Cannabidiol induces programmed cell death in breast cancer cells by coordinating the crosstalk between apoptosis and autophagy

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Running title: CBD induces programmed cell death in breast cancer cells.

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Abbreviations: CBD, cannabidiol; FACS, fluorescence-activated cell sorter; z-VAD-fmk, z-Val-Ala-Asp-fluoromethyl ketone; LC3, microtubule-associated protein light-chain 3; BAF, bafilomycin A1; ER stress, endoplasmic reticulum stress; mTOR, mammalian target of rapamycin; CB, cannabinoid binding receptors; PCD, programmed cell death; PARP, poly ADP ribosyl polymerase; caspases (cysteine-dependent aspartate-directed proteases)

Abstract:

Cannabidiol (CBD), a major non-psychoactive constituent of cannabis, is considered an anti-neoplastic agent based on its *in vitro* and *in vivo* activity against tumor cells. However, the exact molecular mechanism through which CBD mediates this activity is yet to be elucidated. Here, we demonstrated CBD-induced cell death of breast cancer cells, independent of cannabinoid and vallinoid receptor activation. Electron microscopy revealed morphologies consistent with the coexistence of autophagy and apoptosis. Western blot analysis confirmed these findings. We demonstrated that CBD induces ER stress, and subsequently inhibits AKT and mTOR signaling as shown by decreased levels of phosphorylated mTOR and 4EBP1, and Cyclin D1. Analyzing further the crosstalk between the autophagic and apoptotic signaling pathways, we found that Beclin1 plays a central role in the induction of CBD-mediated apoptosis in MDA-MB-231 breast cancer cells. Namely, while CBD enhances the interaction between Beclin1 and Vps34, it inhibits the association between Beclin1 and Bcl-2. In addition, we showed that CBD reduces mitochondrial membrane potential, triggers the translocation of BID to the mitochondria, the release of cytochrome c to the cytosol, and ultimately, the activation of the intrinsic apoptotic pathway in breast cancer cells. CBD increased the generation of reactive oxygen species (ROS), and ROS inhibition blocked the induction of apoptosis and autophagy. Our study revealed an intricate interplay between apoptosis and autophagy in CBD-treated breast cancer cells and highlights the value of continued investigation into the potential use of CBD as an anti-neoplastic agent.

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Introduction:

Breast cancer is the second leading cause of cancer-related death in women in the United States (1). Conventional treatment options are often limited by toxicity or acquired resistance, and novel agents are needed. We analyzed the effects of the *Cannabis sativa* constituent, cannabidiol (CBD), a potent, natural compound with reported activity in breast cancer cell lines, and elucidated its effects on key neoplastic pathways.

Cannabidiol belongs to the cannabinoid family, a group of pharmacologically active compounds that bind to specific G-protein-coupled receptors (2). Phytocannabinoids are plant-derived products from *Cannabis sativa*; endogenous cannabinoids are made in animal and human tissues; and synthetic cannabinoids are laboratory-produced. The G-protein-coupled receptor, CB1, is found mainly in the brain and nervous system, while CB2 is expressed predominantly by immune cells (3). Recent data suggest that some cannabinoids also signal through the vallinoid receptor (4), while others may function in a receptor-independent manner (3). Cannabinoids can modulate signaling pathways central to the growth and spread of cancer. They inhibit cell cycle progression and chemotaxis, and block angiogenesis (5). Recent studies demonstrate that cannabinoids also induce autophagic cell death (6). Δ^9-tetrahydrocannabinol (THC) is one of the best-characterized cannabinoids, however, its therapeutic applications are limited by its psychoactive effects. We focused our work on CBD, a phytocannabinoid devoid of these properties (3).

While CBD is reportedly effective against various tumors, its molecular mechanism of action is not fully characterized. CBD is cytotoxic to gliomas and inhibits tumor cell migration *in vitro* (7-9). In addition, CBD induces apoptosis in human leukemia cell lines

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by activating classical caspase pathways, and enhancing NOX4 and p22 (PHOX) function (10). A recent study reports that CBD inhibits breast cancer growth (11) and downregulates *ID1*, a regulator of metastasis in breast cancer cell lines (12). Furthermore, CBD, in conjunction with THC, induces programmed cell death (PCD) in glioma cells (13).

PCD, a cell suicide program critical to development and tissue homeostasis, can be classified according to the morphology of a dying cell. Apoptosis is a type I PCD involving caspase activation, phosphotidyl serine inversion and DNA fragmentation (14). More recently, autophagy, a process traditionally considered a survival mechanism, was also implicated as a mode of PCD, when excess de novo-synthesized, double membrane-enclosed vesicles engulf and degrade cellular components (15). The relationship between apoptotic and autophagic death is controversial (16). They may cooperate, coexist, or antagonize each other to balance death versus survival signaling (16). We found that CBD induced both apoptosis and autophagy in breast cancer cells, and evaluated further the effects of CBD on the complex interplay between these two types of PCD in breast cancer cell lines. Characterizing more precisely the manner by which CBD kills breast cancer cells will help define the optimal applications of CBD as a cancer therapeutic.

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Materials and Methods:

Cell culture and reagents:

Human cell lines MDA-MB-231, MCF-7, SK-BR-3, ZR-75-1, and MCF-10A (ATCC, Manassas, VA), were cultured in DMEM, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, 5% CO_2. ATCC cell lines are tested for mycoplasma, purity, and contaminants before distribution. All ATCC cell lines were immediately expanded on delivery, and numerous vials of low passage cells were preserved in liquid N_2. No vial of cells was passaged for more than 2 months. CBD (dissolved in ethanol) and Bafilomycin A1 were purchased from Tocris Bioscience (Ellisville, MO) and Sigma (St. Louis, MO), respectively. AM251, AM630, capsazepine, and z-VAD-fmk were purchased from EMD Chemicals (Gibbstown, NJ). LC3, PARP, p-EIF2α, p-AKT, AKT, p-mTOR, mTOR, p-4EBP1, 4EBP1, Cox IV, Bcl-2, Bax, SMAC, Fas-L, and Vps34 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). BID, Beclin1 and caspase antibodies were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). The structures of CBD, AM251, AM630 and capsazepine are provided as Supplemental Figure S1.

Cell viability assay:

Viability was assessed using Cell Proliferation Assay Kit (Promega, Madison, WI) per manufacturer's instructions. Cells were treated with indicated concentrations of CBD in serum-free DMEM. After 24h, cells were incubated with MTS solution prior to measuring absorbance at 490nm (Molecular Devices, Sunnyvale, CA). To analyze the effect of cannabinoid inhibitors on CBD treatment, cells were pretreated for 1h with AM251, AM630 or capsazepine, before CBD incubation.

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Apoptosis assay:

Apoptosis was quantified using FITC Annexin V Apoptosis Detection Kit (BD-Pharmingen, San Diego, CA). Cells were treated with increasing concentrations of CBD for 24h, and Annexin V staining was assessed per manufacturer's instructions, using FACScan flow cytometer (BD Biosciences, Rockville, MD).

