Role of CBD exosomes in Triple negative breast cancer

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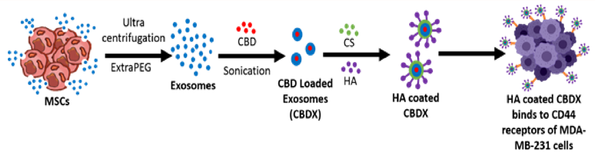
INTRODUCTION

- Breast cancer is the leading cause of cancer-related deaths in women across the world and around 10–15% of all diagnosed breast tumors are triple-negative breast cancer (TNBC) subtype.
- Till date there is no effective specific targeted therapy readily available for TNBC (1).
- Cannabinoids (Δ9-THC and Cannabidiol) exert their anti-cancer effects by regulating various signaling pathways related to proliferation, metastasis, angiogenesis, and differentiation (2, 3).
- Doxorubicin (DOX) is used for the management of TNBC but resistance remains as a concern (4,5).
- Excessive first pass metabolism, poor solubility and increased metabolism by CYP enzymes contribute to poor bioavailability of CBD and limit its clinical usage (6).
- Exosomes (EX) derived from human umbilical cord mesenchymal stem cells (hUCMSCs) are gaining tremendous attention due to their potential clinical applications in various diseases (7).
- EX derived from hUCMSCs also serve as a cell free therapy for regenerative medicine and also as potential delivery tools for various chemotherapeutics (8).

HYPOTHESIS



OBJECTIVES

- To investigate the effects of CBD in 3D spheroid cultures of MDA-MB-231 and MDA-MB-468 TNBC cell lines and organoid cultures
- To determine the novel potential targets affected by CBD treatment through performing next generation RNA sequencing and molecular biology studies
- To depict the possible molecular mechanisms responsible for improved sensitization of DOX by using CBD in TNBC cells
- To prepare and evaluate the anti-cancer effects of CBD loaded exosomes (i.e., exosomes derived from hUCMSCs) in triple negative breast cancer in-vitro and in vivo
- To demonstrate the efficacy of CBD exosomes in improving the sensitivity of doxorubicin (DOX) in triple negative breast cancer in-vitro and in vivo

MATERIALS AND METHODS

CBD (GLP and GMP grade) was purchased from PurisysTM (Athens, GA). DOX was purchased from AK Scientific, Inc (Union City, CA). Dulbecco's Modified Eagle Medium (DMEM) and DMEM/Ham's F12 (1:1 Mixture) media were acquired from Millipore Sigma (St. Louis, MO). TNBC (MDA-MB-231, MDA-MB-468) cells were purchased from ATCC (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Thomas scientific (Swedesborow, NJ).

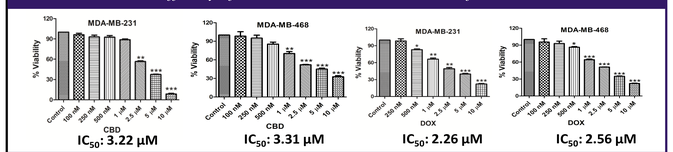
Next generation RNA sequencing was carried out by illumina NovaSeq 6000 system. MTT assay, Western blotting and Quantitative real-time PCR were performed to check cell viability, proteins expression, validation of mRNAs of various genes. ExtraPEG method was used for isolation of exosomes from hUCMSCs. Characterization of EV's (i.e., surface potential, particle size, particle size distribution and particles concentration) by using Nano Zetasizer (PSS) and ZetaView® QUATT- NTA Nanoparticle Tracking -Video Microscope PMX-420. CBD loaded exosomes were prepared by using sonoporation technique.

