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Novel mechanism of cannabidiol-induced apoptosis in breast cancer cell lines

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ABSTRACT

Studies have emphasized an antineoplastic effect of the non-psychoactive, phyto-cannabinoid, Cannabidiol (CBD). However, the molecular mechanism underlying its antitumor activity is not fully elucidated. Herein, we have examined the effect of CBD on two different human breast cancer cell lines: the ER-positive, well differentiated, T-47D and the triple negative, poor differentiated, MDA-MB-231 cells. In both cell lines, CBD inhibited cell survival and induced apoptosis in a dose dependent manner as observed by MTT assay, morphological changes, DNA fragmentation and ELISA apoptosis assay. CBD-induced apoptosis was accompanied by down-regulation of mTOR, cyclin D1 and up-regulation and localization of PPARγ protein expression in the nuclei and cytoplasmic of the tested cells. The results suggest that CBD treatment induces an interplay among PPARγ, mTOR and cyclin D1 in favor of apoptosis induction in both ER-positive and triple negative breast cancer cells, proposing CBD as a useful treatment for different breast cancer subtypes.

1. Introduction

Breast cancer is the most frequently diagnosed cancer in women worldwide. It is the most common cause of cancer-related mortalities among women in developing countries and the second most common cause of cancer-related mortalities among women in developed countries [1,2]. Breast cancer is a heterogeneous disease and is mainly classified, according to the molecular profile of the tumor, into three main subtypes: 1) Estrogen receptor (ER)-positive, which is positive for the biomarker ER alpha, 2) HER2, which is positive for Human Epidermal Growth Factor Two (HER2) and generally negative for ER and the progesterone receptor (PR) and 3) triple-negative breast cancer (TNBC), which is negative for ER, HER2 and PR [3–5]. Endocrine therapy is limited to patients with ER-positive tumors, yet, ~50% of the patients develop resistance to therapy and experience metastatic recurrence of the disease [6,7]. Triple negative tumors, on the other hand, are deficient for well-defined molecular targets making chemotherapy, which is non-specific and cytotoxic, the most common treatment option. Patients with TNBC tend to experience metastasis and early recurrence after treatment as well as lower survival compared to patients with other subtypes of breast cancer [8]. Hence, there is an urgent need for innovative therapeutic interventions to help women with breast tumors.

Cannabinoids are products of the natural plant Cannabis Sativa. They were first introduced as palliative medicinal products, aiding in reducing emesis resulting from chemotherapy for cancer patients [9]. The endocannabinoid system has been studied for its involvement in cancer development, including angiogenesis, apoptosis and metastases. This system has been known to comprise two main endocannabinoid receptors: CB1 that is mainly found in the nervous system, and CB2 that is expressed peripherally and in some central spots such as the brain [9,10]. Cannabidiol (CBD) is a non-psychoactive constituent of cannabis that has been studied for its antineoplastic actions shifting sights towards a new scope of anticancer treatments [11]. Recent studies have found that CBD induces apoptosis in cancer cells via CB1, CB2 and the ion channel receptor TRPV1 [12,13], while others found its apoptotic actions to be receptor independent [14]. CBD was found to induce interplay between apoptotic and autophagy-inducing signals in breast cancer

Abbreviations: CBD, Cannabidiol; ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human Epidermal Growth Factor Two; TNBC, Triple Negative Breast Cancer; IP, Immunoprecipitation; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; mTOR, mammalian target of rapamycin; PPARγ, Peroxisome Proliferator Activated γ.

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cell lines, resulting in a non-receptor mediated programmed cell death [14]. Interestingly, CBD has also been found to enhance the perfusion of chemotherapeutic drugs across barriers through inhibiting the expression of Breast Cancer Resistance Protein in a concentration dependent manner [15].

To understand the antitumor effect of CBD on ER-positive as well as triple negative breast cancer, we examined the effect of CBD on two different human breast cancer cells lines: the ER-positive T-47D and the triple negative MDA-MB-231 cells. Morphological changes of the cells were observed upon treatment with different doses of CBD. This was accompanied by inhibition of cell survival and induction of apoptosis as observed by DNA fragmentation, MTT and ELISA apoptosis assay. The protein expression of the oncogenic and pro-survival markers cyclin D1 and mTOR was inhibited, and the expression as well as nuclear localization of the differentiation marker PPARγ was enhanced. Taken together our results suggest an interplay among mTOR, cyclin D1, and PPARγ as a mechanism of CBD induced apoptosis in ER-positive and triple negative breast cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

Human breast cancer cell lines, T-47D and MDA-MB-231, were purchased from American Type Culture Collection (ATCC, LGC Promochem, Molsheim Cedex, France) and cultured in Roswell Park Memorial Institute (RPMI 1641) and Dulbecco's Modified Eagle's Medium (DMEM), respectively, supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/ml streptomycin. CBD was purchased from (Tocris Bioscience, Bristol, UK). mTOR rabbit polyclonal antibody, cyclin D1 mouse monoclonal antibody, PPARγ mouse monoclonal antibody were purchased from (Santa Cruz Biotechnologies, Inc, CA, USA).