Detecting Reactive Oxygen Species (ROS):

To analyze ROS, cells were incubated with 10μM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen, Eugene, OR) for 30 min, washed, and incubated with 10μM of the ROS scavenger alpha-Tocopherol (TOC) (Acros Organics: Fisher Scientific USA, Pittsburg, PA) and/or CBD (5μM) for 12h. ROS (fluorescent green) were quantified by flow cytometry.

Quantifying autophagy with Acridine orange staining:

Acridine orange (AO) (Sigma) was used to quantify autophagy. Cells were treated with a control or CBD (5μM and 10μM) for 24h, incubated for 15 min with AO (1μg/ml), detached, and analyzed by flow cytometry. The intensity of AO fluorescence (red) reflects the degree of cellular acidity, which positively correlates with autophagy levels.

Electron Microscopy:

Cells were treated with a control or 7.5μM CBD for 16h, then fixed, embedded, sectioned, mounted and stained as previously described (17). Specimens were photographed using a JEOL 1200EX electron microscope (JEOL Limited, Tokyo, Japan).

Analysis of mitochondrial membrane transition:

The mitochondrial membrane potential (MMP) probe 3,3'-dihexyloxacarbocyanine iodide [DiOC$_6$(3)] (Invitrogen) was used to study changes in MMP. Cells were treated

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with CBD (0μM-10μM) for 12h, incubated with 40nM $DiOC_6(3)$ for 20 min, trypsinized, and analyzed by flow cytometry (BD Biosciences).

Isolating mitochondrial and cytosolic cellular fractions:

Mitochondrial and cytosolic fractions were isolated using Mitochondria/Cytosol Fractionation Kit (Biovision, Mountain View, CA) per manufacturer's instructions. A COX IV antibody was used to assess mitochondrial fraction purity.

Western Blot Analysis:

Cells were treated with CBD (0μM-10μM) for 24h, unless otherwise indicated. Protein lysates were separated on NuPAGE precast gels (Invitrogen), transferred to 0.45μm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), probed with specific primary antibodies, and incubated with their respective secondary antibodies. Proteins were detected with Western Lightning Plus ECL Substrate (Perkin-Elmer, Fremont, CA). Membranes were stripped using Re-Blot Plus (Millipore, Billerica, MA), and re-probed using GAPDH as a loading control.

Immunoprecipitation:

Cells were lysed on ice in cell lysis buffer (Cell Signaling Technology). Samples were gently rocked overnight with an anti-Beclin1 antibody at 4°C. The antigen-antibody complexes were extracted using protein A sepharose beads (GE Healthcare, Piscataway, NJ). IP products were boiled in Laemmli sample loading buffer (BioRad), and analyzed by Western blot.

Statistical analyses: Statistical analyses were performed using a standard two-tailed Student's t-test. p-values below 0.05 were considered statistically significant.

Results:

CBD induces concentration-dependent cell death in breast cancer cell lines in a receptor-independent manner. The sensitivity of breast cancer cells to anticancer agents depends in part on estrogen receptor (ER) status (18). We analyzed the effects of CBD on breast cancer cells using both ER+ve (MCF-7 and ZR-75-1) and ER-ve (MDA-MB-231 and SK-BR-3) cell lines with an MTS viability assay. Cells were treated with CBD (0-10μM) for 24 hours. CBD significantly decreased cell viability of both ER+ve and ER-ve cell lines in a concentration-dependent manner (Fig. 1A).

Next, we examined the type of cell death by which CBD mediates its cytotoxic effects on breast cancer cells. To assess whether CBD induced apoptosis in these cell lines, we treated them with a range of CBD concentrations for 24 hours, and quantified Annexin V staining, a marker for early apoptosis, using flow cytometry. A concentration-dependent increase in the Annexin V-positive cells was observed in CBD-treated, ER+ve and ER-ve breast cancer cells indicating induction of apoptosis in all cell lines examined (Fig. 1B).

We also compared the effects of CBD on the viability of MDA-MB-231 breast cancer cells and MCF-10A cells, a non-tumorigenic, human, mammary epithelial cell line (19). Although CBD decreased the viability of both cell lines in a concentration-dependent manner, significantly more MCF-10A cells survived CBD treatment as compared to the breast cancer cells (Fig. 1C), suggesting that CBD may preferentially kill cancer cells vs. normal cells.

To determine if CBD mediates cell death via CB1, CB2 or the vallinoid receptor, we repeated the viability assay with 5μM CBD, and with 1μM and 2μM of the following antagonists: AM251 (selective for CB1), AM630 (selective for CB2), and capsazepine

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(selective for the vallinoid receptor). MDA-MB-231 cells treated with individual antagonists displayed viability levels equivalent to untreated cells (data not shown). While treatment with CBD alone and in combination with either concentration of each antagonist resulted in significant loss of viability compared with untreated cells, viability levels were nearly identical among all treatment combinations (Fig. 1D). We interpret these data to indicate that CBD-induced cytotoxicity in MDA-MB-231 cells occurs independent of these three receptors.

CBD induces both apoptosis and autophagy in MDA-MB-231 breast cancer cells. Recently, THC was reported to induce autophagic cell death in glioma cells (6). To determine if CBD may also be killing cancer cells by autophagy, we examined the morphological changes in CBD-treated MDA-MB-231 cells using electron microscopy. We observed nuclear condensation and margination (Fig. 2A, panel B), characteristics typical of apoptosing cells. In addition, we noted morphologies consistent with autophagy: increased vacuolization and less intracellular organelles (Fig. 2A, panels B & D), membrane whorls and vacuoles containing lamellar structures and/or degrading organelles (Fig. 2A, panel C), double-membraned vacuoles fusing with lysosomes (Fig. 2A, panel D), and swollen mitochondria devoid of cristae (Fig. 2A, panel E). These data suggested the coexistence of apoptosis and autophagy in CBD-treated cells. To confirm the coactivation of these two cell death pathways, we used Western blot analysis to examine the expression of apoptosis- and autophagy-specific proteins (Fig. 2B). During autophagy, microtubule-associated protein 1 light chain 3, LC3-I, is recruited to the autophagosome membrane, attaches to autophagic vacuoles, and is converted into lower migratory form, LC3-II. Since LC3-II formation (LC3 lipidation) is a key step in

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autophagy, it is often considered an autophagy-specific marker (20). Cleavage of PARP occurs downstream of caspase activation and is considered a specific marker of apoptosis (21). Upon examination of these proteins, we observed a concentration-dependent increase of both LC3-II and cleaved PARP (Fig. 2B), indicating the coexistence of autophagy and apoptosis in CBD-treated breast cancer cells.