STATISTICAL ANALYSIS

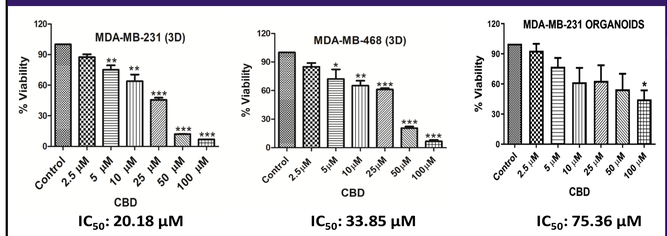
Data were expressed as mean ± S.E.M. Comparison of means between two groups was done using Student's t-test and multiple groups using ANOVA followed by Tukey's post hoc test using GraphPad Prism 5.01. Level of significance used was 5% and ***p<0.001, **p<0.01, *p<0.05 Vs control.

RESULTS

Anti-cancer efficacy of CBD and DOX in 2D cultures of various cell lines

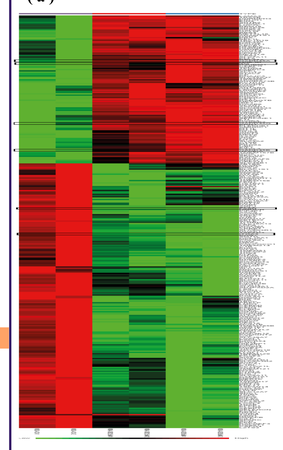


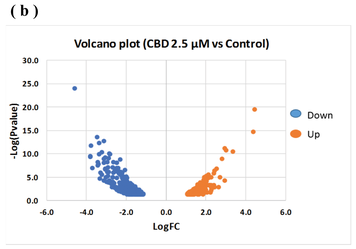
Anti-cancer efficacy of CBD in 3D cultures of various cell lines and organoid cultures



All values were expressed as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 significant vs control.

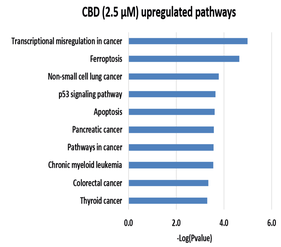
RNA Sequencing and KEGG pathway analysis in CBD treated MDA-MB-231 cells

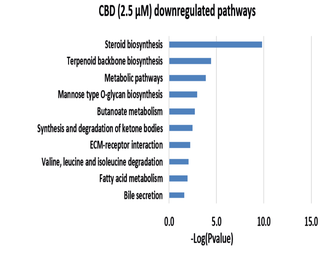




(c)

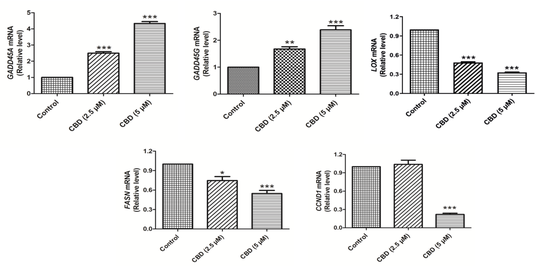
Compared groups	Total number of differentially expressed genes	Number of upregulated genes (FC ≥1)	Number of downregulated genes (FC≥1)
MDA-MB-231 (Control) Vs CBD (2.5 μM)	480	169	311





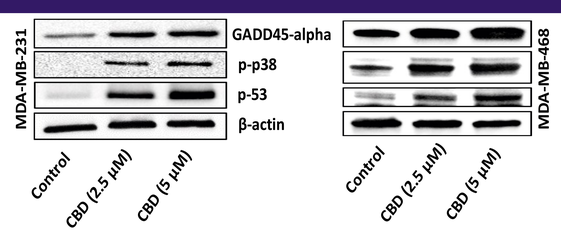
a) Heat map image of RNA sequencing data upon treatment of MDA-MB-231 cells with CBD treatment (n=2). b) Volcano plot showing up- (in orange) and downregulated (in blue) genes with CBD (2.5 μM) treatment in MDA-MB-231 cells when compared to control. c) Table showing the number of differentially regulated genes. d) Bar graphs showing KEGG pathway analysis of upregulated genes with CBD (2.5 μM) treatment in MDA-MB-231 cells. e) Bar graphs showing KEGG pathway analysis of downregulated genes with CBD (2.5 μM) treatment in MDA-MB-231 cells.

