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Effect of combining CBD with standard breast cancer therapeutics

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ABSTRACT

Breast cancer is the most common malignancy in women worldwide. Sixty-five percent of breast cancers are estrogen and/or progesterone receptor positive. Estrogen receptor expression is a prognostic and predictive biomarker of response to endocrine therapy, which consists of the selective estrogen receptor modulator tamoxifen, aromatase inhibitors, and the selective estrogen receptor degrader fulvestrant. Cannabidiol is a phytocannabinoid that is emerging as a potential therapeutic agent. The aim of this study was to investigate the effect of cannabidiol on estrogen receptor-positive and estrogen receptor-negative representative breast cancer cell lines in combination with standard therapeutic agents used in clinical practice. To compare the effects of cannabidiol on breast cancer cell viability, cancer cell lines were exposed to increasing concentrations of cannabidiol. The effects of cannabidiol in combination with the endocrine therapeutics tamoxifen, fulvestrant, and the cyclin-dependent kinase inhibitor palbociclib on breast cancer cell viability were examined. We demonstrated that cannabidiol dose-dependently decreased the viability of all breast cancer cell lines independent of estrogen receptor expression. The addition of cannabidiol to tamoxifen had an additive negative effect on cell viability in ER+ in estrogen receptor positive T-47D line. Cannabidiol did not attenuate the effect of standard treatment of hormone receptor-positive breast cancer with fulvestrant and palbociclib. In addition, cannabidiol did not attenuate the effect of standard treatment of triple-negative breast cancer and human epidermal growth factor receptor 2 positive breast cancer cell lines with trastuzumab and cisplatin.

1. Introduction

Breast cancer (BC) is the most common cancer in women worldwide [1]. Standard immunohistochemical biomarkers for BC include expression of estrogen (ER) and progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) [2]. Sixty-five percent of BCs are ER and/or PR positive and their expression is a prognostic and predictive marker of response to endocrine therapy [3–8]. During treatment of metastatic hormone receptor-positive (HR+) BC, most patients develop resistance to endocrine therapy [9], which consists of selective ER modulators (SERM) tamoxifen [10], aromatase inhibitors (AI) [11] selective ER degraders (SERD) fulvestrant [12], and ovarian suppression with gonadotropin-releasing hormone (GnRH) agonists [13]. The Mechanistic Target of Rapamycin (mTOR) inhibitor everolimus [14] and cyclin-dependent kinase (CDKi) inhibitors are approved in combination with endocrine therapy [15–17]. The combination of CDKi palbociclib and fulvestrant is approved for the treatment of premenopausal and postmenopausal women with HR+ and HER2-negative metastatic BC [18]. Twenty to twenty-five percent of BC tumors have amplification of the HER2 gene and are treated with anti-HER2 therapy (i.e., trastuzumab) [19]. Triple-negative breast cancer (TNBC) accounts for 15–20% of all BC tumors and lacks expression of the ER, PR, and HER2 receptors. Chemotherapy (e.g., cisplatin) and chemotherapy in combination with immunotherapy are the standard treatments for TNBC [20–22].

In humans, the endocannabinoid system consists of genes encoding cannabinoid receptors (CBRs) for endogenously produced cannabinoids

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(endocannabinoids) such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and the enzymes involved in their synthesis and degradation [23]. Cannabinoid receptors (CBRs) are membrane G-protein-coupled receptors [24–26]. The two most important CBRs are cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) [27]. CB1 is one of the most abundant G protein-coupled receptors (GPRs) in the brain and modulates motor function, cognition, memory, analgesia, etc. CB1 is also expressed in the immune system, adrenal gland, heart, lung, prostate, liver, uterus, ovaries, and testes. CB2 is strongly expressed in organs that are part of the immune system, i.e., the spleen, tonsils, and thymus. However, CB2 is also expressed in the central nervous system, but to a much lesser extent than CB1. Cannabinoids also bind to other G protein-binding receptors, e.g., GPR55 [28], GPR18 [29], and GPR119 [30]. Of the family of ion channels, such as transient receptors, the vanilloid receptors (TRPV-1 and TRPV-2) and peroxisomal proliferator-activated receptors (PPAR) [26] have been best studied, as described in Ref. [31] and recently by Walsh et al. [32]. By binding to a variety of CBRs, endocannabinoids regulate various aspects of human physiological, behavioral, immunological, and metabolic functions. In recent years, considerable progress has been made in understanding the distribution and function of endocannabinoid system components in the central nervous system and immune processes. Several pathological conditions have been associated with altered endocannabinoid system signaling, including cancer [33]. Tegger et al. [34] found that endocannabinoids (i.e., anandamide) can reduce the spread of metastases, although often in an inefficient manner. In any case, the authors suggested that monitoring individual endocannabinoid profiles over time may be useful for assessing cancer progression. Numerous publications indicate that several important signaling pathways involved in cell growth, differentiation, metabolism, and apoptosis interact with cannabinoid signaling. Several of these signaling pathways, including AKT, EGFR, and mTOR, are known to contribute to tumor development and metastasis. Cannabinoids can induce apoptosis and autophagy and modulate the immune system [33]. In addition, the endocannabinoid system plays a role in metastasis [34,35]. Therefore, phyto-cannabinoids and synthetic cannabinoids have been extensively studied in the last decade. They all represent a wide range of ligands that interact with CBRs [36,37].