2.2. Cell viability assay

Viability of cells was assessed using Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen Corporation, CA, USA). Briefly, T-47D and MDA-MB-231 cell lines were seeded at densities 10,000 cells per well and let to grow until reached the optimal densities within 48 h. Cells were plated in 96-well plates in complete RPMI-1640 and DMEM medium, respectively then cells were treated with (1–7) μM CBD in serum-free media, while mock cells were cultured in media free serum. After 24hrs, the medium was removed and replaced with 100 μL of the fresh culture medium and then 10 μL of 12 mM MTT stock solution was added to each well. The negative control contained 10 μL of the MTT stock solution and 100 μL of medium alone was incubated at 37 °C for 4 h in the 96-well plate. After that, 100 μL of the prepared SDS-HCl solution were added to each well and mixed thoroughly. The micro plate was incubated at 37 °C for 18 h in the CO_2 incubator. Each sample was mixed again, using a micropipette; the absorbance was read at 545 nm using a micro-plate ELISA reader (Enzyme-linked immunosorbent assay reader, Stat Fax 2100, USA). The IC_{50} of CBD was determined by sigmoidal curve construction.

2.3. Apoptosis assay

Apoptosis induction was quantified using Cell Death Detection ELISA PLUS kit (Roche-Applied Science, Indianapolis, USA). Briefly, cells were seeded at a density 2×10^4/well in a 96 well plate and incubated for 24 h. Treated with 3 and 5 μM CBD for 24 h. Cells were lysed with 2,2′-azino-di (3-ethylbenzthiazolin-sulphuric acid and incubated at room temperature for 2 h with histone biotin and anti-DNA peroxidase, according to manufacturer protocol. The absorbance was measured at 405 nm using ELISA reader (Spectra Max Plus) (Molecular Devices, LLC, Sunnyvale CA, USA).

2.4. Morphological studies

The morphological changes of cells were examined before and after treatment with CBD. Equal number of cells/well was seeded in 12 well plates in DMEM or RPMI medium with different concentrations of CBD, and incubated for 24 h. Cells were washed with PBS, fixed with 10% formalin buffer and examined by an inverted microscope with magnification 400× (Inverted Microscope, Optika, Italy). Digital images were taken, using Kodak microscopic digital camera.

2.5. DNA fragmentation assay

T-47D and MDA-MB-231 cells were cultured in RPMI-1640 and DMEM medium, respectively then cells were treated with (0–5) μM CBD in serum-free media, while mock cells were cultured in media free serum. Trypsinize adherent cells were treated with 1 ml Trypsin/EDTA to detach the cells. Cells were centrifuged for 5 min at 1500 rpm. Cells were resuspend in 1.5 ml ice cold PBS and were centrifuged for 5 min at 1500 rpm and the pelleted cells were resuspended in 200 μL digestion buffer supplemented with proteinases k inhibitor (Bio Basic Inc., NY, USA). Cells in tightly capped tubes were incubated at 55 °C for 18 h with shaking. Samples were extracted with an equal volume of Phenol/Chloroform/Isoamyl alcohol and centrifuged at 4000 rpm for 10 min. Half volume of 3 M ammonium acetate and double volume of 100% ethanol were added to the aqueous solution and centrifuged for 2 min at 4000 rpm. The precipitated DNA samples were washed with 70% ethanol. After centrifugation, samples were air dried, and resuspended in TE buffer. The DNA samples were separated using 1% agarose gel electrophoresis followed by staining with ethidium bromide for visualization under UV (Horizontal Gel Apparatus, Varigel, England).

2.6. Western blot analysis

Cells were plated in 25 cm^2 flask and incubated for 48 h under cell culture conditions (37 °C, 5% CO_2) and were treated with (0–7) μM CBD for T-47D and (0–5) μM CBD for MDA-MB-231. Radio Immunoprecipitation Assay (RIPA) buffer was used for whole cells and also for membrane-bound proteins extracts. Samples are kept on ice at all times and proteinase inhibitors and PMSF were added freshly to the lysis buffer. The tested concentrations were determined according to the MTT assay and IC_{50}. After treatment, the cells were collected and washed with ice-cold PBS buffer, then the PBS was drained, and add ice-cold lysis "RIPA buffer" was added at a concentration of 1 ml per 10^7 cells/100 mm dish/25 cm^2 flasks. Then adherent cells were rapidly scraped using a cold plastic cell scraper, and gently transferred the cell suspension into cooled microfuge tubes and maintained at a constant agitation for 60 min at 4 °C. After that, the cells were centrifuged at 1500 rpm or 15 min. The supernatant were aspirated carefully and placed on ice.

