A STUDY ON THE EFFECT OF MILTEFOSINE DRUG ON NOTCH PATHWAY INDUCING APOPTOSIS IN HUMAN BREAST CANCER CELLS


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ABSTRACT
Breast cancer is one of the leading causes of cancer death in women, for this problem we still needs a new kind of drugs for the treatment, here in this study we had repurposed the miltefosine for the treatment of breast cancer. While on molecular domain Notch proteins play crucial roles in shaping cell fates such as proliferation, differentiation and apoptosis. Role of Notch signaling in human breast cancer has been suggested by the development of adenocarcinomas in the murine mammary gland. However, it is not clear whether Notch signaling is frequently expressed and activated in breast cancers. From this study we had found that Notch signaling was over expressed and highly activated in breast cancer cell lines, on other side we had checked efficacy of miltefosine as anticancer molecule on (MCF-7, MDA-MB-231) breast cancer cell lines that leads to apoptosis. However the genomic & proteomics observations also indicated that miltefosine can downregulate the Akt, Notch (1, 2, 3, & 4) and thus, opening a new gateway to combat multi drug resistant conditions in breast cancer.

KEYWORDS: Notch, Breast Cancer, Akt, Miltefosine.

INTRODUCTION
Miltefosine is a well-known broad-spectrum antimicrobial, anti-leishmanial, phospholipid drug that was originally developed in the 1980s for the treatment of cancer but now it is the only recognized drug effective for treatment of various forms of leishmaniasis, a neglected tropical disease. Despite its excellent activity profile against parasites of trypanosoma,
priority was given for development of a compound as local treatment for cutaneous metastases of breast cancer and eventually led to the approval of miltefosine topical formulation.\(^{[1]}\) The application of oral formulation of miltefosine in the treatment of solid tumor was also evaluated in several Phase II studies with different types of tumors.\(^{[2-4]}\) Breast Cancer is one of the three most common cancers worldwide, along with lung and colon cancers.\(^{[5]}\) In 2019, approximately 268,600 new cases of invasive breast cancer and 62,930 new cases of in situ Breast Cancer were estimated to be diagnosed, along with 41,760 breast cancer related deaths in the United States of America alone. It is a well-diagnosed cancer in Indian population also and accounts for 14% of all cancers in Indian women. The number of new cases of breast cancer registered in India in year 2018 in females of all ages was 1, 62,468 according to Globocan 2018 (WHO). Multiple risk factors are associated with the breast cancer development and these include breast density, reproductive phase (parity and age at first birth), menstruation (menopausal status and age at menarche), and various modifiable lifestyle related factors, like body mass index (BMI), hormone replacement therapy (HRT) and alcohol consumption. Breast cancer is a heterogeneous multifactorial disease on molecular level; its molecular features include activation of human epidermal growth factor receptor 2 (HER2, encoded by ERBB2), activation of hormone receptors (estrogen receptor and progesterone receptor) and BRCA mutations. Most of the signal transduction pathways found deregulated in breast cancer, including Notch which contributes to cancer progression and the recurrence. The Notch transduction pathway is highly conserved from invertebrates to humans and plays a significant role in cell-differentiation, survival, proliferation, stem-cell renewal, and determining cell fate during the development and morphogenesis. Deregulated Notch activity is also involved in the genesis of many other human cancers, such as pancreatic cancer, medulloblastoma and muco-epidermoid carcinoma.\(^{[7]}\) In these tumors, the oncogenic effect of Notch signalling reflects an aberrant recapitulation of the highly tissue-specific function of the cascade during normal development and in tissue homeostasis.\(^{[8]}\) Few studies suggest that cross talk between the Notch signaling and estradiol (a major female sex hormone) has a major role in the progression and development of human breast carcinogenesis and angiogenesis.\(^{[8-11]}\) There are also several studies showing that Notch pathway components are up regulated in early noninvasive stages of breast cancer, including usual ductal hyperplasia (UDH) and ductal carcinoma in situ (DCIS).\(^{[7]}\) These studies indicate that changes in the Notch pathway occur early in breast cancer progression and therefore changes in Notch signaling may play an important role in the initiation of the disease.\(^{[8]}\) Interestingly, one study also demonstrates that
during asymmetric cell division in embryogenesis\cite{9}, the activity of Notch is biologically antagonized by the cell fate determinant Numb (a negative regulator of Notch pathway).\cite{10} The biological antagonism between Notch and Numb controls the proliferative/differentiated balance in development and homeostasis.\cite{11} Although altered Notch signaling has been linked to human diseases, including cancer, evidence for a substantial involvement of Notch in human tumors has remained elusive.\cite{12} Over recent years many approaches have been taken to inhibit the Notch pathway. The first Notch pathway inhibitors used clinically is γ-secretase which prevents release of NICD from plasma membrane by preventing cleavage of NEXT. Current preclinical experiments reveal that Notch pathway inhibitors are unlikely to be effective on their own, but that they should significantly increase the efficacy of current therapies. This implies there is still need to identify patients that are likely to respond to Notch pathway inhibitors.\cite{13,14} The effect of miltefosine on the progression of breast cancer is not well established. An attempt has been made in the present study to validate the therapeutic efficacy of miltefosine in-vitro in breast cancer cell lines and also explored its underlying mechanism of tumor suppression.

