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Targeting ceramide metabolic pathway induces apoptosis in human breast cancer cell lines

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ABSTRACT

The sphingolipid ceramide is a pro apoptotic molecule of ceramide metabolic pathway and is hydrolyzed to proliferative metabolite, sphingosine 1 phosphate by the action of acid ceramidase. Being upregulated in the tumors of breast, acid ceramidase acts as a potential target for breast cancer therapy. We aimed at targeting this enzyme with a small molecule acid ceramidase inhibitor, Ceranib 2 in human breast cancer cell lines MCF 7 and MDA MB 231. Ceranib 2 effectively inhibited the growth of both the cell lines in dose and time dependant manner. Morphological apoptotic hallmarks such as chromatin condensation, fragmented chromatin were observed in AO/EtBr staining. Moreover, ladder pattern of fragmented DNA observed in DNA gel electrophoresis proved the apoptotic activity of Ceranib 2 in breast cancer cell lines. The apoptotic events were associated with significant increase in the expression of pro-apoptotic genes (Bad, Bax and Bid) and down regulation of anti-apoptotic gene (Bcl 2). Interestingly, increase in sub G1 population of cell cycle phase analysis and elevated Annexin V positive cells after Ceranib 2 treatment substantiated its apoptotic activity in MCF 7 and MDA MB 231 cell lines. Thus, we report Ceranib 2 as a potent therapeutic agent against both ER^+ and ER^- breast cancer cell lines.

1. Introduction

Breast cancer is the most common cancer diagnosed in women and it is the leading cause for cancer death in the women aged 20–59 years. As per 2014 estimates, breast cancer accounts for 29% of all new cancers diagnosed among women [1]. In India, it accounts for 25%–31% of all the cancers in women in Indian cities, for every 2 women diagnosed with breast cancer one woman is dying out of it (breast.cancer.india). Based on the expression of hormonal receptors breast cancer can be classified into three subtypes: a) Estrogen Receptor(ER) b) Progestrone receptor (PR) c) Human epidermal growth receptor 2 (HER2), of which 70% of the tumors express estrogen receptor.

Tamoxifen is the drug of choice for ER^+ breast cancer which induces cell death by selectively antagonizing the estrogen mediated cell survival and proliferation [2,3]. However, its significance over cell proliferation has also been reported in ER^- breast cancer cells indicating its ER independent effect [4]. Eventhough, Tamoxifen has been approved to be the gold standard for Breast cancer therapy the major problem encountered with Tamoxifen is resistance after long term treatment [5,6].

Till date, numerous studies have been published to prove the underlying mechanism behind resistance. Recent studies suggest that upregulation of acid ceramidase plays a pivotal role in the development of multi drug resistance in cancer chemotherapy [7–9]. Acid ceramidase is an enzyme of ceramide metabolic pathway and it plays its role by metabolizing the pro-apoptotic ceramide to a proliferative and tumor inducing metabolite, sphingosine 1 phosphate [10–12]. Further, acid ceramidase is over expressed in the tumors of head and neck [13], breast [14,15] and prostate [16,17] whereas, treatment with ceramidase inhibitors or exogenous ceramides induced cell cycle arrest, apoptosis by increasing the ceramide concentration in the cells [18–21]. These findings laid the foundation for the discovery of acid ceramidases a

Abbreviations: ER, estrogen receptor; AO, acridine orange; EtBr, ethidium bromide; GAPDH, glyceraldehyde phosphate dehydrogenase; Bad, Bcl-2 associated death promoter; Bax, Bcl-2 associated X protein; Bid, BH3 interacting-domain death agonist; Bcl 2, B-cell lymphoma 2; ASAH1, acid ceramidase; PI, propidium iodide; D-MAPP, D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol; NOE, n-Oleoyl ethanol amine; D-NMAPPD, N-[(1R, 2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-tetradecanamide.

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potential target for anti cancer therapy.

Our study aimed at targeting ceramide metabolic pathway to induce apoptosis in human breast cancer cell lines. Numerous acid ceramidase inhibitors are available in market and N-Oleoyl ethanolamine, B13, D-MAPP, D-NMAPPD and DM102 have been reported to induce apoptosis in colon, prostate, glioma, melanoma and breast cancer cell lines [22,23]. Ceranib 2, a novel acid ceramidase inhibitor significantly induced apoptosis in human ovarian adenocarcinoma cells [24], rat fibroblast cancer cell line [25] and its effects on other cancer cell lines are yet to be revealed. In this study we have examined the apoptotic activity of Ceranib 2 in human breast cancer cell lines MCF 7, MDA MB 231. Tamoxifen was used as a breast cancer drug control and to the best of our knowledge this is the first paper to report the apoptotic activity of Ceranib 2 in human breast cancer cell lines.

2. Materials and methods

2.1. Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Acridine Orange (AO), Ethidium Bromide (EtBr), Dimethyl Sulphoxide, Dulbecco's Minimum Essential Medium (DMEM), Trypsin, Phosphate Buffered saline (PBS), Ceranib 2 and Tamoxifen were purchased from Sigma Chemical Pvt Ltd, USA. Fetal Bovine Serum was procured from Gibco, USA. DNA Isolation kit was purchased from Qiagen, Reagents for quantitative PCR were procured from Kapa Biosystems (USA). Annexin V and Propidium Iodide were purchased from InVitrogen, USA.