Protein lysates of both T-47D or MDA-MB-231 cell lines were separated, using 7% and 10% polyacrylamide gel according to the molecular weights of the targeted proteins of interest, and transferred onto 0.45-mm nitrocellulose membranes (Bio-Rad Laboratories). The membrane was probed with specific rabbit polyclonal anti-m-TOR, mouse monoclonal anti-Cyclin D1, and mouse monoclonal anti-PPARγ as primary antibodies with concentration of (1:1000). After washing of the membrane with TTBS, it was incubated with their respective secondary antibodies. The transferred

proteins were detected using 3, 3′, 5, 5′ tetramethylbenzidine (TMB) ((Sigma-Aldrich Corp. St. Louis, MO USA) in a dark room for 15 min. Membranes were stripped using blot stripping buffer [Thermo Scientific Restore™ (Thermo Scientific, lL, USA)] and re-probed with anti- β-actin as a control for equal loading.

2.7. Immunoprecipitation

MDA-MB-231 cells were lysed on ice in lysis buffer were gently shacked overnight with an anti mTOR, rabbit polyclonal antibody and anti PPARγ mouse monoclonal antibody with concentration of (1:1000) at 4 °C. The antigen–antibody complexes were extracted via Protein A/G Agarose Plus beads (Santa Cruz Biotech., Dallas, Texas, U.S.A). Immunoprecipitation (IP) products were boiled in Laemmle Sample Loading Buffer (BioRad) and analyzed by Western blot as explained above.

2.8. Immunocytochemistry

T-47D and MDA-MB-231 cells were cultured to be sub-confluent while incubated in 37 °C and then treated with CBD compound for 24 h with the indicated concentrations in culture medium containing fetal bovine serum. Cells were fixed with 2% formalin and incubated for 15 min at room temperature then incubated with Triton ×100 for 20 min at room temperature (Oxford lab. Mumai India) and blocked with Ultra V blocking and incubated for 5 min at room temperature (Thermo Scientific, UK). Cells were fixed on slides and incubated with 1:50 anti-PPARγ mouse monoclonal antibody at 4 °C and were detected, using Pierce Peroxidase IHC Detection Kit (Thermo Scientific, lL, USA). The stained cells were mounted in DPX (Biostatin Ready Reagents Ltd, Manchester,UK) and observed under an light microscope at ×400 magnification.

2.9. Assay of protein content

Protein concentrations were determined using “Bicinchoninic Acid, BCA” (Pierce Biotechnology, Rockford, USA) according to manufacturer's protocol. Briefly, the working reagent of BCA was prepared by adding 50 part of reagent A to 1 part of reagent B, mix gently and was keep at room temperature until use. A serial dilution of Bovine Serum Albumin (BSA) (0–2000 μg/ml) was prepared to set standard curve. 2 ml of working reagent was added to 0.1 ml of unknown protein sample and standards, and incubated at 37 °C for 30 min. The absorbance was measured at 546 nm (ELISA reader, Stat Fax 2100, USA).

2.10. Statistical analysis

Statistical analysis was performed using Graphpad prism 6 software (San Diego, CA). All data represented as mean ± SEM, and results were analyzed using one-was ANOVA. $P < 0.05$ * was considered statistically significant. All experiments were repeated at least three times. Relative band density of western blot was performed using Quantity One analysis software (Bio-Rad).

3. Results

3.1. Cannabidiol inhibits the viability of breast cancer cells

In order to study the effect of CBD on breast cancer cells, T-47D and MDA-MB-231 cells were treated with different doses of CBD. After 24 h of treatment, significant morphological changes were observed starting from 5 μM to 3 μM CBD in T-47D and MDA-MB-231 cells, respectively. CBD treated cells became rounded up, lost contact with neighboring cells, and were more easily detached. The cells exhibited cytoplasmic condensation, shrinkage, tendency to float in the medium, and reduction in size compared to the mock cells (Fig. 1A and B).

To investigate the viability of the cells upon treatment with CBD, MTT assay was performed. The results revealed that CBD inhibits the viability of both cell lines in a dose dependent manner (Fig. 2). The sensitivities of the two cell lines to CBD induced cell death varied. IC50 was calculated showing doses of 5 μM and 2.2 μM for T-47D and MDA-MB-231 cells, respectively. Thus, the TNBC MDA-MB-231 cells are more sensitive to CBD compared to the ER-positive T-47D cells. Taken together CBD induces morphological changes and inhibits the viability of ER-positive and triple negative breast cancer cells in a dose dependent manner.

3.2. CBD induces apoptosis

It has been reported that CBD induces programmed cell death in breast cancer cells [14]. Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis [16]. To find out if the observed inhibition of cell viability, upon CBD treatment, is due to apoptosis, DNA fragmentation assay was performed. T-47D and MDA-MB-231 cells were treated with different concentrations of CBD. In both cell lines, treated cells exhibited DNA fragmentation compared to mock (Fig. 3) and the fragments observed were oligonucleosome sized.

