protein. The effects are different across cancer types. Breast cancer is one of the most common cancers affecting more than one million individuals globally. Although, the chemoprevention is consequently one of the effective ways to cure breast cancer a major concern is potential of drug efflux transporter expression, which can significantly affect treatment efficacy. Here we hypothesized that down-regulation of MDR1 may enhance the effectiveness of chemotherapy. Recent studies demonstrated that verapamil is a calcium ion influx inhibitor and it is a well-known molecule developed against cancer chemo-resistant.

MATERIALS AND METHODS

Monolayer culture

The MCF-7 breast carcinoma cell line was obtained directly from the ATCC (Manassas, VA, USA), and grown in RPMI (Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) at 37°C in a 5% humidified CO_2 incubator. Characterization of cell line was done according to their surface expression phenotype. MCF-7 cell lines were grown for 48 hours with 80% confluency. After that the same cell lines were treated with Verapamil for 72 hours. These cells were used to form 3D tumorospheres along with controls. Verapamil and Cyclophosphamide was purchased from Selleckchem (USA).

Tumorosphere culture

Single cell suspensions were plated in 6-well tissue culture plates covered with poly-2 hydroxyethylmethacrylate (Sigma, St. Louis, MO) to prevent cell attachment, at a density of 1,000 cells/ml in serum free RPMI supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 30% F12 (Sigma), 2% B27 (Thermo Fisher Scientific, Carlsbad, CA), 20 ng/ml recombinant human epidermal growth factor (EGF;

Sigma, St. Louis, MO) and 20 ng/ml recombinant human fibroblast growth factor (FGF; Thermo Fisher Scientific, Carlsbad, CA). The medium was made semi-solid by the addition of 0.5% Methylcellulose (R&D Systems, Minneapolis, MN) to prevent cell aggregation. After 7 days in culture, 3D tumorospheres were collected by gentle centrifugation (200 x g) and dissociated enzymatically (5 min in 1:1 trypsin/RPMI solution at 37°C) and mechanically by passing through a 25G needle (6 strokes). Single cells were re-plated at a density.

Cell kinetic assay

MCF-7 cells were grown in a 96-well tissue culture plate with drugs and appropriate controls. Subsequently they were incubated with the WST-1 reagent (Dojindo Inc, Japan) for 4 hours. After this incubation period, the formazan dye formed was quantitated with a multi well spectrophotometer (ELISA reader). The measured absorbance directly correlates to the number of viable cells.

Cytotoxicity assay

MCF-7 cells cultured as monolayers or tumorospheres were treated with verapamil, cyclophosphamide for 3 days and cell death were measured with LDH cytotoxicity assay (Biovision, USA).

Flow cytometry

PerCP-conjugated CD44, BV711-conjugated CD24 monoclonal antibody was purchased from BD Bioscience. After 3 days of drug treatment the MCF-7 cells were dissociated with 0.25 % trypsin- EDTA (1 mM) (Invitrogen) for 3 min and washed with Calcium and magnesium free dulbecco phosphate buffered saline solution by spinning at 400g for 7 minutes. Then these cells were diluted in 100 μl FACS buffer (PBS containing 1 % fetal calf serum) and then incubated for 1 hr at 4° C in FACS buffer with the corresponding mAb: anti-CD44-PerCP, CD24-BV711. Flow cytometry analysis was performed with a BD FACS Caliber II flow cytometer (BD Biosciences).

Statistical Analysis

Data was expressed as mean standard deviation. Statistical packages for social sciences (SPSS) (SPSS UK Ltd, Woking, Surrey, UK) was used for data analysis. Chi-square analysis was used to assess significant changes between different groups. The level of significance was set at $P \leq 0.05$ and analysis of variance (one-way ANOVA) was used to compare the means of different groups.

4. RESULTS

CPA promotes P-gp expression

We examined the expression of P-gp in MCF-7 cell line with or without CPA. We treated the MCF-7 cell line with CPA for 48 hrs and we checked the P-gp expression using flow cytometry. We noticed significant increase in P-gp expression when compared to the control. However, in control the expression of P-gp was unchanged.
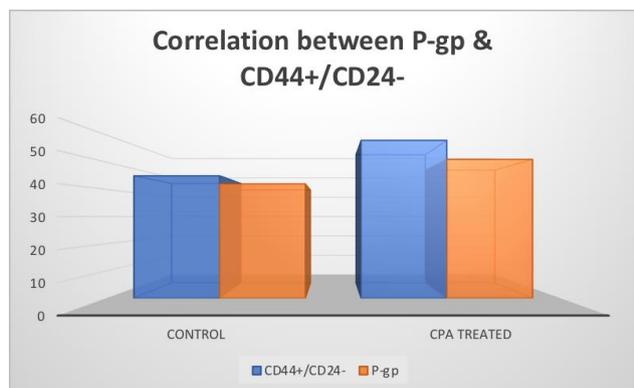
Figure 1: Increased level of CSC in CPA treated cells and in turn the expression of P-gp in MCF-7 cell lines.