2.2. Cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-231 were procured from National Centre for Cell Sciences (NCCS Pune, India) and were cultured in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100 U/ml) and Streptomycin (100 μg/ml). The cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and 80% confluent cells were sub cultured by trypsinization.

2.3. Cell viability assay

Cell viability assay was performed to determine the cytotoxic effect of Ceranib-2 and Tamoxifen. Cell viability was assessed using MTT, a modified colorimetric technique in which the live cells have the ability to convert MTT (yellow Tetrazolium derivative) to purple formazan product by mitochondrial reductases. MCF-7 and MDA-MB-231 were seeded per well in 96 well plates and were allowed to attach overnight in the CO_2 incubator. After attachment, the cytotoxic effects of the drugs were determined after 48 and 72 h by adding MTT to each well followed by incubation at 37 °C for 4 h. The medium was removed completely and the formazan crystals were dissolved in DMSO. The optical density was measured using an ELISA reader at 570 nm. The cell viability was calculated using the following formula: % Cell Viability = (A_{570} sample/A_{570} control) × 100%.

2.4. Acridine orange (AO)/ethidium bromide (EtBr) staining

MCF-7 and MDA-MB-231 cells were treated with Ceranib 2 and Tamoxifen. After 24 h, cells were stained with AO (100 μg/ml) and EtBr (100 μg/ml). The cells were visualized under florescent microscope at a magnification of 200× to discriminate live, apoptotic and necrotic cells.

2.5. DNA gel electrophoresis

MCF-7 and MDA-MB-231 cells were treated with Cereanib-2 and Tamoxifen for 24 h. DNA was extracted from untreated and treated cells using Qiagen Mammalian Genomic DNA isolation kit. The genomic DNA samples were separated in 1.2% Agarose gel by electrophoresis and the gel stained with Ethidium Bromide was visualized under UV Trans illuminator.

2.6. Quantitative RT-PCR

The expression of pro-apoptotic genes Bax, Bid, Bad and anti-apoptotic gene Bcl-2 were determined by Real time PCR. Total RNA was isolated from the samples after 24 h of drug treatment and was reverse transcribed using Reverse transcript cDNA synthesis kit according to manufacturer's instructions. The primers were designed using the program primer BLAST and the primers used for amplification are given in the Table 1. GAPDH was taken as internal control. cDNA was mixed with Kappa SYBER Green mastermix, primers, ROX dye and the reaction volume was made to 10 μl with PCR grade water and the quantification was performed using Step One RT-PCR machine at an annealing temperature of 60 °C. The specificity of the amplified product was determined using melt curve analysis for each primer pair and the data was analyzed by comparative CT method. The fold change in expression was calculated using the formula $2^{-}(-\Delta\Delta CT)$.

2.7. Cell cycle phase analysis

MCF 7 and MDA MB 231 Cells were exposed to Ceranib 2 and Tamoxifen for 24 h and the cell pellets were re-suspended in 300 μl PBS. The cells were fixed in 70% ethanol, washed in PBS, re-suspended in PBS. Propidium Iodide (PI) and RNAase A were added to each tube to maintain the final concentration of 50 μg/ml. The tubes were incubated in dark at 37 °C for 30 min. The cells were analyzed by Fluorescent activated cell sorting in FACS Calibur instrument (BD Calibur, San Jose, CA) and PI fluorescence was detected using FL 2 filter (585 nm).

2.8. Annexin V/propidium iodide (PI) staining

Annexin V/PI staining was performed to quantify the percentage of live, apoptotic cells and necrotic cells after drug treatment. MCF 7 and MDA MB 231 Cells were treated with Ceranib 2, Tamoxifen for 24 h and the apoptosis was assessed using Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 & Propidium Iodide (PI) according to manufacturer's protocol. The stained cells were analyzed in FACS calibur measuring the fluorescence emission at 530 nm using 488 nm excitation.

2.9. Statistical analysis

Data were expressed in mean ± SD 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.

3. Results

3.1. Cell viability assay to determine IC_{50}

Ceranib 2 and Tamoxifen significantly reduced the viability of cells after 72 and 48 h in dose and time dependant manner. IC_{50} values of Ceranib 2 was found to be 5 μM and 10 μM respectively in MCF 7 cells (Fig. 1A) whereas, for Tamoxifen the IC_{50} values were

Table 1
Quantitative RT PCR primers.

SL NO	Primers	Sense (5′–3′)	Anti sense (5′–3′)
1	GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCAACC
2	Bad	AGAGTTTGAGCCGAGTGAGC	GACTCCGGATCTCCACAGC
3	Bax	TTCATCCAGGATCGAGCAGG	GGAAAAAGACCTCTCGGGGG
4	Bid	GTTCTGACAACAGCTTCCGC	GCCTGGCAATATTCCGGATG
5	Bcl 2	CTTTGAGTTCGGTGGGGTCA	GAAATCAAACAGAGGCCGCA