RT-qPCR validation of genes in CBD treated MDA-MB-231 cells



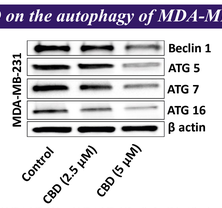
Quantitative real-time PCR of genes like GADD45A, GADD45G, LOX, FASN and CCND1 after treatment of MDA-MB-231 cells with CBD for 48 h. All values were expressed as mean ± S.E.M. *p < 0.05 **p < 0.01 ***p < 0.001 significant vs control.

CBD increased the protein expression of GADD45 alpha, p-p38 and p53 in MDA-MB-231 and MDA-MB-468 cells



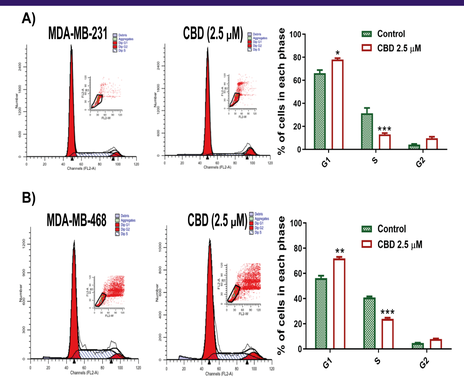
Western blots of GADD45-alpha, p-p38 and p53 in CBD treated MDA-MB-231 and MDA-MB-468 cells for 48 h. All values were expressed as mean ± S.E.M.

Effect of CBD on the autophagy of MDA-MB-231 cells



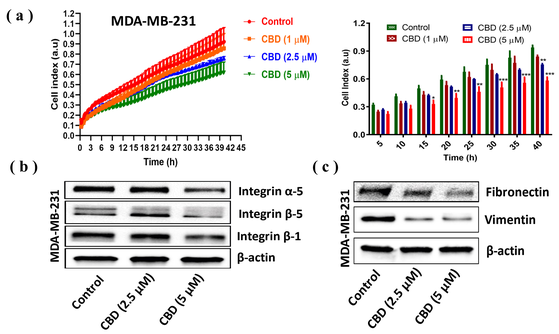
Western blots of GADD45-alpha, p-p38 and p53 in CBD treated MDA-MB-231 and MDA-MB-468 cells for 48 h. All values were expressed as mean ± S.E.M.

Flow cytometry of cell cycle analysis in MDA-MB-231 and MDA-MB-468 cells



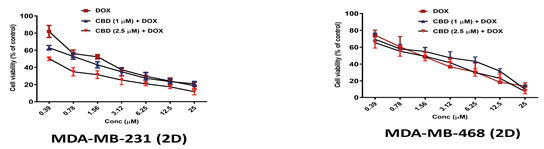
Flow cytometry of cell cycle analysis. A & B) Representation of the flow histograms and bar graphs showing cell cycle analysis after staining with propidium iodide (PI) in MDA-MB-231 and MDA-MB-468 cells treated with CBD (2.5 μM) and compared to control. All values are expressed as mean ± SEM (n=3). *p<0.05, ***p< 0.001 v.s Control.

Effect of CBD on the migration and the invasion of MDA-MB-231 cells



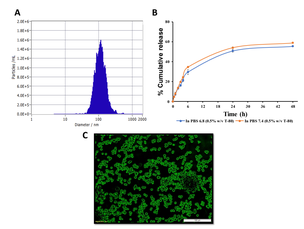
a) MDA-MB-231 cells treated with various concentrations of CBD were seeded in an xCelligence CIM-16 plate, and migration of the cells was monitored for 40 h and finally compared to untreated MDA-MB-231 cells; a.u represents arbitrary units. b) Effect of CBD on the protein expression of integrin-α5, integrin -β5 and integrin-β1 in 2D cultures of MDA-MB-231 cells. c) Effect of CBD on the protein expression of fibronectin and vimentin in 2D cultures of MDA-MB-231 cells.