In order to find out if the observed fragmentation is due to apoptosis, Cell Death Detection ELISA PLUS, which is a photometric enzyme immunoassay used for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after apoptotic induced cell death, was used. T-47D and MDA-MB-231 cells were treated with 5 μM and 3 μM, respectively. The doses were chosen depending on the IC50 results obtained for each cell line (Fig. 2). Fig. 4 represents histone release in T-47D and MDA-MB-231 cells treated with CBD in comparison to mock cells. CBD treatment significantly enhanced histone release in both cell lines. Thus, the observed inhibition of cell survival upon CBD treatment is due to apoptosis.

3.3. Cannabidiol inhibits protein expression of mTOR and cyclin D1 in breast cancer cells

Mammalian Target of Rapamycin, known as mTOR, is a serine-threonine kinase that functions as a principle coordinator of cell proliferation and survival [17,18]. It has been reported that CBD inhibits oncogenic mTOR phosphorylation as well as the protein expression of the cell cycle regulator, Cyclin D1, in MDA-MB-231 cells [14]. In order to investigate the effect of CBD on mTOR and cyclin D1 protein expression, T-47D and MDA-MB-231 cells were treated with different concentrations of CBD for 24 h. Protein expression of mTOR was determined using Western Blotting and IP in T-47D and MDA-MB-231 cells, respectively (Fig. 5A and B). In both cell lines, CBD inhibited mTOR expression levels in a dose dependent manner compared to mock. Consistent with the previously reported results in MDA-MB-231 cells [14], CBD inhibited the protein expression of Cyclin D1 in T-47D cells (Fig. 5C). Taken together, CBD inhibits the pro-survival and oncogenic mTOR and cyclin D1 protein expression in ER-positive and triple negative breast cancer cells. The results suggest a role of both mTOR and Cyclin D1 in CBD-induced apoptosis.

3.4. Cannabidiol enhances protein expression and nuclear localization of PPARγ in breast cancer cells

It has been reported that Peroxisome Proliferator-Activated Receptor Gamma (PPARγ), a member of the nuclear receptor