To determine if different CBD concentrations resulted in an altered ratio of apoptosis and autophagy, we incubated MDA-MB-231 cells with increasing concentrations of CBD for 24 hours. Using flow cytometry, we quantified the percentage of autophagic cells with acridine orange, and the percentage of apoptotic cells with Annexin V. We observed low, equivalent levels of apoptosis and autophagy in untreated MDA-MB-231 cells (Fig. 2C). Both apoptosis and autophagy increased in a CBD-induced, concentration-dependent manner (Fig. 2C). At 5µM CBD, there was significantly more autophagy than apoptosis in MDA-MB-231 cells (Fig. 2C). Although both forms of PCD continued to rise with increasing CBD concentration, their overall levels were not significantly different at 10µM CBD (Fig. 2C). We interpret these data to indicate that autophagy may precede apoptosis in CBD-treated breast cancer cells; however, this must be confirmed by examining the apoptosis/autophagy ratio at various times after treatment with a single CBD concentration (e.g. 5µM).

CBD mediates autophagy and apoptosis by inducing ER stress in breast cancer cells and inhibiting AKT/mTOR/4EBP1 signaling. In eukaryotic cells, endoplasmic reticulum (ER) stress plays an important role in the induction of autophagy in response to multiple cellular stressors. An earlier study revealed that CBD treatment of MDA-MB-231 cells led to Ca^{++} efflux, a physiology commonly resulting in ER stress (11). To assess

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whether CBD can cause ER stress in MDA-MB-231 cells, we examined by Western blot analysis, the phosphorylation status of EIF2α, a putative marker of ER stress. Within 2 hours of treatment with 5μM CBD, EIF2α phosphorylation increased significantly, indicating the induction of ER stress by CBD (Fig. 3A).

AKT is a survival molecule, and its decreased phosphorylation precedes the induction of both autophagy and apoptosis (22). We observed decreased levels of phosphorylated AKT between 4 and 24 hours after treatment with CBD (Fig. 3A). LC3-II levels increased between 16 and 24 hours post-CBD treatment, representing the induction of autophagy. Between 16 and 24 hours post-treatment, we observed a significant drop in pro-PARP levels and a significant increase in cleaved PARP (Fig. 3A), reflecting the induction of apoptosis. To confirm the time-dependent, CBD-induced increase in apoptosis in MDA-MB-231 cells, we quantified Annexin V positivity under conditions identical to that used for the Western blot analysis. Consistent with the data attained by Western blotting, we observed a significant increase in Annexin V positive MDA-MB-231 cells 16 and 24 hours after CBD treatment (Fig. 3B). These data suggest that CBD promotes autophagy and apoptosis in breast cancer cells by inducing ER stress and downregulating AKT signaling.

The AKT/mTOR/4EBP1 signaling pathway is frequently activated in human cancers. It modulates breast cancer metastasis, cancer cell proliferation and acquired drug resistance. Additionally, autophagy can be mediated by inhibiting the mTOR pathway (22). Since we observed decreased phosphorylation of AKT and the induction of PCD in CBD-treated breast cancer cells, we examined by Western blot analysis the phosphorylation status of mTOR and its downstream effector 4EBP1, in MDA-MB-231 cells (Fig. 3C).

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We observed a concentration-dependent decrease in the phosphorylation of both proteins after CBD treatment (Fig. 3C), indicating a decrease in AKT/mTOR/4EBP1 signaling. Eukaryotic initiation factor 4E (eIF4E) is a downstream effector of 4EBP1 in this signaling pathway. It binds to the mRNA 7-methyl guanosine cap structure and facilitates the translation of various proto-oncogenic proteins such as c-Myc and cyclin D1 (23). In an earlier publication, we demonstrated that cyclin D1 is downstream of eIF4E in the AKT/mTOR/4EBP1 pathway (24). Therefore, we analyzed the protein levels of cyclin D1 in CBD-treated breast cancer cells. We observed a significant decrease in cyclin D1 expression in the CBD-treated- vs. control-treated breast cancer cells (Fig. 3C). These results support our finding that CBD inhibits AKT/mTOR/4EBP1, a critical oncogenic pathway.

CBD induces apoptosis in MDA-MB-231 cells via the mitochondria-mediated signaling pathway. Since we observed apoptosis in CBD-treated breast cancer cells, we explored further the molecular mechanisms involved in the CBD-mediated apoptosis of MDA-MB-231 cells. Signaling by both mitochondria-mediated and death receptor-mediated pathways converge at caspases -7, -9 and/or -3 (25). Thus, we examined the effect of CBD on the cleavage of procaspases -3, -7 and -9 into the smaller caspases -3, -7 and -9, respectively, by Western blot analysis. We observed a concentration-dependent increase in all three cleaved caspases after CBD treatment (Fig. 4A).

Mitochondria play a central role in apoptotic cell death, which is mediated by outer membrane permeabilization in response to death triggers such as DNA damage and growth factor deprivation (25). Mitochondrial membrane permeabilization induces the release of the apoptotic molecules cytochrome c and Smac/DIABLO to the cytosol,

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which leads to caspase-dependent apoptosis (25). Hence, we analyzed mitochondrial membrane potential (MMP) in control-treated and CBD-treated MDA-MB-231 cells using $DioC_6(3)$ (Fig. 4B). We observed dissipation of MMP in a concentration-dependent manner as reflected by an increased percentage of cells with low $DioC_6(3)$ fluorescence, indicating enhanced mitochondrial membrane permeability (Fig. 4B).

Upstream of mitochondrial membrane permeabilization, pro-caspase-8 is activated by proteolytic cleavage. BID is a pro-apoptotic Bcl-2 family member and a substrate for activated caspase-8 which resides in both the cytosol and mitochondria of cells as an inactive precursor (25). Proteolytic cleavage of BID by caspase-8 converts cytosolic BID to its active form, t-BID, whereupon it translocates to the mitochondria (26). Moreover, cleaved BID can cooperate with other proapoptotic Bcl-2 members, including Bax and/or Bak, to enhance mitochondrial membrane permeabilization (25). In this study, we observed a concentration-dependent, CBD-induced elevation of Bax (Fig. 4C), however Bak levels did not change (data not shown).

In addition to intracellular death signaling, mitochondria-based apoptosis can be initiated by engaging various death signaling receptors on the cell surface, including Fas-L and TRAIL (27) . To characterize more precisely the mechanisms of CBD-induced apoptosis, we examined by Western blot analysis the levels of Fas-L, TRAIL, pro-caspase-8, caspase-8, BID and t-BID in whole cell lysates of MDA-MB-231 cells after treatment with various concentrations of CBD. We observed a concentration-dependent increase in Fas-L expression (Fig. 4C), but TRAIL levels did not change (data not shown). There was a reduction in pro-caspase-8 levels in the whole cell lysates (Fig. 4C), and the concurrent appearance of activated, cleaved caspase-8 after treatment with CBD (Fig.

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4C). We also observed the appearance of t-BID in the total cell lysates after treatment with similar CBD concentrations (Fig. 4C).