Of the exogenous phyto-cannabinoids, tetrahydrocannabinol (THC) and cannabidiol (CBD) are isolated from *Cannabis sativa* or produced synthetically, as previously described by Pacher et al. [37]. THC is a psychoactive compound that is very abundant in the *Cannabis sativa* plant. In addition to THC, CBD and other minor cannabinoids have also been shown to be responsible for many cannabinoid effects, such as euphoria, pain relief, and anti-inflammatory effects [38–40]. THC is a nonselective agonist of CBRs and binds with higher affinity to CB1 and lower affinity to CB2 [37,38,41]. CBD has a lower affinity for CBRs and acts as an inverse agonist at CB2 [36,40]. THC, CBD, and other exogenous cannabinoids affect several features of tumor progression, as observed in several cancers, particularly gliomas/glioblastomas [42], followed by carcinomas of the skin, liver, colon, prostate, and breast [43–45]. Elbaz et al. [46] found that modulation of the tumor microenvironment is a novel anti-tumor mechanism of CBD in BC. Opitz et al. [47] also pointed out the P-glycoprotein transporter facilitates multiple drug resistance by excreting anticancer drugs. The expression of P-glycoprotein was decreased by THC and CBD [48,49]. Studies on various BC subtypes have shown that phyto-cannabinoids effectively arrest the cell cycle and induce cell death through pathways such as apoptosis and autophagy [46,50,51]. In a recent review, Kisikova et al. [52] showed that CBD induces BC cell death through autophagy, leading to apoptosis in vitro, and inhibits proliferation, invasion, and angiogenesis of BC cells *in vivo*. Other synthetic CBR agonists also inhibit tumor growth and metastasis of BC. In one study, CBD was the most cytotoxic of all cannabinoids tested in ER+ and ER- cell lines [53–56]. Most recent studies have focused on the combination of THC and CBD, as these two compounds target different receptors and may act synergistically [57–59]. There are many potential interactions between cannabinoids and HR+ BC: cannabinoids interact with the hypothalamic-pituitary-gonadal axis, and there is evidence that the molecular pathways between CBRs and estrogens overlap [60]. In addition, cannabinoids may have direct effects on endocrine and targeted therapies, including fulvestrant, aromatase inhibitors, and palbociclib [61]. Although the use of THC in combination with CBD is well established in palliative care as a second- or third-line therapy for difficult-to-control symptoms such as nausea, appetite, pain, mood swings, and sleep disturbances [52,62], the use of THC in the clinical setting is limited because of the psychoactive properties of THC and for legal reasons, as explained by Abrams [63]. Therefore, in this study, we only investigated the effect of nonpsychoactive CBD on cell viability of representative ER+ (MCF-7 and T-47D) and ER- (HER2+ SK-BR-3) and TNBC (MDA-MB-231) cell lines alone and in combination with standard therapeutic agents, i.e., tamoxifen, fulvestrant, palbociclib, trastuzumab, and cisplatin.

2. Materials and methods

2.1. Cell lines

BC cell lines ER+ MCF-7, and ER+ T-47D, HER2+ SK-BR-3, and TNBC MDA-MB-231 were obtained from ATTC [58]. Cell lines were grown in T75 cell culture flasks in basal media (DMEM (11965092): MCF-7 and T-47D, DMEM F12 (11320033): MDA-MB-231, and McCoy's 5A (26600023): SK-BR-3 (all media from Thermo Scientific, USA) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin, in humidified atmosphere containing 5% (v/v) CO2. After reaching approximately 90% confluence, cells were detached with trypsin. Cell pellets were washed twice with PBS and stored at −80 °C.