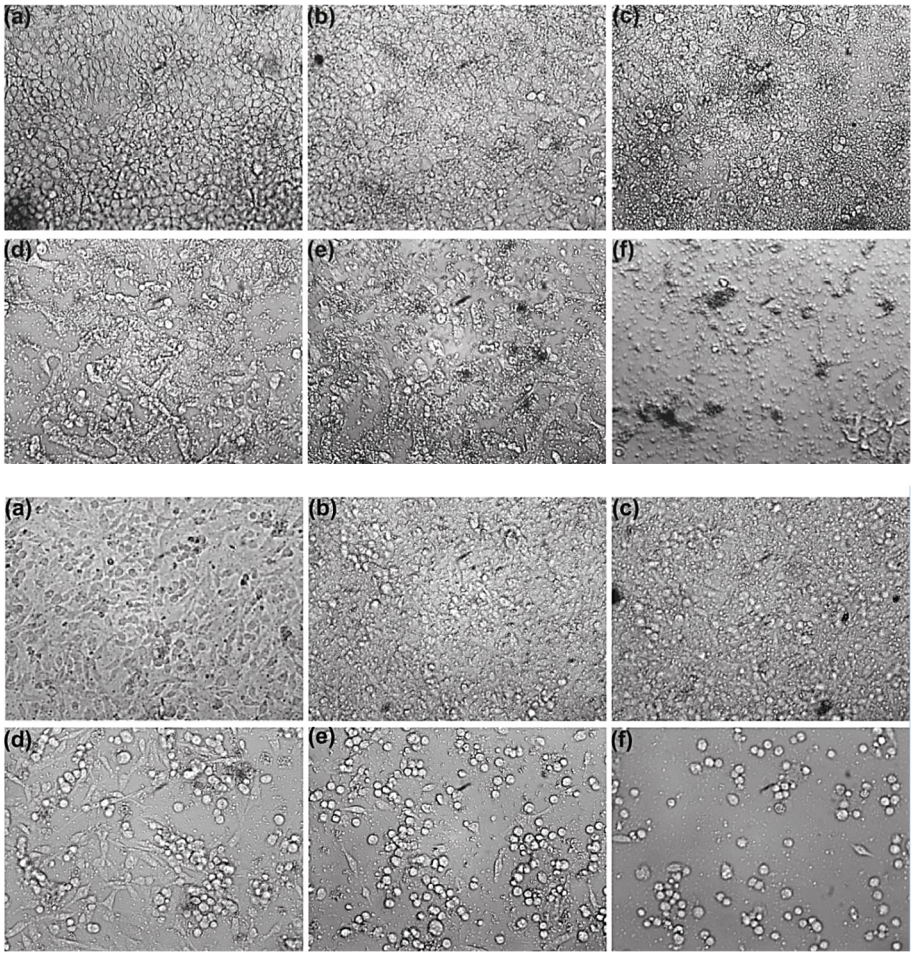

Fig. 1. Cannabidiol induces morphological changes in breast cancer cells. (A) T-47D cells were treated with different concentrations of CBD for 24 h: (a) Mock, (b) 1 μM CBD, (c) 3 μM CBD, (d) 5 μM CBD, (e) 7 μM CBD, (f) 10 μM CBD. (B) MDA-MB-231 cells were treated with CBD at different concentrations for 24 h: (a) Mock, (b) 1 μM CBD, (c) 2 μM CBD, (d) 3 μM CBD, (e) 5 μM CBD, and (f) 7 μM CBD. (A, B) Cells were washed with PBS and fixed with formalin then examined at 200× using an inverted microscope. The experiment has been performed in triplicates.

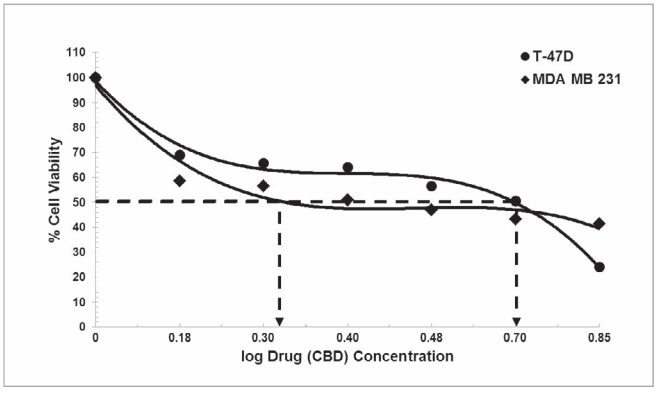

Fig. 2. Cannabidiol inhibits the survival of breast cancer cells. Cells were treated with different doses of CBD (1, 1.5, 2, 2.5, 3, 5, 7 μM) for 24 h. Percentage cell viability was calculated compared to mock. The calculated anti-log concentration signifies CBD IC50 of 5 μM (anti-log 0.70 = 5 μM) for T-47D and IC50 of 2.2 μM (anti-log 0.34 = 2.2 μM) for MDA-MB-231 cells.

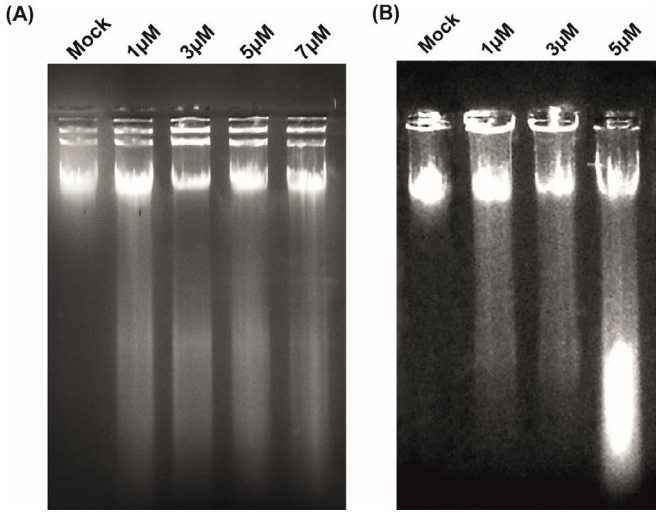

Fig. 3. Cannabidiol induces DNA fragmentation in breast cancer cells. T-47D and MDA-MBA-231 cells were treated with CBD (1–7 μM) and (1–5 μM), respectively for 48 h, then DNA fragmentation was detected using agarose gel electrophoresis. The figures are representative of three independent experiments.

superfamily, mediates CBD-induced apoptosis in human lung cancer cells [19]. To investigate the effect of CBD on PPARγ protein expression, cells were treated with different concentration of CBD

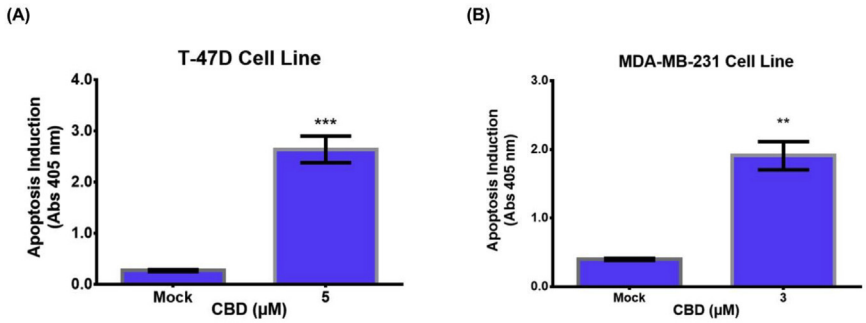

Fig. 4. Cannabidiol induces apoptotic cell death in breast cancer cells. T-47D and MDA-MB-231 cells were treated with 5 μM and 3 μM CBD, respectively, for 24 h (**$p < 0.01$ and ***<0.1 compared to the mock using the unpaired Student t-test). Error bars represent standard error. Each data point was an average of results from three independent experiments performed in triplicates.