We examined protein expression in the cytosolic and mitochondrial fractions of MDA-MB-231 cells after CBD treatment. In the cytosolic fraction, levels of BID decreased in a concentration-dependent manner (Fig. 4D), and at 10μM CBD, we detected t-BID in the mitochondrial fraction (Fig. 4D). The release of cytochrome c to the cytosol is an authenticated marker for mitochondria-mediated apoptosis (25). Therefore, we examined cytochrome c levels in the cytosol of CBD-treated and control-treated MDA-MB-231 cells by Western blot analysis. After CBD treatment, we observed an increase in the cytosolic levels of cytochrome c over basal levels in the control-treated cells (Fig. 4D). SMAC (second mitochondria-derived activator of caspases) competes with caspases for binding to the IAPs (inhibitor of apoptosis proteins), thereby blocking apoptosis and facilitating apoptosome formation (28). We observed transiently elevated levels of SMAC in the cytosol following CBD treatment (Fig. 4D). Taken together, these data demonstrate that CBD induces mitochondria-mediated apoptosis as a result of the convergence of intrinsic and extrinsic death signaling stimuli.

ROS generation is required for CBD-induced apoptosis and autophagy in breast cancer cells. Impaired mitochondrial function results in increased ROS production (29), and recent studies implicate elevated ROS levels in the induction of autophagy (30). In addition, ROS plays a critical role in the CBD-mediated cell death of human glioma and leukemia cells (8, 10). Therefore, we examined the effect of CBD on ROS generation in MDA-MB-231 cells. After incubating the cells with 5μM CBD for 12 hours, we observed a significant increase in ROS (Fig. 5A). Pretreatment with α-Tocopherol (TOC), an

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antioxidant ROS scavenger, significantly inhibited CBD-induced ROS generation (Fig 5A). To examine the role of ROS in CBD-induced apoptosis and autophagy, we examined by Western blot analysis the levels of pro-PARP, cleaved PARP, LC3-I and LC3-II, with and without pretreatment with TOC (Fig. 5B). CBD enhanced the levels of LC3-II and cleaved PARP (Fig. 5B). Pretreatment with TOC significantly decreased the CBD-induced elevation of these proteins (Fig. 5B), which suggests that ROS contributes to CBD-induced apoptosis and autophagy in breast cancer cells. To confirm the functional effects of blocking ROS generation, we examined apoptosis levels (with an Annexin V assay) in MDA-MB-231 cells after incubation with CBD, TOC or both. We found that inhibiting ROS generation with TOC, attenuated a significant portion of CBD-induced apoptosis (Suppl. Fig. S2), confirming a pivotal role for ROS in CBD-induced PCD.

Caspase and autophagy inhibitors alter the levels of CBD-induced apoptosis in MDA-MB-231 cells. The contribution of caspase activation to apoptosis is well characterized while its relation to autophagic death is still unclear. To address the role of caspases in our studies, we used z-VAD-fmk, a general caspase inhibitor (CI), and evaluated the percentage of apoptotic cells with an Annexin V assay. We pretreated MDA-MB-231 cells with 50μM CI for 1 hour, and then incubated them for 24 hours in 5μM CBD. We observed a small, albeit significant, decrease in apoptosis in the cells pretreated with CI before CBD incubation as compared to those incubated with CBD alone (Fig. 5C, lanes "CI+CBD" and "CBD"). These data indicate that CBD-induced apoptosis is dependent, at least in part, on caspase activation, however, alternative,

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currently undefined mechanism(s), may also facilitate CBD-induced apoptosis when caspase cleavage is blocked.

To address the interplay between apoptosis and autophagy in CBD-induced cell death we employed Bafilomycin A1 (BAF), a specific inhibitor of acidic vacuole formation and function during autophagy. BAF suppresses autophagy in irradiated cancer cells, thereby favoring apoptosis (31). We pretreated MDA-MB-231 cells with 5μM CBD for 16 hours, followed by an 8-hour incubation with 10nM BAF. The population of Annexin V-positive cells increased by ~15% after BAF incubation as compared to cells treated with CBD alone (Fig. 5C, lanes "BAF+CBD" and "CBD"). We interpret these data to indicate that apoptosis levels increase in CBD-treated breast cancer cells when autophagy is inhibited.

To corroborate these findings, we examined under the same conditions, the levels of cleaved PARP and cleaved caspase-8 by Western blot analysis. We observed a decrease in cleaved PARP in MDA-MB-231 cells pretreated with a general caspase inhibitor (CI) before incubation with CBD, as compared to those treated with CBD alone (Fig. 5D, lanes "CI+CBD" and "CBD"). These data demonstrate that CBD-induced apoptosis in breast cancer cells depends in part on caspase activation, a finding consistent with our Annexin V data. We also observed elevated levels of cleaved caspase-8 in the cells treated with a combination of BAF and CBD as compared to those treated with CBD alone (Fig. 5D, lanes "BAF+CBD," and "CBD"), suggesting a concomitant rise in apoptosis when autophagy is blocked. These data illustrate further that CBD-induced cell death results in a complex interplay between apoptosis and autophagy in MDA-MB-231 breast cancer cells.

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CBD-induced molecular crosstalk during autophagy and apoptosis in breast cancer cells. Autophagy and apoptosis can be triggered by overlapping signaling mechanisms (32). The Bcl-2 interacting protein Beclin1 is a key autophagy signaling molecule. Beclin1 functions as a scaffold for the assembly of a multi-protein complex by binding to phosphatidylinositol-3-kinase class-III/vacuolar protein sorting-34 (Vps34), UV resistance associated gene (UVRAG) and activating molecule in Beclin1-regulated autophagy (AMBRA1) (33). This complex induces autophagosome formation during nutrient deprivation-induced autophagy, although the precise mechanism by which it induces autophagy is not known (34). A recent *in vitro* study suggests that Beclin1 is cleaved by caspases which render it incapable of inducing autophagy (35, 36). We observed cleavage of Beclin1 after incubation with 5μM, 7.5μM and 10μM CBD (Fig. 6A). This finding is consistent with caspase-mediated cleavage of Beclin1, and implicates a role for Beclin1 in CBD-mediated cell death.

Bcl-2 helps maintain mitochondrial integrity, protects cells from apoptosis, and when bound to Beclin1, it can inhibit autophagy as well (37). Dissociation of Beclin1 from the Bcl2/Bcl-xl/Mcl-1 complex results in unchecked autophagy and apoptosis (32). The binding of Beclin1 to Vps34 is required for the initiation and nucleation phase of autophagy. Moreover, elevated levels of Vps34 stimulate autophagy in mammalian cells (33). When we examined levels of Bcl-2 and Vps34 by Western blot analysis in MDA-MB-231 cells after incubation with various concentrations of CBD, we observed a significant decrease in Vps34 following 10μM CBD treatment (Fig. 6A).