**METHODOLOGY**

**Cell culture**

MCF-7 (estrogen receptor positive breast cancer cell lines), MDA-MB-231 (triple negative breast cancer cell lines and they are about 15% to 20% of all breast cancer cases and hard to treat them & exhibit the MDR condition), cell lines were purchased from the National Centre for Cell Science, Pune, India. The cells were cultured in DMEM and RPMI (Dulbecco’s modified Eagle’s medium) containing 10% fetal bovine serum (FBS) in a sterilized and humid incubator (5% CO₂).

**Cell Viability Assay**

The cell viability of miltefosine was assessed by MTT assay. The treated cells with miltefosine were seeded in 96-well plates at a concentration of 3000 cells per well and incubated for 5 days. Then, 20 μL of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl blue) dye solution was added to each well, and the plates were cultured for 4 h at 37°C. The MTT solution was removed, and 150 μL of DMSO was used to solubilize the MTT formazan. The results were measured spectrophotometrically at a wavelength of 490 nm.
Detection of morphological apoptosis with Hoechst 33342 staining
To determine morphological apoptosis in cells we performed Hoechst 33342 staining and fluorescence microscopy. Cells were grown to 50% confluence on cover slip and left for 18-20h in incubator until full morphology is attained. Next day-old media was replaced with fresh media and cells were treated with different concentration of miltefosine. After 24h cells were fixed with 4% par formaldehyde and permeabilize with 0.3% Triton-X100 in PBS. Cells were then stained with Hoechst 33342 at a final concentration of (0.5µg/ml) in PBS and incubated for 5 min at 25°C. Brightly stained, condensed nuclei with characteristic features of apoptotic cells were counted using a fluorescence microscope. A minimum of 5 fields of at least 100 cells per field was counted. Images were captured at 20X magnification.

Annexin V-FITC assay
To analyze apoptosis, cells were seeded in 6-well plates at a density of 2.0 × 10^5 cells/well and allowed to grow overnight. Next, the cells were treated with miltefosine at different concentrations for 48 h at 37 °C. Untreated cells were used as the control. After incubation, the cells were harvested and washed twice with cold PBS. Next, 1 × 10^5 cells will be dispersed in 100 µL of 1 × Annexin V binding buffer. Subsequently, 5 µL of Annexin V-FITC and 5 µL of PI were added, and the cells were incubated at room temperature in the dark for 15 min. Finally, 400 µL of 1 × Annexin V binding buffer was added under gentle mixing, and the samples were analyzed by flow cytometry (BD Biosciences, San Jose, CA) & FlowJo software.