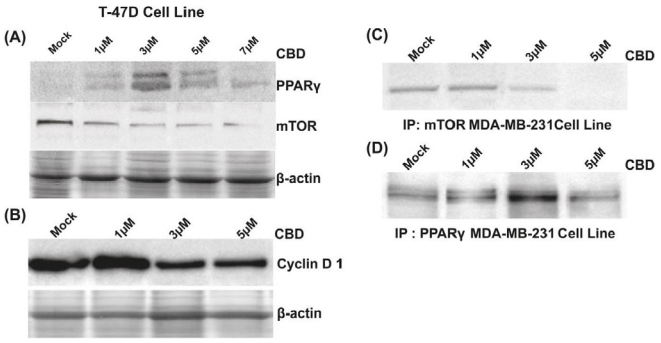

Fig. 5. Cannabidiol down-regulates mTOR and Cyclin D1 and up-regulates PPARγ protein expression levels in breast cancer cells. (A, C) T-47D cells were treated with different concentrations of CBD for 24 h. Whole cell extract was prepared, and m-TOR, Cyclin D1 (A) and PPARγ (C) expression was detected by Western Blotting. (B, D) MDA-MB-231 cells were treated with different concentrations of CBD for 24 h. Cells were lysed with lysis buffer, and mTOR (B) and PPARγ (D) were immunoprecipitated and then subjected to Western Blotting.

for 24 h and the protein expression was estimated using Western Blotting and IP for T-47D and MDA-MB-231 cells, respectively. CBD treatment enhanced PPARγ protein expression in T-47D and MDA-MB-231 cells respectively (Fig. 5A and D). Nuclear and cytoplasmic localization of PPARγ were also investigated in T-47D and MDA-MB-231 breast cancer cell lines respectively, using immunocytochemistry as described under materials and methods section. CBD treatment enhanced PPARγ expression and localization in the nuclei of the treated cells compared to untreated control that showed very low to no detected PPARγ expression in both tested cell lines, T-47D (Fig. 6; upper panel) and MDA-MB-231 (Fig. 6; lower panel), suggesting a role of the transcription factor PPARγ in CBD-induced apoptosis of ER-positive and triple negative breast cancer cells.

4. Discussion

As the need for new innovative antineoplastic drugs is growing, studies have found Cannabis Sativa extracts, the cannabinoids, to possess anticancer activity [9,10,14,20,21]. The present study was performed to investigate the effect of CBD, a phtyo-cannabinoid, on two types of human breast cancer cell lines: 1) the well differentiated ER-positive T-47D cells and 2) the poorly differentiated highly invasive triple negative MDA-MB-231 cells. CBD was found to inhibit cell survival and induce apoptotic cell death in both cell lines. This was concomitant with the down-regulation of mTOR and Cyclin D1 and the up-regulation of PPARγ protein expression levels. Both cell lines have shown enhanced nuclear localization of PPARγ upon treatment with CBD. Together these findings propose CBD as a useful treatment for ER-positive as well as triple negative breast cancer and suggest a novel mechanism underlying CBD-induced apoptosis.

Our findings demonstrate that CBD inhibits the viability of both T-47D and MDA-MB-231 cells. The observed inhibition is in agreement with previous studies reporting that CBD selectively inhibits the viability and induces apoptosis in ER-positive (MCF-7 and ZR-75-1) and ER-negative (MDA-MB-231 and SK-BR-3) cells [16], and that CBD inhibits the growth of MDA- MB-231 human breast cancer cell line xenografts [21]. Apoptosis is marked at its early stages with cell size loss [16]. This is consistent with our data where the treatment with CBD conferred morphological changes and gradual loss of size in a dose dependent manner. The monolayer cells became rounded up, shrank and exhibited cytoplasmic condensation as well as loss of contact with neighboring cells. The loss of chromatin compaction and its degradation are regarded as long-standing hallmarks for apoptosis where free histones and DNA particles are released as nucleosomes fall apart during the process [16,22]. Our data reveal that CBD induces DNA fragmentation in a dose dependent manner. Moreover, CBD induces the release of histones as detected by ELISA in T-47D and MDA-MB-231 cells. Per our knowledge, CBD-induced apoptosis in T47-D cells is reported for the first time in the present study. Interestingly, MTT assay revealed that the IC50 for MDA-MB-231 and T-47D cells were 2.2 μM and 5 μM, respectively. Thus, TNBC MDA-MB-231 are more sensitive to CBD compared to the ER-positive T-47D cells.