By immunoprecipitation we examined also the effect of CBD on the physical interactions of Vps34 and Bcl-2 with Beclin1 in MDA-MB-231 cells (Fig. 6B). We observed a strong

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interaction between Beclin1 and Vps34 after 5µM CBD treatment (Fig. 6B), however, at

10µM CBD, the interaction between these proteins was attenuated (Fig. 6B). There was

also less interaction between Beclin1 and Bcl-2 at 10µM CBD as compared with control

(Fig. 6B). These data support strongly our observation that at higher CBD concentrations,

MDA-MB-231 breast cancer cells favor apoptotic cell death, and the decreased

association between Beclin1 and Bcl-2 marks an intersection between CBD-induced

autophagic and apoptotic PCD.

In a recent report, Wirawan et al. proposed that Beclin1 cleavage is the physiological link

between autophagic and apoptotic signaling (36). Upon caspase cleavage, Beclin1 can no

longer induce autophagy. Moreover, one of the resulting protein fragments, Beclin1-C,

translocates to the mitochondria where it enhances apoptosis by inducing the release of

cytochrome c (36). We observed a significant increase of cleaved Beclin1 in the

mitochondrial fraction of the 10µM CBD-treated cells (Fig. 6C). We hypothesize that by

this mechanism CBD coordinates the balance between the modes of programmed cell

death by simultaneously inhibiting autophagy and inducing mitochondria-mediated

apoptosis in MDA-MB-231 cells. Although both types of cell death are present over a

range of CBD concentrations, when MDA-MB-231 cells are incubated with 10µM CBD,

apoptosis supersedes autophagy as the predominant mechanism of programmed cell death

(Fig. 6).

Discussion:

There is an urgent need to develop innovative ways to treat breast cancer that has become

resistant to established therapies. We sought to identify novel agents by examining

natural products with validated, anti-cancer properties. We focused our study on the

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cannabinoid CBD, which induces cytotoxicity in human glioma, leukemia and breast cancer cells *in vitro* (5, 8-12, 38) and inhibits the metastasis of breast cancer cells (12, 38). We explored the molecular mechanisms by which CBD induced programmed cell death (PCD) in breast cancer cells lines, and examined the intricate relationship between CBD-induced apoptosis, autophagy and ROS generation.

We found that CBD inhibited the survival of both ER positive and ER negative breast cancer cell lines, and induced apoptosis in a concentration-dependent manner. Moreover, at these concentrations, CBD had little effect on MCF-10A cells, non-tumorigenic, mammary cells (19). These data enhance the desirability of CBD as an anti-cancer agent, since they suggest that CBD preferentially kills breast cancer cells, while minimizing damage to normal breast tissue.

Many cannabinoids mediate their effects by binding to CB1, CB2 or the vallinoid receptor (3, 4), however, our results indicate that CBD induces PCD independent of these receptors. This is a promising area for future investigation in that identifying the receptor through which CBD mediates its anti-cancer effects may better inform the design of novel drugs with a mechanism of action similar to CBD.

In this study we demonstrated that CBD induced both apoptosis and autophagy-induced death in breast cancer cells. While PCD by apoptosis is well-documented, autophagy-mediated cell death is a relatively recent discovery, and there is much to be learned about the factors that prejudice autophagy toward self-protection or self-destruction. Recent studies support growing evidence that ER stress can trigger autophagic cell death (39, 40). We observed the induction of ER stress in MDA-MB-231 cells within 2 hours of CBD treatment, followed by LC3-II accumulation, leading us to conclude that CBD-

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induced autophagy in breast cancer cells does not maintain homeostasis. Rather, in this context, autophagy is cytodestructive, and leads to programmed cell death.

Based on recent publications demonstrating that the AKT/mTOR pathway can inhibit autophagy, and that ER stress can block this pathway, we examined the effect of CBD on AKT/mTOR signaling, which is dysregulated in many neoplasms (22). We found that CBD decreased the phosphorylation of AKT, mTOR and 4EBP1, and decreased Cyclin D1 levels in breast cancer cells. Moreover, concurrent with inhibiting AKT/mTOR signaling, CBD elevated cleaved PARP and LC3-II levels, markers for the induction of apoptosis and autophagy, respectively, suggesting that CBD may induce PCD in breast cancer cells by inhibiting AKT/mTOR signaling.

Apoptosis can be induced either by the intrinsic pathway (which is mediated by the mitochondria) or signaling from death receptors at the cell surface (the extrinsic pathway) (14, 27). We found that CBD induced the activation of caspase-8, the generation and translocation of t-BID to the mitochondria, the release of cytochrome c and SMAC into the cytosol, and increased levels of Fas-L. These data support our hypothesis that CBD induces PCD through mitochondria-mediated apoptosis; however, CBD appears to initiate this form of PCD with both internal (t-BID translocation) and external stimuli (Fas-L, death receptor engagement).

Recent publications demonstrate that reactive oxygen species (ROS) play a central role in the CBD-induced death of glioma and Jurkat cells (8, 10). Consistent with these studies, we observed CBD-enhanced ROS generation in breast cancer cells. Moreover, when we limited ROS levels with TOC, CBD-induced expression of protein markers for both

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apoptosis and autophagy decreased, and levels of apoptosis decreased, suggesting that ROS play a critical role in the CBD-induced PCD of breast cancer cells.

We explored the relationship between CBD-induced apoptosis and autophagic cell death by blocking each form of PCD with specific caspase and autophagy inhibitors. While caspase inhibition decreased levels of CBD-induced apoptosis and protein markers in breast cancer cells, inhibiting autophagy enhanced levels of CBD-induced apoptosis and protein marker expression. These data suggest that blocking CBD-induced autophagy may affect a compensatory rise in apoptosis as an alternative means of programmed cell death. We hypothesize that CBD may mediate this ratio between apoptosis and autophagy in CBD-induced PCD via Beclin-1. Beclin-1 can block autophagy when it forms a protein complex with Bcl-2 (34). However, if Beclin-1 is cleaved, this complex dissociates and autophagy is induced (37). Moreover, this cleavage product translocates to the mitochondria and induces apoptosis by enhancing the release of cytochrome c (36). We observed a CBD-induced translocation of Beclin-1 to the mitochondria, which suggests that a potential mechanism by which CBD affects the balance between autophagy and apoptosis in breast cancer cells, is by inducing the cleavage and translocation of Beclin-1.

In summary, we demonstrated that CBD, a plant-derived cannabinoid, preferentially kills breast cancer cells by inducing ER stress, inhibiting mTOR signaling, enhancing ROS generation, and mediating a complex balance between autophagy and mitochondria-mediated apoptosis in MDA-MB-231 breast cancer cells. These findings support the continued exploration of CBD as an alternative agent for breast cancer treatment.