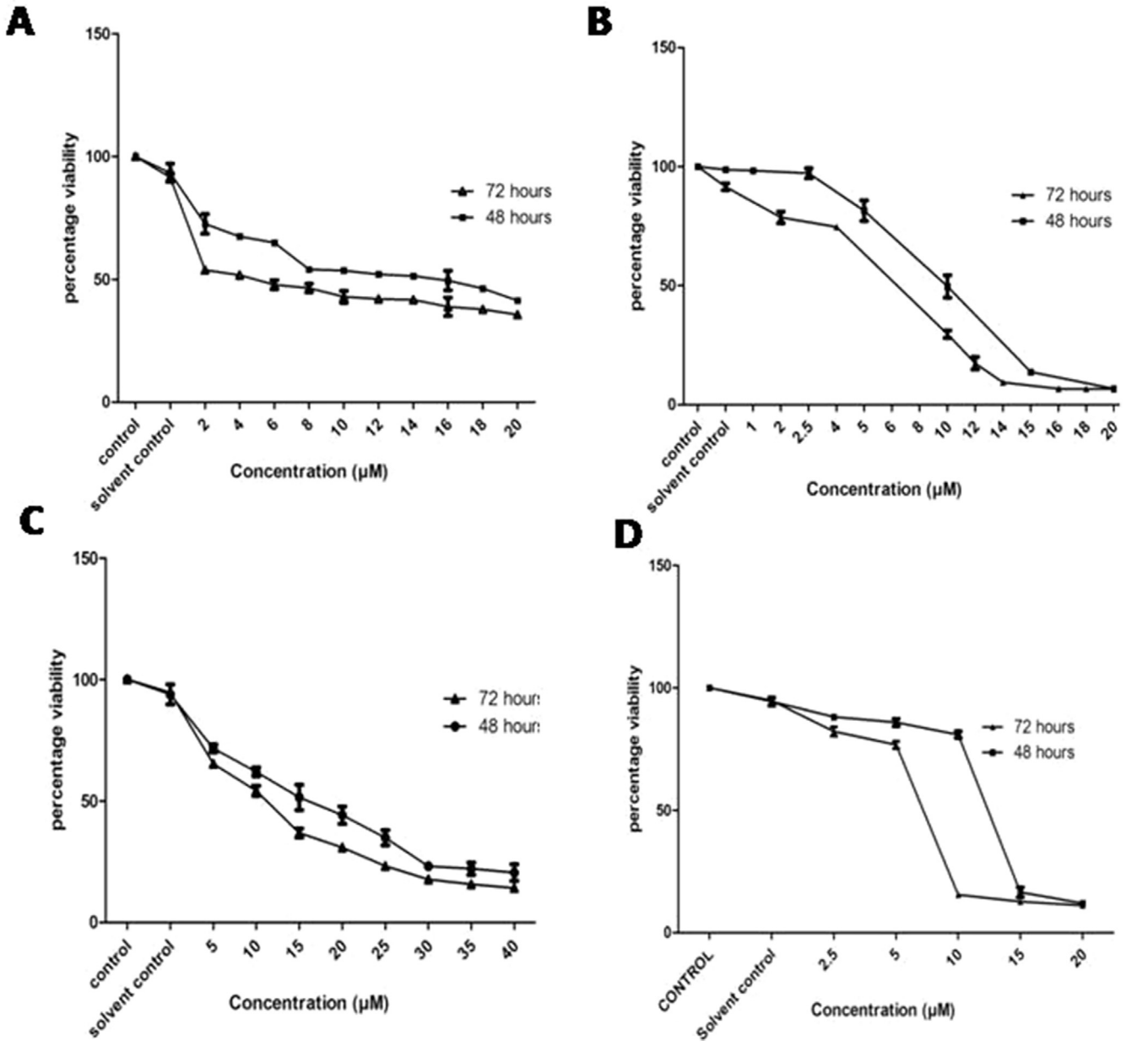

Fig. 1. Effects of Ceranib 2 and Tamoxifen on human Breast cancer cell lines to determine cell viability. A) MCF 7 cells treated with Ceranib 2 B) MCF 7 cells treated with Tamoxifen C) MDA MB 231 cells treated with Ceranib 2 D) MDA MB 231 cells treated with Tamoxifen. Each bar represents mean ± SD of three independent experiments and the results were highly significant with p value < 0.001.

9 μM and 10 μM respectively (Fig. 1B). In MDA MB 231 cells, after Ceranib 2 treatment IC_{50} was attained at 10 and 15 μM respectively (Fig. 1C), whereas after Tamoxifen treatment IC_{50} values were 9.5 and 13.5 μM respectively (Fig. 1D). These results indicate the cytotoxic effect of ceranib 2 and Tamoxifen in both the breast cancer cell lines and values were standardized for further analysis.

3.2. AO/EtBr staining to detect nuclear changes

Significant changes in morphology were observed after Ceranib

2 and Tamoxifen treatment whereas such changes were not observed in the control group (Fig. 2A, B left panel). Nuclear changes were visualized under fluorescent microscope using AO/EtBr staining. Acridine orange stains both live and dead cells whereas Ethidium bromide stains only the dead cells. Live cells and early apoptotic cells will fluoresce green but early apoptotic cells contain highly condensed chromatin and fragmented nuclei whereas late apoptotic cells and necrotic cells fluoresce orange. Cell shrinkage, condensed chromatin, fragmented nuclei, late apoptotic bodies were observed after drug treatment (Fig. 2A, B right panel). These results revealed the apoptosis inducing ability of Ceranib 2 in MCF 7 and MDA MB 231 cell lines.

3.3. DNA gel electrophoresis

DNA fragmentation is a key event in apoptosis in which the chromatin DNA is cleaved by the action of endonucleases and subsequently the fragmented DNA will show a ladder pattern in the gel electrophoresis. DNA ladder pattern was observed after Ceranib 2 and Tamoxifen treatment whereas, the DNA remained intact in both the control groups (Fig. 3A).