Anti-metastatic Property by Wound healing assay
To evaluate the effects of miltefosine on cell migration, wound-healing assay were performed. In the wound-healing assay, HTPF dose-dependently significantly decreased the migration of the MDA-MB-231 cells compared with the untreated control. Treatment with miltefosine at 3h and 6h inhibited MDA-MB-231 cells migration significantly. The wound-healing assay suggested that miltefosine suppresses the migration of breast cancer cells.

Reverse Transcription Quantitative Polymerase Chain Reaction (qRT-PCR)
Total RNA was isolated by the TRIZOL method, cDNA synthesis by High-Capacity cDNA Reverse Transcription Kit (4368814), all the primers were designed by IDT software, validated by other web tools and purchased from IDT. RT-PCR protocol was followed by PowerUp SYBR green master mix (ABI- A25741), and the result of RT-PCR was analyzed by DataAssist software (Thermo scientific). q-PCR primers are shown below;
Primer used for qRT-PCR

Notch-1;
Forward primer: 5’-GTCAACGCCTAGATGACC-3'
Reverse primer: 5’-TTGTTAGCCCGTTCTTCAG-3'

Notch-2;
Forward primer: 5’-TCCACTTCATACTCAGTTGA-3'
Reverse primer: 5’-TGGTTCCAGAGAAAACATACA-3'

Notch-3;
Forward primer: 5’-GGGAAAAAGGCAATAGGC-3'
Reverse primer: 5’-GGAGGGAGAAGCCAAAGTC-3'

Notch-4
Forward primer: 5’-AACTCCTCCCCAGGAATCTG-3'
Reverse primer: 5’-CCTCCATCCAGCAGGT-3'

Akt
Forward primer: 5’-CTTCGTGAACATTAACGACGGC-3'
Reverse primer: 5’-AATGGCCACCTGACTAAGAGTG G-3’ GAPDH
Forward primer: 5’-TGAAGGTAGAGTGCAACGGATTTG-3'
Reverse primer: 5’-CATGTGGCCATGAGGCT-3'.

Western Blot Analysis; for protein expression analysis, total protein was extracted according to the RIPA buffer protocol and detected by BCA kit. Western blotting was performed by using antibody (anti-akt1- Ab28422, anti-tubulin-Ab6160, anti-notch1- EP1238Y, hrp conjugated secondary antibody-Ab6721) and followed the Sambrook & Russel protocol for the detection of the expression of the proteins.

Statistical Analysis; All the data are presented as means ± SD and were analyzed by using SPSS 17.0 software. The significance of differences was assessed using one-way ANOVA combined with Student’s t-test (*p < 0.05; **p < 0.01; ***p < 0.001) and Pearson correlation.
RESULT

Cell characterization, viability and proliferation

Human breast cancer cells were grown to 70% confluence and treated with miltefosine for 24h the photographs were taken at 20X magnification using a phase-contrast inverse microscope (Nikon i-3 Eclipse). From the microscopic images it was observed that with increase in dose concentration from 0.5 μm to 10 μm, cell viability decreases. The miltefosine showed dose-dependent inhibition of cell proliferation. The absorbance was recorded at 540 nm by a microplate reader (BIORAD-680). From this we had calculated the IC-50 value which indicated that 1μm conc. of miltefosine is very effective against the breast cancer cells. The percentage viability was calculated by using the formula:

\[
\% \text{ Growth inhibition} = 100 - \% \text{Cell viability}^* 
\]

Where *% Cell viability = \[\text{OD of treated})/(\text{OD of control})\] X 100 (OD = optical density)

![Microscopic images of human breast cancer cells](image1)

Fig. 1: Microscopic examination of human breast cancer cell (MDA-MB 231) concentration range (0.5 μm/ml to 5 μm) against control cells for 24 hours.