Mammalian Target of Rapamycin, known as mTOR, is a kinase that integrates intracellular and extracellular signals mediating the activity of growth factor receptors and functioning as a principle coordinator of metabolism, growth, proliferation and survival [17,18]. A crucial signaling node, mTOR is often deregulated in human cancer including breast cancer [18,23,24], and the use of mTOR inhibitors has been investigated in the clinic [18,25,26]. In breast cancer, mTOR expression correlates for worse prognosis [23], and studies reveal that the specific mTOR inhibitor, Rapamaycin, induces apoptosis in breast cancer cells [27,28]. CBD has been reported to inhibit mTOR phosphorylation in MDA-MB-231 cells [14]. In the present study, we report that CBD exerts an anti-survival effect through the inhibition of mTOR protein expression in both T-47D and MDA-MB-231 breast cancer cell lines.

mTOR activation controls cell growth via the m-TOR dependent cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E (eIF4E) pathways [29]. Cyclin D1, a key regulator of the G1/S transition of the cell cycle [30], is downstream of eIF4E

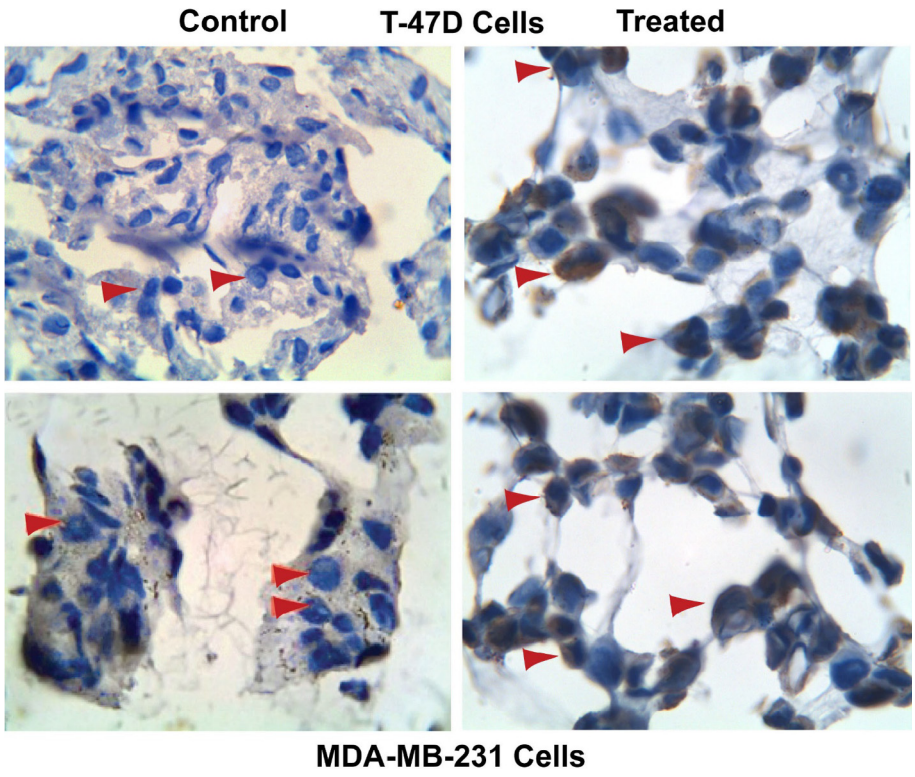

Fig. 6. Cannabidiol enhances the expression of nuclear and cytoplasmic PPARγ in breast cancer cells. T-47D cells (upper panel) and MDA-MB-231 cells (lower panel) that were treated with (3 μM) CBD (right panel) for 24 h compared to untreated control (left panel). Nuclei pointed by red arrows showing differences between mock and treated cells brown staining of stained protein vs counter staining blue stain. Images were taken using light microscope at 1000×.

in the AKT/mTOR/4EBP1 pathway [31]. It has been reported that mTORC signaling regulates the protein expression of cyclin D1 in ER-positive MCF-7 breast cancer cells [32]. Cyclin D1 is required for oncogene-induced tumorigenesis in breast cancer. Studies reveal that cyclin D1 plays an important role in cell survival and induces resistance against apoptosis through the reduction of the expression of pro-apoptotic protein, Bax [33]. Interestingly, it is reported that CBD induces significant decrease in cyclin D1 protein expression in MDA-MB-231 cells [14]. Thus, we examined the effect of CBD on cyclin D1 protein expression in T-47D cells. Our results demonstrate that CBD significantly inhibits cyclin D1 protein expression in T-47D cells. This suggests that CBD inhibits both mTOR expression and signaling in ER-positive and triple negative breast cancer cells.