Targeting CSC by using Verapamil

We confirmed that CPA alone is inducing the expression of both P-gp and CSC in MCF-7 cell line. So, we have decided to supress the expression of CSC by using P-gp inhibitor. We have treated the MCF-7 cell line with and without verapamil and we have maintained the cells for 9 days to check the formation of tumorosphere. The tumorosphere culture showed that the MCF-7 cell line treated with verapamil has failed to form tumorosphere and on the other hand the control cells formed tumorosphere.
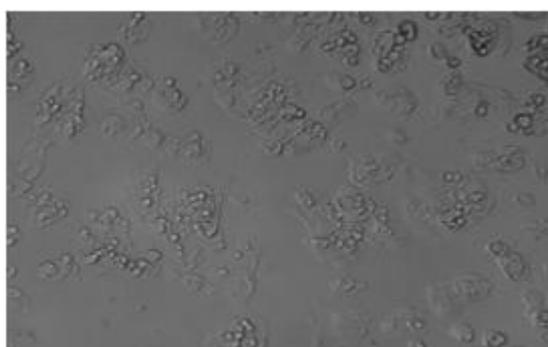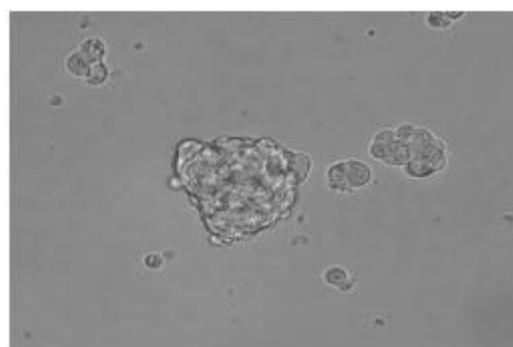
Figure 2: Verapamil a treated cell fails to form 3D tumorospehere against untreated

Correlation between P-gp and CSC

We then thought to correlat the expression of P-gp with cancer stem cell markers (CD44+ and CD24-). For that we have treated the MCF-7 cell line with CPA for 2 days and we checked the expression of both P-gp and

CD44+/CD24- using flow cytometry. We observed that in control arm, the expression of both P-gp and CD44+/CD24- were unchanged. However, in the CPA treated arm the expression of P-gp and CD44+/CD24- were significantly increased when compared untreated arm.
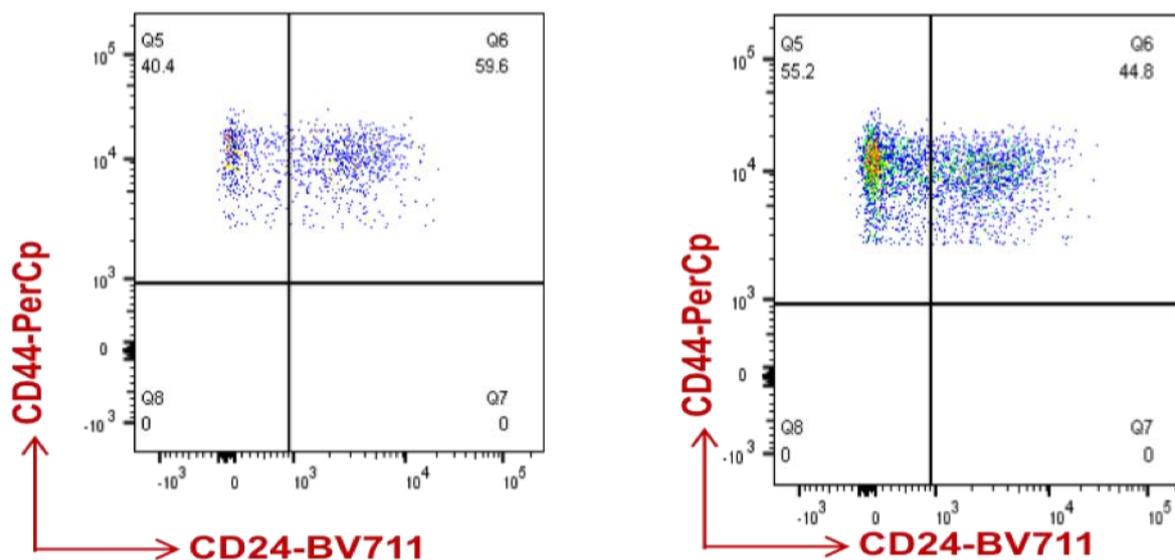

Figure 3: CPA treated cells increases the CD44+/CD24- expression against control

Targeting P-gp by using Verapamil

We confirmed that CPA alone is inducing the expression of P-gp and CSC in MCF-7 cell line. To supress the expression of P-gp and CSC, we have chosen Verapamil (P-gp inhibitor). We have treated the MCF-7 cell line with and without verapamil for 48 hours and we have carried out the flow cytometry analysis from the verapamil treated MCF-7 cell line. The flow cytometry data demonstrated that the MCF-7 cell line treated with verapamil has significant decrease in P-gp expression and also noticed decreased expression of CSC from the verapamil treated arm. But in the verapamil untreated arm, the expression of both P-gp and CD44+/CD24+ were unchanged in MCF-7 cell line.

