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Evaluation of Synergistic effect of Ceranib 2 and Tamoxifen in human breast cancer cells

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ABSTRACT

Breast cancer is the most common cancer diagnosed among Indian women. Despite the targeted therapies, the major problem in breast cancer therapy is the resistance developed after prolonged treatment. Recent studies explored a novel target acid ceramidase which plays crucial role in drug resistance. Therefore in our previous study we aimed at targeting the enzyme with an inhibitor, Ceranib 2. Though the apoptotic effect of Ceranib 2 was reported previously its effect in combination with Tamoxifen is unknown. In this study, MCF-7 and MDA MB-231 cell lines were treated with a combination of Ceranib 2 and Tamoxifen at different doses. Based on their cytotoxic effect two doses were standardized to determine the morphological and DNA changes. Moreover, when given as a combination the IC_{50} doses of both the drugs were lowered. Notable morphological changes and nuclear changes indicating DNA fragmentation were observed in the drug treatment groups with respect to the control group. These results revealed the synergistic effect of Ceranib 2-Tamoxifen in MCF-7 and MDA MB-231 cells.

Keywords: ceranib 2, tamoxifen, breast cancer.

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INTRODUCTION

Breast cancer is the leading cause of death among women and is expected to be 30% of new cancer diagnosis by 2017 [1]. In India, the incidence rate is higher among the women aged 40-50 years. Though the incidence rate is not as high as United states, mortality rate is higher i.e. 50% in India but in United states its only 20% [2]. Tamoxifen is considered to be the gold standard for the treatment of ER+ breast cancer [3] but its effect on ER- cell lines has also been reported [4]. However, the major problem encountered with Tamoxifen or chemotherapeutics is the resistance developed after prolonged treatment [5].

Numerous studies describe the underlying mechanism behind drug resistance. But recent studies unveiled a novel target acid ceramidase, which play crucial role in the development of drug resistance [6]. Acid ceramidase is the key enzyme of ceramide metabolic pathway metabolizes the pro-apoptotic ceramide to sphingosine and further sphingosine-1-phosphate [7]. Sphingosine-1-phosphate signals for cell survival, proliferation and angiogenesis [8, 9]. Acid ceramidase is aberrantly expressed in breast cancers [10, 11] and treatment with acid ceramidase inhibitors DM102, NOE and B13 significantly induced apoptosis and cell cycle arrest of MCF-7, MDA MB-231 and BT-474 [12]. Due to the poor bioavailability of DM102, NOE and B13 novel small molecule acid ceramidase inhibitors Ceranib 1 and Ceranib 2 were synthesized by Draper et al [13]. As Ceranib 2 was more potent than Ceranib 1 we aimed at targeting the enzyme with a small molecule acid ceramidase inhibitor, Ceranib 2 to induce apoptosis.

Our previous studies determined the apoptotic effect of Ceranib 2 and Tamoxifen in human breast cancer cell lines, MCF-7 and MDA MB-231 cells [14]. Anti-cancer effects of chemotherapeutics can be enhanced by a combination of chemotherapeutic agents [15]. Though significant apoptotic effect was attained with Ceranib 2 and Tamoxifen their effect as a combination is elusive. Therefore, in the present study we report the synergistic effect of Ceranib 2 and Tamoxifen in MCF-7 and MDA MB-231 cells.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's Minimum Essential Medium , Trypsin, Phosphate Buffered saline (PBS), MTT, Ceranib 2 and Tamoxifen were procured from Sigma, USA. Fetal Bovine Serum was purchased from Gibco, USA. DNA isolation kit was procured from Qiagen, Germany.

Cell culture

MCF-7 and MDA MB-231 cell lines were procured from NCCS Pune, India and the cells were cultured in Dulbecco's Minimum Essential Medium with 10% Fetal bovine serum. The cells were maintained at 37ºC in CO_2 incubator and the cells were sub-cultured with Trypsin after attaining 80% confluency.

MTT assay

MTT assay was performed to study the cytotoxic effect of Ceranib 2 + Tamoxifen in MCF-7 and MDA MB-231 cell lines. Cells were seeded in 96 well plates and after attachment they were treated with different doses of Ceranib2 + Tamoxifen as shown in Figure 1. After 24, 48 and 72 hours of treatment 10µl of MTT (5mg/ml) was added to each well and incubated at 37 ºC for 3 hours. The medium was removed completely and the formazan crystals were dissolved in DMSO. The absorbance was measured in an ELISA reader at 570 nm and the percentage viability was calculated using the formula, Percentage cell viability = Absorbance570 sample / Absorbance570 control× 100 %

Morphological Examination

MCF-7 and MDA MB-231 cells were seeded in 24 well plate and after attachment cells were treated with a combination of Ceranib2 and Tamoxifen (2.5 µM Ceranib 2 +2.5 µM Tamoxifen and 5 µM Ceranib 2 + 2.5 µM Tamoxifen) for 24 hours. The morphological changes were observed under phase contrast microscope at 200X magnification and photographed.

DNA gel electrophoresis

DNA gel electrophoresis was performed to visualize the changes in DNA upon apoptosis induction. MCF-7 and MDA MB-231 cells were treated with a combination of Ceranib2 and Tamoxifen (2.5 µM Ceranib 2 +2.5 µM Tamoxifen and 5 µM Ceranib 2 + 2.5 µM Tamoxifen) for 24 hours. After 24 hours cells were pelleted and the DNA was isolated using Qiagen DNA isolated kit. The genomic DNA samples were separated in 1.2% Agarose gel containing Ethidium bromide. The gel was visualized under UV transilluminator and photographed.