3.4. Quantitative gene expression profile

Quantitative expressions of pro-apoptotic and anti-apoptotic genes were examined to study the relative expression of such genes with respect to the constitutively expressed gene GAPDH. 2 fold increase in expression of Bid and Bad genes were observed after Ceranib 2 treatment in MCF 7 cells, but the fold change of Bax (3.6) was higher than the other two pro-apoptotic genes. In contrast, the fold change was not as high as Ceranib 2 after Tamoxifen treatment but a notable decrease in expression of anti-apoptotic gene, Bcl 2 was observed in both Ceranib 2 and Tamoxifen treated MCF 7 cells. The relative decrease in Bcl 2 expression was much significant in Tamoxifen (0.3) compared to Ceranib 2 (0.7) treatment (Fig. 3B). In MDA MB 231 cells, the fold change was found to be 4.5 for Bad, but the fold change was not as high as Bad for Bax and Bid genes after Ceranib 2 treatment. Moreover, no significant increases in expression of pro-apoptotic genes were observed after Tamoxifen treatment. However, the relative decrease in expression of anti-apoptotic gene Bcl 2 was highly significant after Tamoxifen (0.0007) treatment compared to Ceranib 2 (0.08) treatment (Fig. 3B). These results signify the apoptotic activity of Ceranib 2 in MCF 7 and MDA MB 231 cell lines.

3.5. Cell cycle phase analysis

In MCF 7 cells, percentage of cells in sub G1 phase increased from 4.35% to 47.35% and 38.95% after Ceranib 2 and Tamoxifen treatment whereas the percentage of cells in S phase was decreased from 19.76% to 11.24% and 18.75% respectively after treatment with Ceranib 2 and Tamoxifen. Similarly, in MDA MB 231 cells, the sub G1 population increased from 13.15% to 78.40% and 69.16% after treatment with Ceranib 2 and Tamoxifen but there was a suppression in the percentage of S phase cells from 24.15% to 3.29% and 3.27% respectively. (Fig. 4A, B) Thus increase in sub G1 population and decrease in S phase population of cells further revealed the apoptotic effect of Ceranib 2 and Tamoxifen in breast cancer cells.

3.6. Annexin V/PI staining

The percentage of Apoptotic and necrotic cells were quantified using Annexin V/PI staining. Annexin V forms a complex with phosphatidyl serine found on the outer membrane of apoptotic

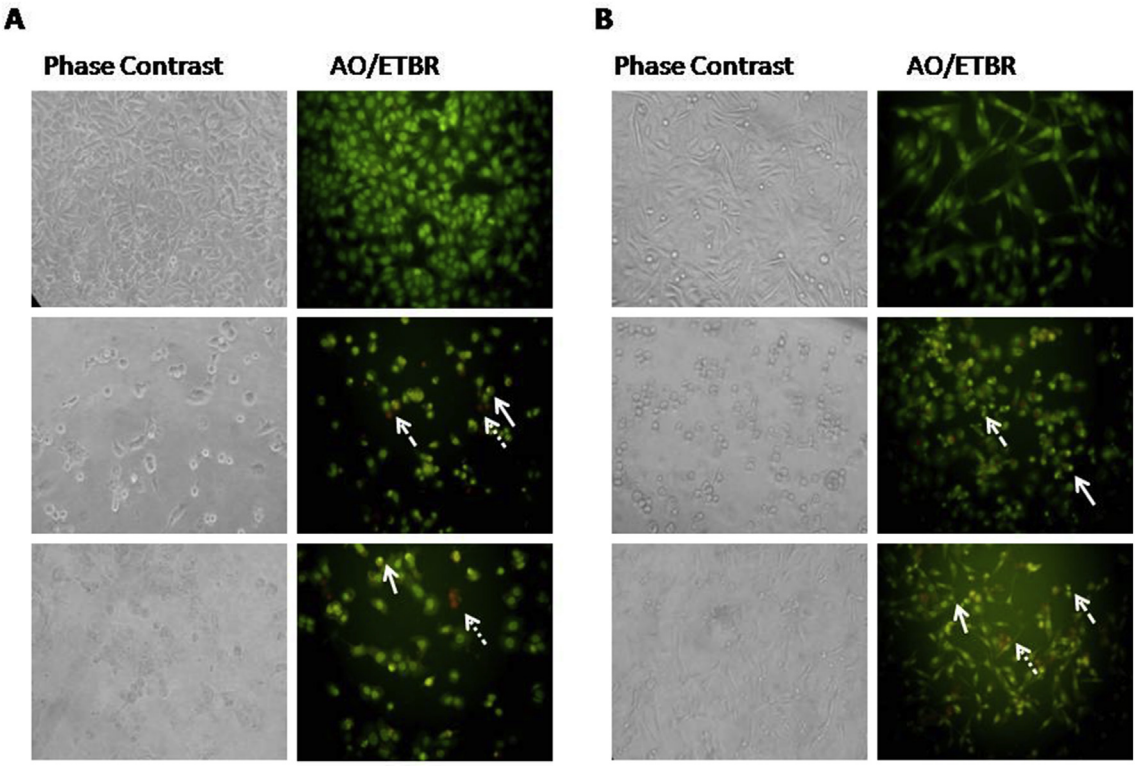

Fig. 2. Morphological and Nuclear changes observed in Phase contrast microscope and fluorescent microscope after AO/EtBr staining at 200× magnification. A) MCF 7 Cells Top panel: Control, Middle panel: Ceranib 2 treated, Bottom panel: Tamoxifen treated B) MDA MB 231 Cells Top panel: Control, Middle panel: Ceranib 2 treated, Bottom panel: Tamoxifen treated (straight arrows indicate condensed chromatin, dotted arrows indicate fragmented nuclei, round dotted arrows indicate late apoptotic and necrotic cells.