CBD increased the sensitization of DOX in MDA-MB-231 and MDA-MB-468 cells



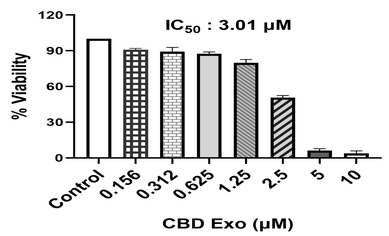
- IC_{50} values of DOX alone is 2.26 μM
- IC_{50} values of DOX in CBD (1 μM and 2.5 μM) pretreated MDA-MB-231 cells were found to be 952 nM and 300 nM respectively
- IC_{50} values of DOX alone is 2.56 μM
- IC_{50} values of DOX in CBD (1 μM and 2.5 μM) pretreated MDA-MB-468 cells were found to be 1.72 μM and 1.02 μM respectively

Synthesis and characterisation of CBD loaded exosomes

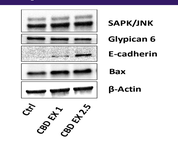


A) NTA analysis showing mean particle size distribution graph for CBD loaded exosomes B) *In-vitro* CBD release from exosomes in PBS at pH- 6.8 and 7.4 representing % cumulative CBD released Vs time, C) Fluorescently labelled exosomal uptake in MDA MB 231 cells.

Anti-cancer efficacy of CBD loaded exosomes in MDA-MB-231 cells

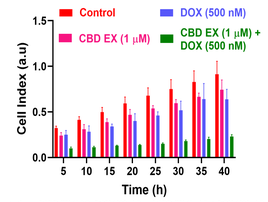


Effect of CBD exosomes on the migration and apoptosis of MDA-MB-231 cells



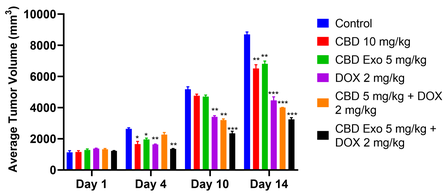
Western blots of SAPK/KNK, Glypican 6, E-cadherin and Bax in CBD EX treated MDA-MB-231 cells for 48 h. All values were expressed as mean ± S.E.M.

CBD exosomes and DOX combination decreased the migration of MDA-MB-231 cells



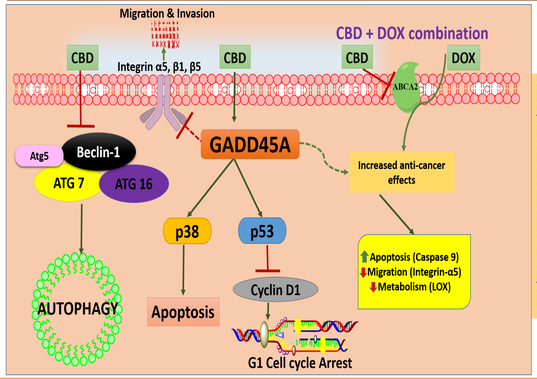
MDA-MB-231 cells treated with various concentrations of CBD EX (1 M), DOX (500 nM) and CBD EX (1 μM) + DOX (500 nM) were seeded in an xCelligence CIM-16 plate, and migration of the cells was monitored for 40 h and finally compared to untreated MDA-MB-231 cells; a.u represents arbitrary units.

Effect of CBD exosomes in an MDA-MB-231 Triple-Negative Breast Cancer Xenograft Model



All values were expressed as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 significant vs control.

Summary



CONCLUSIONS

- CBD decreases the proliferation and migration of MDA-MB-231 and MDA-MB-468 cells through activation of GADD45alpha/p38 signalling pathway mediated apoptosis.
- CBD induces G1 phase cell cycle arrest and downregulation of integrins-α5, -β5, -β1, fibronectin and vimentin.
- CBD inhibits autophagy in MDA-MB-231 and MDA-MB-468 cells.
- CBD increased the sensitivity of DOX in MDA-MB-231 and MDA-MB-468 cells, which might be due to CBD induced-decrease in ABCA2, autophagy and integrin beta-1 proteins
- The concept of CBD sensitization of DOX in TNBC can also be investigated in multiple types of cancers where anti-cancer drugs show resistance

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