Interestingly, the down-regulation of both cyclin D1 and mTOR, upon treating the cells with CBD, occurred simultaneously with the up-regulation of Peroxisome Proliferator-activated Receptor Gamma (PPARγ) protein expression in both cells lines. PPARγ is a ligand binding transcription factor that belongs to the nuclear receptor superfamily and functions by regulating gene transcription via binding to consensus DNA sequences located in the promoter regions of target genes. PPARγ plays a role in different biological processes, such as cell differentiation, proliferation as well as apoptosis [34]. Studies report that PPARγ ligands inhibit the proliferation and induce apoptosis in human breast cancer cells [14,35,36]. PPARγ was also found to stop the progression of 7,12-dimethylbenz[a]anthracene-mediated breast tumors in vivo in PPARγ haploinsufficient mice [37]. Moreover, ligand activated-PPARγ has been found to induce differentiation and reduce malignancy, growth rate and clonogenic capacity of human breast cancer cells [38].

It is reported that CBD bind to and activate PPARγ in rat aorta [37]. CBD has also been shown to bind with PPARγ in 3T3-1L fibroblasts and increase its transcriptional activity leading to the differentiation into adipocytes [39]. In human lung cancer cells, CBD up-regulates mRNA and protein expression of PPARγ and COX-2. CBD thus enhances the levels of COX-2-dependent prostaglandins causing translocation of PPARγ to the nucleus and inducing PPARγ-dependent apoptosis [19]. Our results indicate that CBD enhances the protein expression and nuclear localization of PPARγ in ER-positive and triple negative breast cancer cells, suggesting that CBD activates PPARγ which then plays a role in the CBD-induced apoptosis. The induction of PPARγ is associated with cyclin D1 inhibition, suggesting a correlation between the two proteins, as supported by several published studies [40–42]. In MCF-7 cells, PPARγ agonists resulted in concentration and time-dependent decrease in Cyclin D1 and ER protein expression and this was accompanied by the inhibition of cell proliferation and G_1-G_0 to S phase progression of the cell cycle [40]. Cyclin D1, on the other hand, represses binding of PPARγ ligands, transactivation, promoter activity, and expression [41]. It was demonstrated that cyclin D1-deficient mouse embryonic fibroblasts possess increased tendency for adipocytes differentiation; this tendency is liable for reversal through the reintroduction of cyclin D1 to the cells [41,42].

Hence, there is a strong evidence that there is a crosstalk between mTOR, Cyclin D1, and PPARγ, aiding cancer cells through their survival mechanism. In the present study, we propose that in both ER-positive and triple negative breast cancer cells, CBD enhances and activates protein expression of PPARγ, leading to inhibition of protein expression and activity of mTORC, which in turn reduces the protein expression of Cyclin D1 and alleviates the inhibitory effect that possesses over PPARγ transcriptional activation (Fig. 7).

All in all, we have established the anticancer effect of CBD on

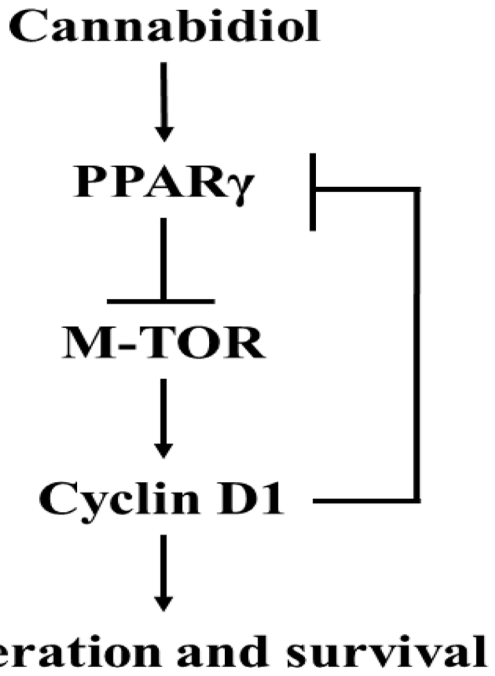

Fig. 7. Proposed mechanism of CBD-induced apoptosis in breast cancer cells. Treating breast cancer cells with CBD up-regulates the protein expression of PPARγ and activates the receptor. PPARγ then inhibits protein expression and activity of mTORC. This results in the down-regulation of protein expression of Cyclin D1 and hence alleviates its inhibitory effect on PPARγ and consequently increases PPARγ transcriptional activity.

two types of human breast cancer cell lines, T-47D and MDA-MB-231. CBD induces apoptosis in breast cancer cells, and the results suggest an interplay between different cancer-related signals (mTOR, cyclin D1, and PPARγ) as a mechanism underlying CBD-induced apoptosis.

Conflict of interest disclosure statement

The authors have no financial conflicts to disclose.

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