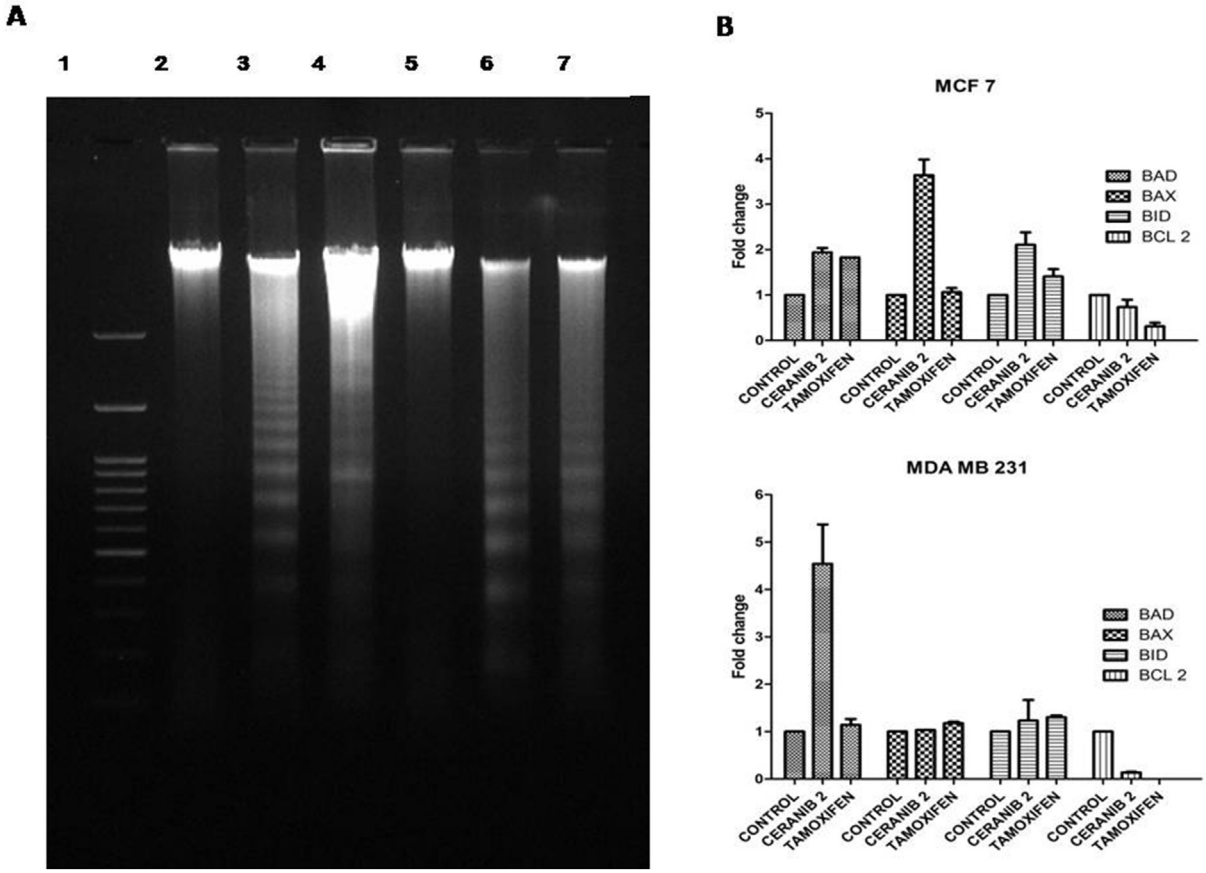

Fig. 3. A) **DNA Ladder pattern observed in gel electrophoresis**. Lane 1: 100 bp ladder, Lane 2: MCF 7 control, Lane 3: MCF 7 Ceranib 2 treated, Lane 4: MCF 7 Tamoxifen treated, Lane 5: MDA MB 231 control, Lane 6: MDA MB 231 ceranib 2 treated 7) MDA MB 231 Tamoxifen treated B) **Expression profile of Bad, Bax, Bid and Bcl 2 in MCF 7 and MDA MB 231 cells**. Each bar represents mean ± SD of three independent experiments and the results were highly significant with p value < 0.001.

cells and the percentage of apoptotic cells is directly proportional to Annexin V fluorescence whereas, PI stains necrotic and dead cells. In MCF 7 cells, the percentage of early apoptotic cells after Ceranib 2 and Tamoxifen treatment were 36.21% and 11.32% respectively whereas, the percentage of late apoptotic cells were 15.65% and 5.37% respectively. In case of MDA MB 231 cells, the percentage of early apoptotic cells after Ceranib 2 and Tamoxifen treatment were 36.14% and 15.88% respectively whereas, the percentage of late apoptotic cells were 34.35% and 3.91% respectively (Fig. 4C, D). Increased population of Annexin V positive cells confirmed the apoptotic effect of Ceranib 2 in MCF 7 and MDA MB 231 cells.