Viability of MCF-7 Cell line in the presence of Verapamil (P-gp inhibitor)

After 3 days of verapamil treatment, MCF-7 cell lines were measured for the viability by WST-1 method. We observed very minimal or negligible difference between control and verapamil treated cell lines, and the same was observed in the morphological images under inverted microscope (data not shown here). This result indicates, verapamil alone play a vital role in suppressing the P-gp inhibitor and it did not affect the viability of the cells.
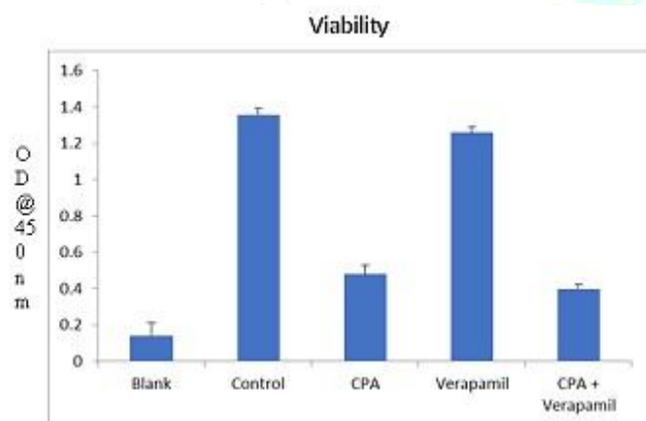

Figure 4: Viability of CPA and CPA + Verapamil treated cells

Combination of Chemo with verapamil improves Cytotoxicity

We have got convincing data that verapamil alone has not affected the viability of MCF-7 cells. So, further we treated the MCF-7 cell line in combination with verapamil and CPA. Then we measured the cell death by LDH cytotoxicity assay. This experiment showed us that, combination of both P-gp inhibitor and chemotherapy drug drastically affect the viability of MCF-7 cell line.
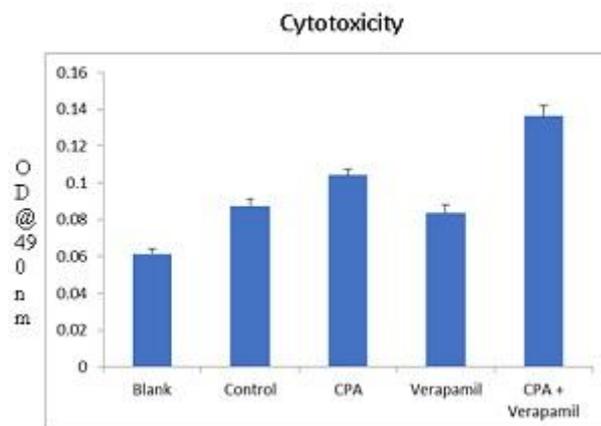

Figure 5: Cytotoxicity of CPA Vs CPA+ Verapamil treated cells
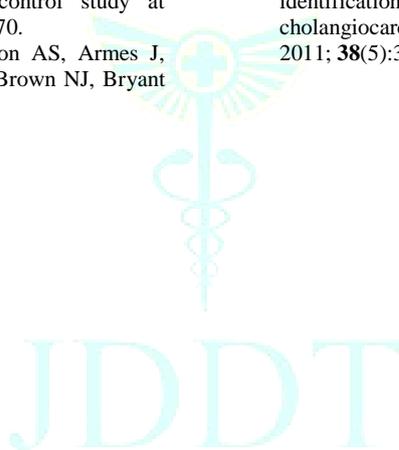
DISCUSSION

Like in most of the cancers, breast cancer also fails to respond to their current chemotherapies (12). The intrinsic and extrinsic resistance are contributed by P-glycoproteins. Here we set out to understand the relationship between the conventional chemotherapy (cyclophosphamide) and an inhibitor of transmembrane flux of calcium ions (verapamil). When combined with chemotherapeutic agent verapamil can help to promote intracellular drug accumulation. Treatment of cells along with P-gp inhibitor represents a potential strategy to improve the clinical outcomes.

Recent understanding of the drug resistance facilitated mechanisms underlying the treatment failures in breast cancer (16). Treating breast cancer using conventional chemotherapy along with P-.gp inhibitor like verapamil increases the drug sensitiveness. In this context, verapamil decreases P-glycoprotein and cancer stem cell expressions in MCF-7 cell lines. The same was shown in tumorospheres as well. When verapamil used the viability was not reduced, whereas when verapamil and CPA used combined the viability was drastically decreased. Deregulation of P-glycoprotein may be an important clinical and pathologic feature of breast cancer and a predictor of poor overall survival (15). In this study, we treated MCF-7 cell lines with verapamil, an inhibitor of p-glycoprotein that resulted in decreased levels of the stem cell markers CD44+/CD24-.

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