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Figure legends:

Figure 1: CBD induces concentration-dependent cell death and apoptosis in breast cancer cell lines in a receptor-independent manner. (A) Cell survival (as measured by MTS assay) of various breast cancer cell lines, treated with increasing concentrations of CBD for 24h. (B) Percentage of apoptosis (as measured by % of Annexin V-positive cells) in various breast cancer cell lines, treated with increasing concentrations of CBD for 24h. (C) Cell survival (as measured by MTS assay) of MDA-MB-231 breast cancer cells and MCF-10A, non-tumorigenic mammary, epithelial cells, treated with increasing concentrations of CBD for 24h. (**p $\leq$ 0.01). (D) Cell survival (as measured by MTS

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assay) of MDA-MB-231 cells treated with 0μM or 5μM CBD alone, and pretreated for 1h with indicated concentrations of the CB1 antagonist AM251, the CB2 antagonist AM630 or the vallinoid antagonist capsazepine, and then CBD. Data from Fig. 1 (A, B, C and D) represent the mean ± SD of at least 3 independent experiments.

Figure 2: CBD induces both apoptosis and autophagy in MDA-MB-231 breast cancer cells. (A) Representative electron micrographs of untreated MDA-MB-231 cells (panel A) and MDA-MB-231 cells treated with 7.5μM CBD for 16h (panels B, C, D, and E); N = nucleus; For panels A and B, scale bars = 2μm. For panels C, D, and E, scale bars = 500 nm. Panel C: Left arrow indicates membrane whorls. Right arrow indicates vacuole filled with degrading organelles. Panel D: Double-membraned vacuole fusing with lysosome (indicated by double-headed arrow). Arrow heads indicate empty vacuoles. Panel E: * indicates swollen mitochondrion devoid of cristae. (B) Representative Western blot analysis of PARP cleavage and LC3 lipidation in MDA-MB-231 cells, treated with increasing concentrations of CBD for 24h. GAPDH used as loading control. (C) Percentage of autophagy (as measured by % of acridine orange-positive cells) and percentage of apoptosis (as measured by % of Annexin V-positive cells) in MDA-MB-231 cells, treated with increasing concentrations of CBD for 24h. Data represent the mean ± SD of at least 3 independent experiments. (**$p \leq 0.01$).

Figure 3: CBD mediates autophagy and apoptosis by inducing ER stress in breast cancer cells and inhibiting AKT/mTOR/4EBP1 signaling. (A) Representative Western blot analysis of p-EIF-2α, p-AKT, total AKT, LC3-I, LC3-II, pro-PARP and cleaved PARP expressed in MDA-MB-231 cells at various times following 5μM CBD treatment. GAPDH used as loading control. (B) Percentage of apoptosis (as measured by the % of

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Annexin V-positive cells) in MDA-MB-231 cells at various times following 5μM CBD treatment. Data represent the mean ± SD of at least 3 independent experiments. (*p ≤ 0.05; **p ≤ 0.01). (C) Representative Western blot analysis of p-mTOR, mTOR, p-4EBP1, 4EBP1 and Cyclin D1 expressed in MDA-MB-231 cells, treated with increasing concentrations of CBD for 24h. GAPDH used as loading control.

Figure 4: CBD induces apoptosis in MDA-MB-231 cells via the mitochondria-mediated signaling pathway. (A) Representative Western blot analysis of pro-caspases -9, -7, -3 and cleaved caspases -9, -7, -3 in MDA-MB-231 cells, treated with increasing concentrations of CBD for 24h. GAPDH used as loading control. (B) Mitochondrial membrane potential (ΔΨ)m as assessed by flow cytometry, using $DiOC_6(3)$ in MDA-MB-231 cells, treated with increasing concentrations of CBD for 12h. Data represent the mean ± SD of at least 3 independent experiments. (*p ≤ 0.05; **p ≤ 0.01). (C) and (D) MDA-MB-231 cells were treated with increasing CBD concentrations for 24h. (C) Representative Western blot analysis of Fas-L, pro-caspase-8, cleaved caspase-8, BID and t-BID (cleaved BID) in whole cell lysates. GAPDH used as loading control. (D) Representative Western blot analysis of BID, t-BID, cytochrome c, SMAC and COX IV in cytosolic and mitochondrial fractions. COX IV used to confirm the purity of subcellular fractions.

Figure 5: The effect of ROS-, caspase-, and autophagy inhibitors on CBD-induced apoptosis and autophagy in MDA-MB-231 breast cancer cells. (A) Fold change in ROS levels as measured by DCF-DA and flow cytometry, after incubation with CBD, α-Tocopherol (TOC), a ROS scavenger/inhibitor, or both vs. untreated control. MDA-MB-231 cells were incubated with DCF-DA (10μM) and CBD (5μM) in the presence or
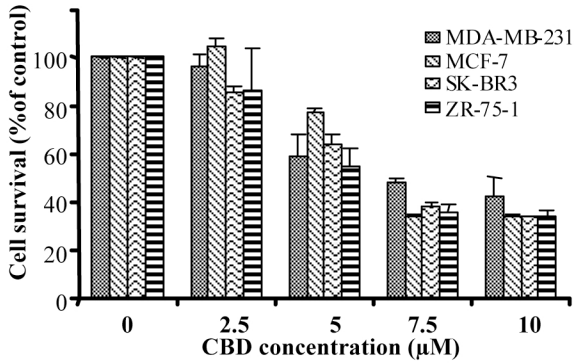
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absence of TOC (10μM), and analyzed by flow cytometry. Data represent the mean ± SD of at least 3 independent experiments. (**p ≤ 0.01). (B) Representative Western blot analysis of pro-PARP, cleaved PARP, LC3-I and LC3-II in MDA-MB-231 cells after incubation with CBD (5μM) and/or TOC (10μM). GAPDH used as loading control. (C) Percentage of apoptosis (as measured by % of Annexin V-positive cells) in MDA-MB-231 cells after treatment with 5μM CBD, 50μM CI (general caspase inhibitor z-VAD-fmk) and 10nM BAF (autophagy inhibitor BafilomycinA1) alone, or in designated combinations. Data represent the mean ± SD of at least 3 independent experiments. (*p ≤ 0.05). (D) Representative Western blot analysis of pro-caspase-8, cleaved caspase-8, pro-PARP and cleaved PARP in MDA-MB-231 cells after treatment with CBD (5μM), CI (50μM) and BAF (10nM) alone, or in designated combinations. GAPDH used as loading control.

Figure 6: Molecular crosstalk between CBD-induced autophagy and apoptosis.