4. Discussions

Acid ceramidase a key enzyme involved in the conversion of pro apoptotic ceramide to proliferative metabolite, sphingosine 1 phosphate has been up regulated in the tumors of head, neck, prostate and breast [13–17]. Further, targeting Acid ceramidase with inhibitors significantly induced cell death by increasing the intracellular ceramide concentration. Numerous acid ceramidase inhibitors have been proven to posses anti cancer activity in wide range of cancer cell types. Moreover, the gold standard for breast cancer Tamoxifen, has significantly inhibited acid ceramidase irreversibly and Tamoxifen was reported to be the most potent drug compared to other acid ceramidase inhibitors NOE, B 13 and DM102 in breast cancer cell lines [26]. However, Ceranib 2 a novel acid ceramidase inhibitor has been reported to be the most potent drug compared to D-MAPP, D-NMAPPD, and Ceranib 1 with an IC50 of 0.73 μM in SKOV3 cells [24]. Further, the apoptotic activity of Ceranib 2 has been demonstrated in 5RP7 cell line [25] and these significant outcomes prompted us to study the apoptotic activity of acid ceramidase inhibitor, Ceranib 2 in human breast cancer cell lines.

Cell viability studies revealed the cytotoxic effect of Ceranib 2 and Tamoxifen in MCF 7 and MDA MB 231 cells. Ceranib 2 with an IC_{50} of 5 μM was found to be more potent in inhibiting the proliferation of MCF 7 cells than Tamoxifen whereas, the IC_{50} doses of Ceranib 2 and Tamoxifen were closer to each other in MDA MB 231 cells. In both the cell lines, significant reduction in cell viability was attained at lower concentrations after Ceranib 2 treatment whereas, the percentage viability decreased abruptly above 8 μM upon Tamoxifen treatment. The drugs were dissolved in Dimethyl sulphoxide and slight reduction in cell viability was observed in the solvent control group.

Significant morphological changes were observed in both the cell lines after 6 h of Ceranib 2 treatment (pictures not shown) but such changes were observed in Tamoxifen only after 24 h of treatment. Nuclear changes such as chromatin condensation and fragmented nuclei were analyzed with AO/EtBr staining. Cell shrinkage, chromatin condensation and nuclear fragmentation (karyorrhexis) are some of the morphological hallmarks of apoptosis [27] and these changes were observed after Ceranib 2 treatment indicating its apoptotic role in breast cancer cell lines. Further, previous reports suggested that these morphological events are due to the proteolytic cleavage of key proteins by the activated caspases [28]. DNA fragmentation is the biochemical