2.2. Immunofluorescence

Expression of CBRs was confirmed by immunofluorescence ER+ cell lines MCF-7 and T-47D. Cell nuclei were stained with DAPI (blue) and CB antibodies, including rabbit polyclonal anti-CB1 (ab23703), anti-CB2 (ab45942), and anti-CB3 antibodie (ab203663), respectively (Abcam, UK) with Alexa Fluor 488 dye (green). Nonspecific background staining was blocked with normal goat serum (Dako) and 0.1% Triton-X. Primary antibodies diluted in PBS containing 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) were applied to the sections overnight at 4°C. After washing the sections in PBS containing 1% BSA, secondary antibodies conjugated with Alexa Fluor 488 anti-rabbit (Life Technologies, Carlsbad, CA, USA) were applied to the sections at a PBS dilution of 1:200 for 1 h at room temperature. Sections were covered with Prolong Gold mounting medium with DAPI (Life Technologies, Carlsbad, CA, USA). Control incubations were performed with blocking peptides for CB1 and CB2 (ab50542 and ab45941, Abcam, UK) and in the absence of primary antibodies for the GPR55. For slide analysis, we used a fluorescent Nikon Eclipse Ti inverted microscope with NIS -Elements AR 4.13.04 software (Nikon Instruments, Melville, NY, USA).

2.3. Cell viability assay and statistical analysis

The ER+ T-47D cell line was treated with CBD alone or in combination with tamoxifen. The HER2+ SK-BR-3 cell line was treated with CBD alone or in combination with trastuzumab, and the TNBC MDA-MB 231 cell line was treated with CBD alone or in combination with cisplatin. The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, USA, MO) was used to measure cell viability at 48 h for the T-47D and SK-BR-3 cell lines and at 72 h for the MDA-MB 231 cell line according to the manufacturer's instructions. In brief, cells were grown overnight in 96-well plates (8000 cells/well). Cells were treated with various concentrations of CBD, fulvestrant, palbociclib, trastuzumab, tamoxifen, and cisplatin. CBD (Tocris, Bristol, UK) was dissolved in

70% ethanol at a 50 mM stock concentration. Tamoxifen (Sigma-Aldrich, USA, MO) was dissolved in methanol at a stock concentration of 12.9 mM. Cisplatin (Sigma-Aldrich, USA, MO) was dissolved in DMSO at a stock concentration of 3.3 mM. Trastuzumab (from hospital) was prepared in saline at a stock concentration of 144 μM. Fulvestrant (Selleckchem, USA, TX) was dissolved in DMSO at a stock concentration of 10 mM. Palbociclib (Selleckchem, USA, TX) was dissolved in PBS at a stock concentration of 50 mM. The control incubations contained the same amount of vehicle solvents, DMSO (≤0.4%), ethanol (≤0.04%), and methanol (≤0.02%). After 48 h or 72 h of treatment, MTT substrate was added, and after 3 h of incubation, absorbance was measured at ΔOD 570/690 352 nm after addition of DMSO using a microplate reader (Synergy™ HT, Bio-Tec Instruments Inc., Winooski, VT, USA). GraphPad Prism software was used for analysis (GraphPad Software, San Diego, 354 CA, USA).

3. Results

3.1. Expression of cannabinoid receptors in breast cancer cell lines

To detect the expression of CBRs in selected ER+ BC cell lines T-47D and MCF-7, we used immunofluorescence and confirmed that both T-47D and MCF-7 expressed CB1 and CB2, but only the T-47D line was positive for the expression of GPR55 (Fig. 1). Blocking peptides against CB1 and CB2 epitopes were used as controls (Fig. S1). In particular, T-47D is strongly positively labeled for CB1 and CB2.

3.2. Cannabidiol affects viability of estrogen receptor-positive and negative breast cancer cell lines

To compare the effect of CBD on ER+ vs. ER- BC subtypes, selected BC cell lines were exposed to increasing concentrations of CBD. The viability of ER+ (T-47D) and ER- (SK-BR-3, and MDA-MB-231) cell lines was decreased in a dose-dependent manner. The ER+ T-47D line, which we confirmed to be CB1-, CB2-, and GPR55-positive (Fig. 2A), was most affected at lower CBD concentrations compared with the SK-BR-3 and MDA-MB-231 lines, with IC50 values of ~ 24 μM for SK-BR-3 and T-47D cell lines after 48 h and ~ 40 μM for MDA-MB-231 after 72 h of treatment (Fig. 2A, B, 2C).

3.3. Cannabidiol affects the viability of estrogen receptor-positive breast cancer cell lines

Estrogen receptor-positive BC cell lines were exposed to increasing concentrations of CBD in combination with endocrine therapy (tamoxifen, fulvestrant) and targeted therapy (palbociclib).