(A) Representative Western blot analysis of Beclin1, cleaved Beclin1, Vps34 and Bcl-2 in whole cell lysates of MDA-MB-231 cells treated with indicated concentrations of CBD or a control for 24h. GAPDH used as loading control. (B) Representative Beclin1 immunoprecipitation/Western blot analysis of Vps34 and Bcl-2 in MDA-MB-231 cells treated with indicated concentrations of CBD for 24h. Beclin1 used as loading control. (C) Representative Western blot analysis of Beclin1 and cleaved Beclin1 in cytosolic and mitochondrial fractions of MDA-MB-231 cells treated with indicated concentrations of CBD or a control for 24h. COX IV used to confirm the purity of subcellular fractions.
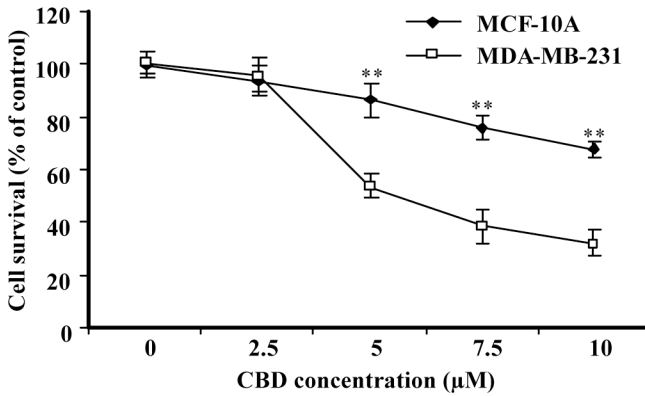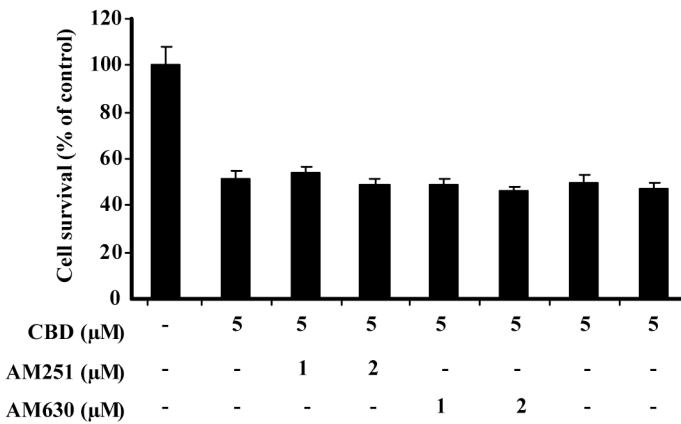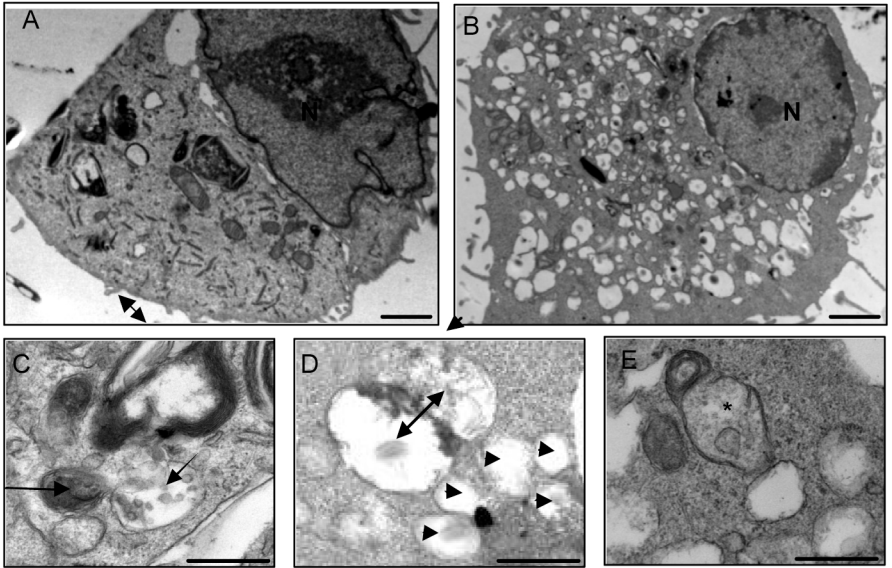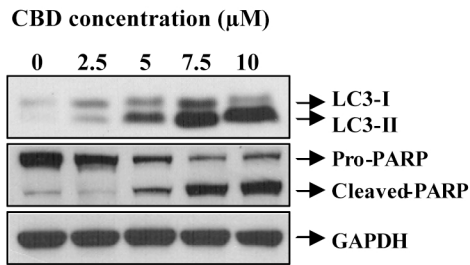
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Figure 1

Figure 2

A

B

C

Figure 3

A

Time post-CBD treatment (hours)

0   1   2   4   8   16   24

→ p-EIF-2a (Ser51)

→ p-AKT (Ser473)

→ AKT

→ LC3-I
→ LC3-II

→ Pro-PARP
→ Cleaved-PARP

→ GAPDH

B

% Apoptosis

Time post-CBD treatment (hours)

C

CBD concentrations (μM)

0   2.5   5   7.5   10

→ p-mTOR (Ser2448)

→ mTOR

→ p-4EBP1 (Ser65)