MTT assay

![MTT assay graphs](image2)

Fig.2 Anti-proliferative effect of miltefosine (24 h, 48 h, 72 h) against human breast cancer cell line MDA-MB -231.
Fig.3 Anti-proliferative effect of miltefosine (24 h, 48 h, 72 h) against human breast cancer cell line MCF-7.

Detection of morphological apoptosis with Hoechst 33342 staining

Cells stained with Hoechst 33342 at a final concentration of (0.5µg/ml) in PBS and under which the brightly stained cells shown the condensed nuclei with characteristic features of apoptotic cells inverted microscope, the morphology of the cells shows a complete loss and blebbed which is identical to the apoptotic cells at different conc. of miltefosine. As the dose of miltefosine increased the apoptotic cells were found be more in higher conc. of drug as shown in fig 4. & 5.

Fig. 4: Morphological apoptosis with Hoechst 33342 staining in MCF-7 at concentration .5 µM, 1µM, 5 µM, 10 µM and control.
Fig. 5 Morphological apoptosis with Hoechst 33342 staining in MDA-MB-231 at concentration .5 µM, 1µM, 5 µM, 10 µM and control.

Flow Cytometry analyzes Apoptosis (Annexin V)
Miltefosine treated breast cell lines were double stained with PI/ annexin V-FITC and quantified with flow cytometry. Treatment with drug induced apoptosis in breast cell line as shown in figure 6. Result obtained from FACS was analyzed by using FlowJo software, quadrant R5 show secondary necrotic cells, R6 late apoptotic cells, R7 early apoptotic cells, and R8 showed live cells, the result analyzed by t-test from where we got the p value 0.012. Miltefosine treatment allows maximum cell death in breast cancer cell line.

Fig. 6: Miltefosine causing apoptosis in breast cancer cells.

Anti-metastatic Property by Wound healing assay
In the wound-healing assay, HTPF dose-dependently significantly decreased the migration of the MDA-MB-231 cells compared with the untreated control (Fig. 7). Treatment with
miltefosine at 3h and 6h inhibited MDA-MB-231 cells migration significantly. The wound-healing assay suggested that miltefosine suppresses the migration of breast cancer cells.

![Images showing wound healing assay results](image)

**Fig. 7:** Anti-metastatic effect of miltefosine significantly decreased the migration of the MDA-MB-231 cells in a dose-dependent manner compared with the untreated control. Treatment with miltefosine at 3h, 6h and 12h inhibited MDA-MB-231 cells migration significantly.

**Miltefosine target the notch protein (RT-PCR &Western Blotting)**

Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems™, USA) instrument was used for the quantitative gene expression study by using SYBR- Green chemistry. All reactions were carried out in duplicate in a 10 µl volume per well with No Template control (NTC). Beta Actin (β-actin) was used as endogenous control. Amplification condition for PCR reaction were: 50°C for 20 s, 95°C for 10min, followed by 40 cycles of 95°C for 15s, and 60°C for 1min. Dissociation curve was generated to distinguish specific amplicon from unspecific amplicon. RT-PCR result shows that there is decrease in expression level of notch family and Akt1 by miltefosine as shown in fig 8.

Western blot analysis was performed by total protein extraction using the Lysis buffer (Pierce, Rockford, IL, USA) and quantified through the Bradford method. Each 50-µg protein sample was separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), and the membranes were incubated with antibodies against (1:500 dilution, Cell Signaling Technology) AKT and anti-tubulin overnight at 4°C. After
incubation, the bound proteins were determined by ECL (Pierce) using a Bio Imaging System (UVP Inc., Upland, CA, USA). The result of western blot shows that by increasing concentration of miltefosine there is significantly decrease in expression level of AKT and Notch1 as shown in fig 9.