Statistical Analysis

Data were expressed in mean ± SD of three independent experiments and the results were analyzed using two-way ANOVA for comparison between treatment groups and control in graphPad Prism 5 software, $p < 0.5$ was considered to be statistically significant

RESULTS

Cytotoxic effect of Ceranib 2 + Tamoxifen in MCF-7 and MDA MB-231 cells

In MCF-7cells, IC50 attained at 2.5 + 2.5 µM of Ceranib 2 + Tamoxifen after 72 hours and the percentage decrease in viability ranges from 51% to 62% in drug treated groups with respect to control group. But, the decrease in percentage viability in the different treatment groups after 48 and 24 hours of drug treatment were 27% to 44% and 4% to 26% respectively (Fig. 1). In MDA MB-231 cells, IC50 attained at 2.5+2.5 µM of Ceranib 2 + Tamoxifen after 72 hours and the percentage decrease in viability ranges from 50% to 73% in the drug treated groups with respect to control group. After 48 hours, IC_{50} dose attained at 7.5 + 5 µM of Ceranib 2 and Tamoxifen and the percentage decrease in viability in the drug treated groups with respect to control group ranges from 35% to 57% .The percentage decrease in viability ranges from 7% to 39% in drug treated groups with respect to control groups after 24 hours (Fig. 2). For subsequent assays 2.5 + 2.5 µM and 5 + 2.5 µM of Ceranib 2 +Tamoxifen were used.

Effect of Ceranib 2 + Tamoxifen in the morphology of MCF-7 and MDA MB-231 cells

Notable morphological changes were observed in both the cell lines upon treatment with two different doses of Ceranib 2 + Tamoxifen. The cells became rounded and detached from the surface of the plate in drug treated groups whereas the control cells remained intact (Fig 3 A&B).

Effect of Ceranib 2 + Tamoxifen in the apoptosis of MCF-7 and MDA MB-231 cells

DNA fragmentation is a key event in apoptosis and treatment with Ceranib 2 + Tamoxifen induced remarkable changes in the DNA of MCF-7 and MDA MB-231 cells. Though the fragmentation pattern was not clear in both the cell lines treated with 2.5+2.5 µM of Ceranib 2 + Tamoxifen the smear in agarose gel electrophoresis indicates the DNA damage. MCF-7 and MDA MB-231 cells treated with 5+2.5 µM of Ceranib 2 and Tamoxifen showed ladder pattern in gel electrophoresis indicating DNA fragmentation. But the DNA remained intact in the control groups (Fig. 4).

DISCUSSION

Breast cancer is the common cancer of breast wherein the breast cells no longer behave in a normal manner and undergoes uncontrolled proliferation [16]. Based on the expression of Estrogen receptor (ER), Progesterone receptor (PR), Human Epidermal Growth factor receptor (HER) in breast cancer cells numerous targeted therapies are available for the treatment of breast cancer [17]. Tamoxifen, the selective estrogen receptor modulator is commonly used for the treatment of early and advanced ER^+ breast cancers [18]. However, previous studies showed it ER independent action in ER^- cell lines [4]. Despite its beneficial role over ER^+ and ER^- breast cancers, the major problem encountered is the resistance developed after prolonged treatment. Recent research in cancer explored a novel target acid ceramidase which plays crucial role in the development of resistance [6]. In our previous studies we have evaluated the apoptotic effect of Tamoxifen and Acid ceramidase inhibitor (Ceranib 2) in breast cancer cells. When chemotherapeutics given as a

combination induces cell death at low doses we were interested in studying the synergistic effect of Ceranib 2 and Tamoxifen in human breast cancer cell lines MCF-7 and MDA MB-231.

Synergistic effect was observed in both the cell lines after treatment with a combination of Ceranib 2 and Tamoxifen after 24, 48 and 72 hours of treatment. The IC_{50} dose of Tamoxifen was declined to 2.5 µM from 9 µM (MCF-7) and 9.5 µM (MDA MB-231) when given as a combination with 2.5 µM Ceranib 2. Similarly, the IC_{50} dose of Ceranib 2 was decreased to 2.5 µM from 5 µM (MCF-7) and 10 µM (MDA MB-231). In MCF-7 cells, synergistic effect was more pronounced in 48 and 72 hour treatment groups than 24 hours but in MDA MB-231 cells notable synergistic effect was observed in all the treatment groups irrespective of different time points. Therefore, we report maximal synergistic effect of Ceranib 2 and Tamoxifen in MDA MB-231 cells.

Cell shrinkage and detachment of cells in the Ceranib 2 + Tamoxifen treated groups are the morphological hallmarks of apoptosis [19]. The morphological changes are due to the proteolytic cleavage of key proteins by activated caspase proteases [20]. DNA fragmentation observed under gel electrophoresis is the biochemical hallmarks of apoptosis [19]. The fragmentation of DNA is due to the action of endogenous DNases which degrades internucleosomal regions into double stranded DNA fragments of 180 – 200 basepairs [21].

CONCLUSION

Taken together, combination of Ceranib 2 and Tamoxifen significantly induces apoptosis of human breast cancer cell lines and Tamoxifen when given as a combination with Ceranib 2 improved its cytotoxic effect in both the cell lines. Further, synergistic effect was highly pronounced in MDA MB- 231 than MCF-7.

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