→ 4EBP1

→ Cyclin D1

→ GAPDH
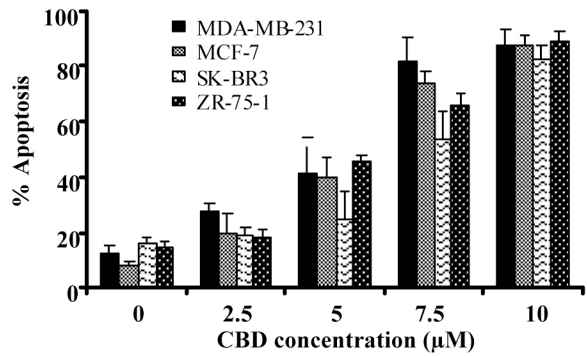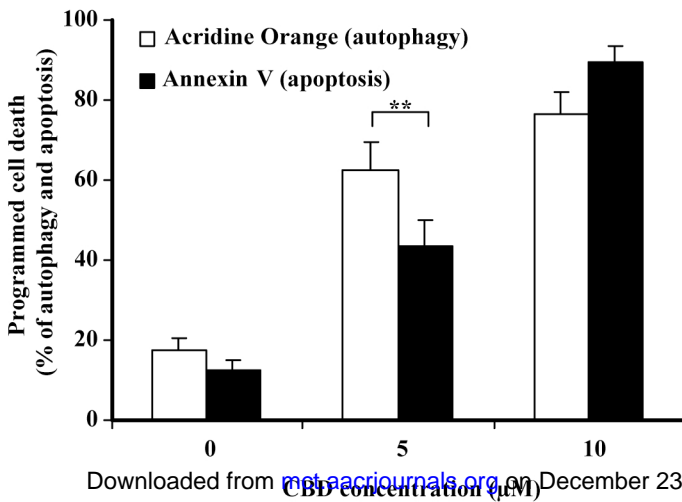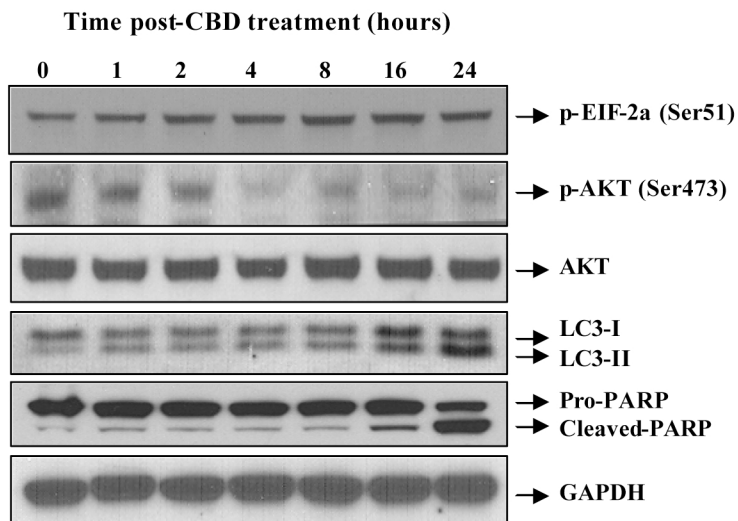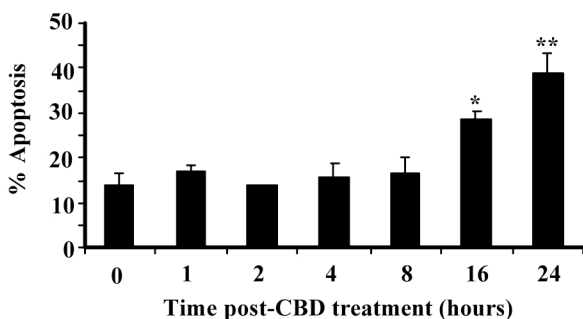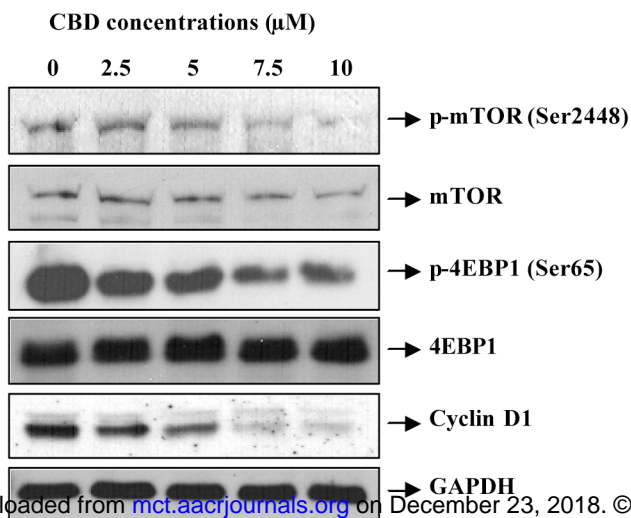
Figure 4

A

CBD concentration (μM)

| 0 | 2.5 | 5 | 7.5 | 10 |

→ Pro-Casp-9
→ Cleaved Casp-9
→ Pro-Casp-7
→ Cleaved Casp-7
→ Pro-Casp-3
→ Cleaved Casp-3
→ GAPDH

B

C

CBD concentration (μM)

0　2.5　5　7.5　10

→ Fas-L
→ Pro-Casp-8
→ Cleaved Casp-8
→ Bid
→ t-Bid
→ Bax
→ GAPDH

D

CBD concentration (μM)

| 0 | 2.5 | 5 | 10 | | 0 | 2.5 | 5 | 10 |

→ Bid
→ t-Bid
→ Cytochrome c
→ SMAC
→ COX IV

Cytosol　　　　**Mitochondria**

Figure 5

A

B

C

D

Figure 6

A

CBD concentration (µM)

0   2.5   5   7.5   10

→ Beclin1
→ Cleaved Beclin1
→ Vps34
→ Bcl-2
→ GAPDH

B

IgG ctrl   CBD (µM)   + ctrl

0   2.5   5   10

IP: Beclin1, WB: Vps34

IP: Beclin1, WB: Bcl-2

IP: Beclin1, WB: Beclin1

C

CBD concentration (µM)

0   2.5   5   10   0   2.5   5   10

→ Beclin1
→ Cleaved Beclin1
→ COX IV

Cytosol   Mitochondria

American Association
for Cancer Research
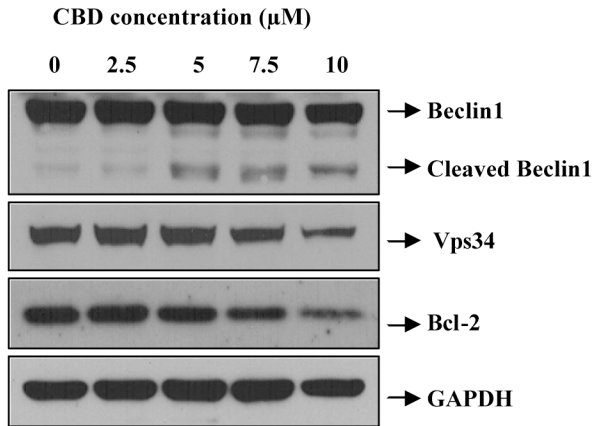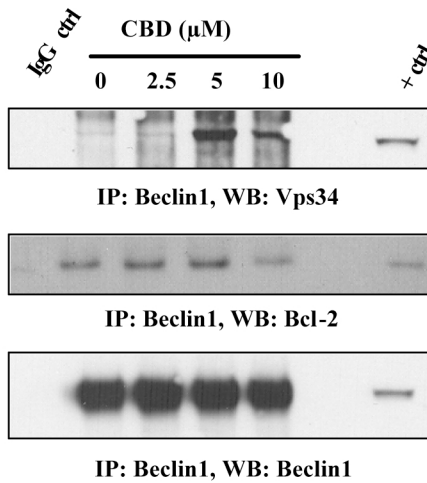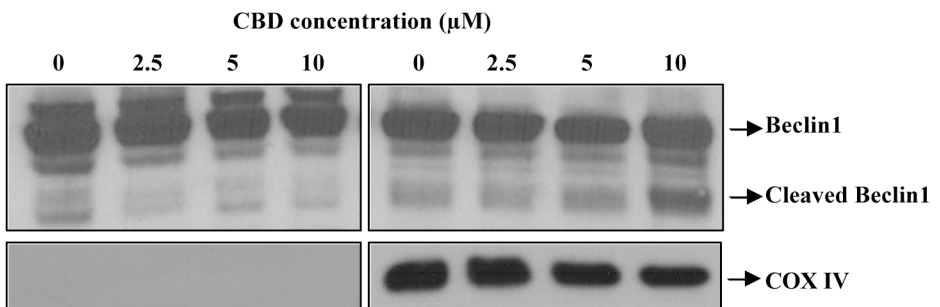
Molecular Cancer Therapeutics

Cannabidiol induces programmed cell death in breast cancer cells by coordinating the crosstalk between apoptosis and autophagy

Ashutosh Shrivastava, Paula M. Kuzontkoski, Jerome Groopman, et al.

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