![Western blot result showing the role of miltefosine on breast cancer cell line.](image)

**Fig. 9:** Western blot result shows that role of miltefosine on the breast cancer cell line.

DISCUSSION

Several modes of action of miltefosine and the structurally related alkyl lysophospholipids including direct cytotoxic activity\(^{17}\), inhibition of phosphatidylcholine biosynthesis\(^{18}\),
induction of differentiation\textsuperscript{[19]} and interference with signal transduction processes by inhibition of the PI3K/Akt /PKB pathway have been discussed. All these mechanisms lead to reduced cell survival or increased apoptosis mediated through inducing intracellular stress (reactive oxygen species) by blocking essential survival signals or by inducing various pro-apoptotic cells signalling pathways.\textsuperscript{[20-23]} It also supports that miltefosine, as a known human PI3K/Akt inhibitor, may influence the susceptibility of host cell infection through this pathway. However, the precise mechanism of the tumour-inhibiting activity of miltefosine in breast cancer has not been explored. In the present study, our results revealed that miltefosine decreases the percentage of viable MCF-7, MDA-MB-231 breast cancer cells in a concentration-dependent manner that has not been reported in available literature. However, there was no accessible information regarding the effect of miltefosine on migration and invasion of MCF-7 and MDA-MB-231 breast cancer cells and associated signaling pathways. The results of wound-healing assay indicated that miltefosine decreases the migration and invasion of viable MDA-MB-231 and MCF-7 breast cancer cells and these effects were concentration-dependent. 0.5 µm to 10µm concentration of miltefosine was used in the present study and it was established that the 1um concentration is more effective to trigger the apoptosis in breast cancer cell lines. MTT assay result showed that the IC-50 value of drug was found to be effective at 1.0 µm concentration which had been further used throughout the study, and wound healing result indicate that after the treatment with miltefosine the migratory effect of breast cancer cells were inhibited significantly. The treatment of breast cancer cell with miltefosine exhibits significant inhibition of number of colonies formed when compared with untreated control whereas, the FACS results showed that there was significant reduction in the cell proliferation as shown in figure 6 where control consist of 1.22% early apoptotic cells in R7 quadrant whereas miltefosine induces 81.8% apoptosis in breast cancer cells . Quadrant R6 represent late apoptotic cells where control cell shows 1.4% apoptosis which is increased by 11.8 % in miltefosine treatment. It could be concluded that miltefosine has capability to induce the apoptosis in breast cell lines significantly. It has been widely recognized that increased Notch signaling is one of the main drivers of cellular malignancies in breast cancer. In recent years many domains had been in consideration to inhibit the Notch pathway in which the first inhibitor used clinically was γ-secretase which prevents release of NICD from plasma membrane by preventing cleavage of NEXT but it had limitations to provokes the MDR condition in breast cancer. Current preclinical experiments reveal that Notch pathway inhibitors were unlikely to be effective on their own, but they could significantly increase the efficacy of current therapies. RT-PCR
result showed that there is decrease in expression level of notch family (NICD, notch1, 2, 3, & 4) and akt-1 by miltefosine targeting, whereas the relative fold change indicates that there is significant down expression of notch1, 2, 3, &4 and akt1 genes by miltefosine (-3 to -10 RQ value on log 2 graph). On the other hand, the western result also indicates that there is decrease in protein expression level of notch-1, 2, 3, 4 and akt-1. The sensitivity of tumors to miltefosine is likely to depend on drug distribution (and the spatial distribution of clonogenic cells within the tumor) and intrinsic drug sensitivity which is an important and neglected cause of clinical resistance to chemotherapy. Yet studies are needed to document the paramount importance of role of miltefosine as anticancer agent in breast cancer cells. Present observations conclude that miltefosine might act as promising anti-cancerous drug for breast cancer as it has significant antiproliferative activity and has antimigratory property and the distribution and bioavailability of miltefosine in breast cancer cells is also found to be significant.

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**Conflict-of-interest disclosure**

The authors declare no conflicts of interest.

**Compliance with Ethical Standards**

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