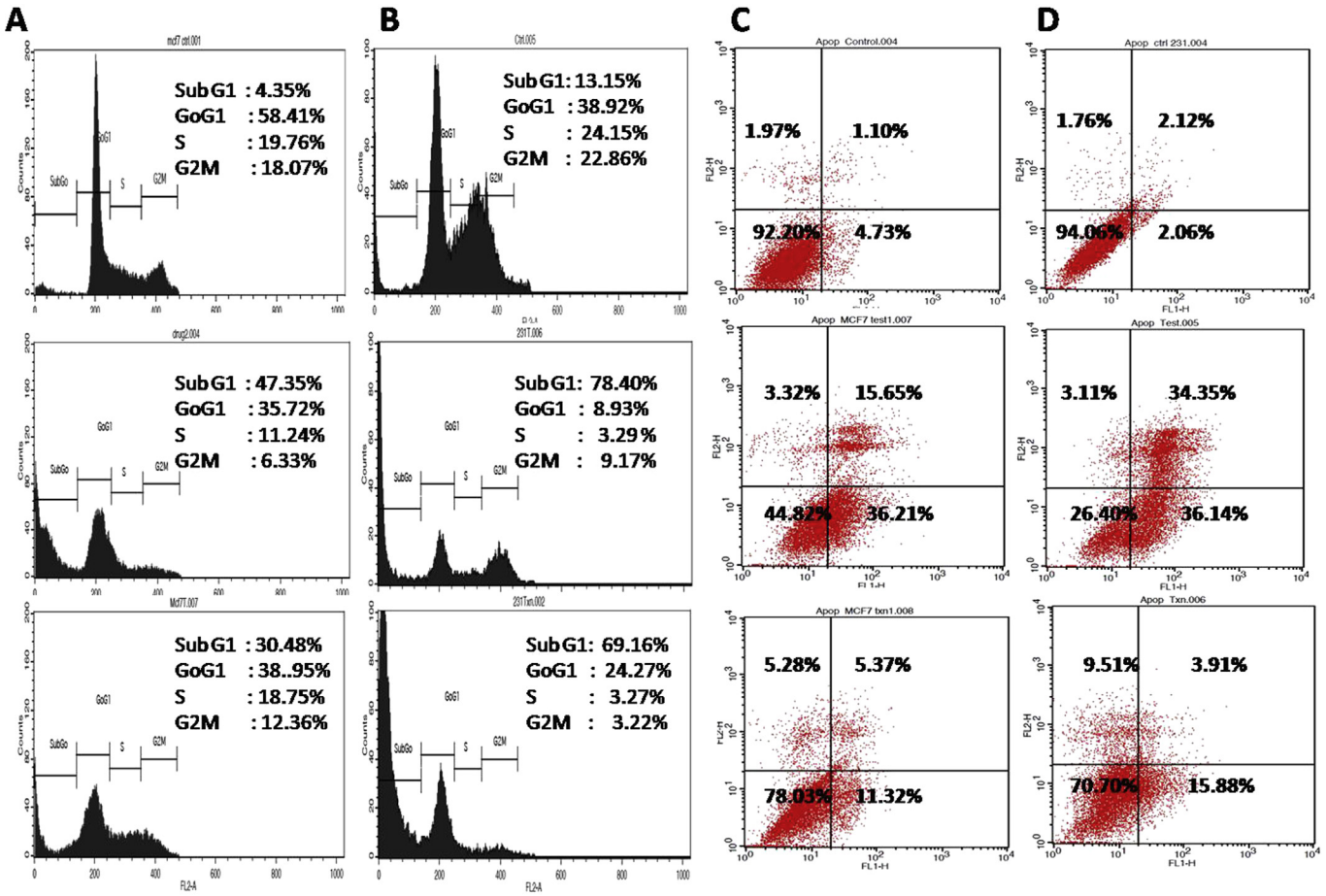

Fig. 4. The percentage DNA content in sub G1, Go/G_1S, G_2M phases were analyzed using Propidium Iodide (PI) in FACS A) MCF 7 Cells Top panel: Control, Middle panel: Ceranib 2 treated, Bottom panel: Tamoxifen treated B) MDA MB 231 Cells Top panel: Control, Middle panel: Ceranib 2 treated, Bottom panel: Tamoxifen treated. **The percentage of live, early apoptotic, late apoptotic and necrotic cells quantified using Annexin V and Propidium Iodide(PI)** C) MCF 7 Cells Top panel: Control, Middle panel: Ceranib 2 treated, Bottom panel: Tamoxifen treated D) MDA MB 231 Cells Top panel: Control, Middle panel: Ceranib 2 treated, Bottom panel: Tamoxifen treated Lower left quadrant: Live cells Annexin V^-/PI^-, Lower right quadrant: Early apoptotic cells Annexin V^+/PI^-, Upper right quadrant: Late apoptotic cells/Necrotic cells Annexin V^+/PI^+, Upper left quadrant: Late necrotic cells Annexin V^-/PI^+.

hallmark of apoptosis and it is characterized by the formation of double strand DNA breaks of 180–200 bps by the action of endogenous DNAses [29]. A typical ladder pattern of fragmented DNA observed after Ceranib 2 treatment further confirms its apoptotic effect against MCF 7 and MDA MB 231 cells.

Bcl 2 family of genes plays a major role in deciding the cell fate, of which Bax acts as pro-apoptotic effector, Bid and Bad act as pro-apoptotic activators whereas Bcl2 inhibits the pro-apoptotic role of Bax, Bid and Bad [30,31]. Pro-apoptotic effectors are closely associated with mitochondrial membrane and by the action of pro-apoptotic activators promote pore formation in the membrane initiating the apoptotic process. In contrast, Bcl 2 inhibits the pore formation either by preventing the interaction of pro-apoptotic activators with effectors or by directly interrupting the effectors [32]. In our study, treatment with Ceranib 2 up regulated the mRNA expression of pro-apoptotic genes but Bcl 2 expression was down regulated. Moreover, treatment with Tamoxifen successfully inhibited the expression of Bcl 2 but little effect was observed in the mRNA expression of pro-apoptotic genes and these results are consistent with a previous report which down regulated only Bcl 2 after Tamoxifen treatment in MCF 7 cells [33].

Accumulation of sub G1 population in cell cycle phase analysis is considered to be the best marker for apoptosis and this is due to the decreased DNA content in apoptotic cells [34]. Further, loss of S phase population is another important indicator of apoptosis. Thus increase in sub G1 population and decrease in S phase population of cells further revealed the apoptotic effect of Ceranib 2 in Breast cancer cells. Moreover, translocation of phosphatidyl serine from the inner side of plasma membrane to outer surface is another key event in apoptosis and Annexin V has high binding affinity for phosphatidyl serine and when labeled with a fluorochrome easily detects apoptotic cell population [35]. In our study, significant percentage of cells underwent this apoptotic process after Ceranib 2 and Tamoxifen treatment.

Thus we conclude that acid ceramidase is a potential target for breast cancer and the ability of Ceranib 2 to induce apoptosis in MCF 7 and MDA MB 231 cell lines revealed its significant effect in both ER^+ and ER^- cancer subtypes. Further, we suggest that Ceranib 2 may become a therapeutic agent for breast cancer in future.

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Transparency document

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References

[1] Rebecca Siegel, et al., Cancer statistics, 2014, CA Cancer J. Clin. 64 (2014) 9–29.
[2] V. Craig Jordan, Optimising endocrine approaches for the chemoprevention of breast cancer: beyond the study of tamoxifen and raloxifene (STAR) trial, Eur. J. Cancer 42 (2006) 2909–2913.
[3] Victor G. Vogel, et al., Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial, JAMA 295 (2006) 2727–2741.
[4] Chun-Yu Liu, et al., Tamoxifen induces apoptosis through cancerous inhibitor of protein phosphatase 2A–dependent phospho-Akt inactivation in estrogen receptor–negative human breast cancer cells, Breast Cancer Res. 16 (2014) 431.
[5] Paromita Raha, et al., Combined histone deacetylase inhibition and tamoxifen induces apoptosis in tamoxifen-resistant breast cancer models, by reversing Bcl-2 overexpression, Breast Cancer Res. 17 (2015) 26.
[6] Wenwen Zhang, et al., The novel role of miRNAs for tamoxifen resistance in human breast cancer, Cell. Mol. Life Sci. (2015) 1–10.
[7] Saeed Elojeimy, et al., Role of acid ceramidase in resistance to FasL: therapeutic approaches based on acid ceramidase inhibitors and FasL gene therapy, Mol. Ther. 15 (2007) 1259–1263.
[8] Ana Ramírez de Molina, et al., Acid ceramidase as a chemotherapeutic target to overcome resistance to the antitumoral effect of choline kinase α inhibition, Curr. Cancer Drug Targets 12 (2012) 617–624.
[9] Joseph C. Cheng, et al., Radiation-induced acid ceramidase confers prostate cancer resistance and tumor relapse, J. Clin. Invest. 123 (2013) 4344.
[10] Naoya Hirata, et al., Sphingosine-1-phosphate promotes expansion of cancer stem cells via S1PR3 by a ligand-independent Notch activation, Nat. Commun. 5 (2014).
[11] Leyre Brizuela, et al., Osteoblast-derived sphingosine 1-phosphate to induce proliferation and confer resistance to therapeutics to bone metastasis-derived prostate cancer cells, Mol. Oncol. 8 (2014) 1181–1195.
[12] Thomas H. Beckham, et al., Acid ceramidase promotes nuclear export of PTEN through sphingosine 1-phosphate mediated Akt signaling, Plos. One 8 (2013) 76593.
[13] Ikuhiro Maeda, et al., Rapid screening of specific changes in mRNA in thyroid carcinomas by sequence specific-differential display: decreased expression of acid ceramidase mRNA in malignant and benign thyroid tumors, Int. J. Cancer 81 (1999) 700–704.
[14] Nicole Sanger, et al., Acid ceramidase is associated with an improved prognosis in both DCIS and invasive breast cancer, Mol. Oncol. 9 (2015) 58–67.
[15] E. Ruckhäberle, et al., Acid ceramidase 1 expression correlates with a better prognosis in ER-positive breast cancer, Climacteric 12 (2009) 502–513.
[16] Thomas H. Beckham, et al., Acid ceramidase-mediated production of sphingosine 1-phosphate promotes prostate cancer invasion through upregulation of cathepsin B, Int. J. Cancer 131 (2012) 2034–2043.
[17] Xiang Liu, et al., Acid ceramidase upregulation in prostate cancer: role in tumor development and implications for therapy, Expert. Opin. Ther. Targets 13 (2009) 1449–1458.
[18] Xiang Liu, et al., Acid ceramidase inhibition: a novel target for cancer therapy, Front. Biosci. 13 (2007) 2293–2298.
[19] Margaret Flowers, et al., C6-ceramide and targeted inhibition of acid ceramidase induce synergistic decreases in breast cancer cell growth, Breast Cancer Res. Treat. 133 (2012) 447–458.
[20] Sehamuddin Galadari, et al., Tumor suppressive functions of ceramide: evidence and mechanisms, Apoptosis 20 (2015) 689–711.
[21] Lan Yang, et al., C6 ceramide dramatically enhances docetaxel-induced growth inhibition and apoptosis in cultured breast cancer cells: a mechanism study, Exp. Cell. Res. 332 (2015) 47–59.
[22] Krishna P. Bhabak, et al., Effective inhibition of acid and neutral ceramidases by novel B-13 and LCL-464 analogues, Bioorg. Med. Chem. 21 (2013) 874–882.
[23] Denny Proksch, Jan Jasper Klein, Christoph Arenz, Potent inhibition of acid ceramidase by novel B-13 analogues, J. Lipids 2011 (2010).
[24] Jeremiah M. Draper, et al., Discovery and evaluation of inhibitors of human ceramidase, Mol. Cancer Ther. 10 (2011) 2052–2061.
[25] Djanan Vejselova, et al., Cytotoxic and apoptotic effects of ceranib-2 offering potential for a new antineoplastic agent in the treatment of cancer cells, Turk. J. Biol. 38 (2014) 916–921.
[26] Samy AF. Morad, et al., Novel off-target effect of tamoxifen—inhibition of acid ceramidase activity in cancer cells, Biochim. Biophys. – Mol. Cell Biol. 1831 (2013) 1657–1664.
[27] John FR. Kerr, Clay M. Winterford, Brian V. Harmon, Apoptosis. Its significance in cancer and cancer therapy, Cancer 73 (1994) 2013–2026.
[28] Seamus J. Martin, Douglas R. Green, Protease activation during apoptosis: death by a thousand cuts? Cell 82 (1995) 349–352.
[29] Carl D. Bortner, Nicklas BE. Oldenburg, John A. Cidlowski, The role of DNA fragmentation in apoptosis, Trends. Cell Biol. 5 (1995) 21–26.
[30] Michael Certo, et al., Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members, Cancer Cell 9 (2006) 351–365.
[31] Michael C. Wei, et al., Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death, Science 292 (2001) 727–730.
[32] Fabien Llambi, et al., A unified model of mammalian BCL-2 protein family interactions at the mitochondria, Mol. Cell 44 (2011) 517–531.
[33] Guo-Jun Zhang, et al., Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-XL, without alteration of p53 protein levels, Clin. Cancer Res. (1999) 2971–2977.
[34] Donald Wlodkowic, Joanna Skommer, Zbigniew Darzynkiewicz, Flow Cytometry-Based Apoptosis detection. Apoptosis, Humana Press, 2009, pp. 19–32.
[35] G. Koopman, et al., Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis, Blood 84 (1994) 1415–1420.