3.3.1. The combination of cannabidiol and tamoxifen

The addition of CBD to tamoxifen in the ER+ T-47D BC cell line did not reduce the efficacy of tamoxifen (Fig. 3A). CBD in combination with tamoxifen had an additive negative effect on cell viability in the T-47D cell line, but the same effect was not observed in the MCF-7 cell line (Fig. 3B).

The inhibitory concentration factor (FIC) for the interactive responses between tamoxifen and CBD in the T-47D cell line was calculated based on the combinations that produce half-maximal inhibition, according to method 2 of Deng et al. [55]. The FIC factor distinguishes between the additive and synergistic effects of two drugs. The FIC efficacy of the combination is defined as: Synergistic (FIC <0.5), Additive (0.5 < FIC <4) and Antagonistic (FIC >4). Thus, the tamoxifen concentration was fixed and the dose-response curve of the inhibitory effect of CBD was plotted in a range of dose-response concentrations. The IC50 of CBD (in the presence of the fixed tamoxifen concentration) was then calculated using GraphPad Prism software. FIC analysis showed that tamoxifen in combination with CBD resulted in an additive response that was 1.77 (Fig. 3).

3.3.2. The combination of cannabidiol, fulvestrant, and palbociclib

CBD had no negative effect on standard BC treatments with palbociclib and fulvestrant (Fig. 4A–B, D-E), except when T-47D BC cells were exposed to a combination of 5 μM fulvestrant and 5 μM CBD (Fig. 4B), in which case CBD increased the viability of T-47D cells. However, this

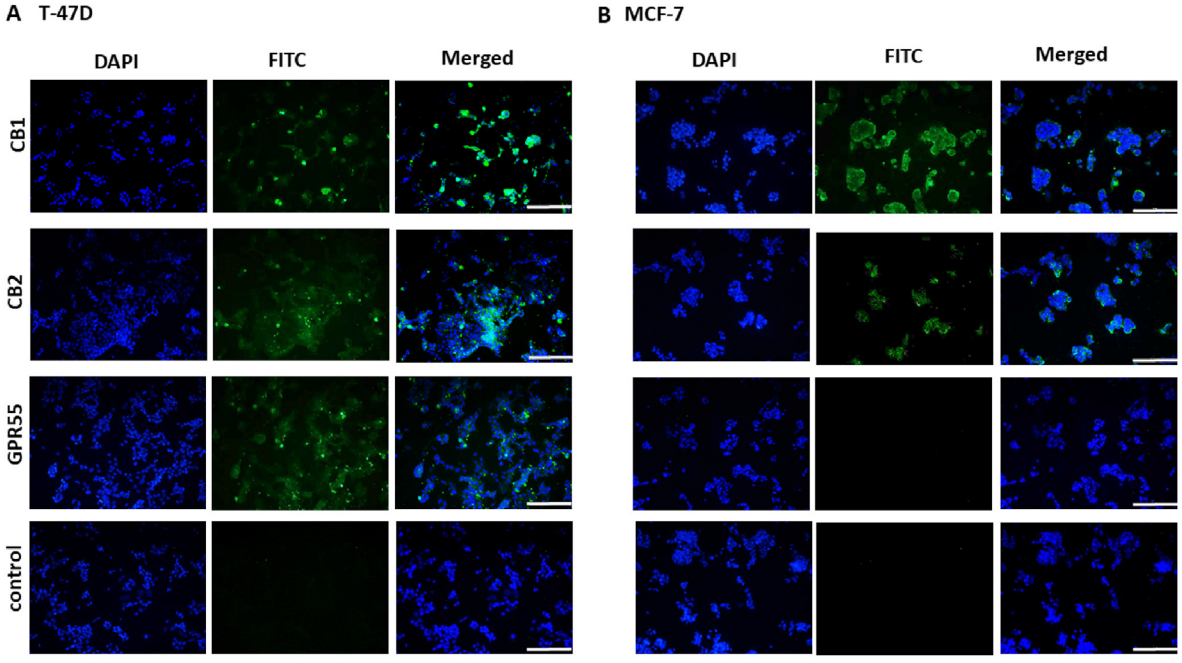

Fig. 1. Immunofluorescence staining of CBRs. Staining of CB1, CB2, and GPR55 in A) T-47D and B) MCF-7. Nuclei were stained with DAPI (blue) and CB1, CB2, and GPR55 were stained with Alexa Fluor 488 (green). Merged images show co-localization of nuclei and CBRs. Microscopy was performed at 20× objective magnification. The scale corresponds to 50 μm. Legend: Anti- CB antibody (CB1 and CB2), anti-G protein-coupled receptor 55 antibody (GPR55). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

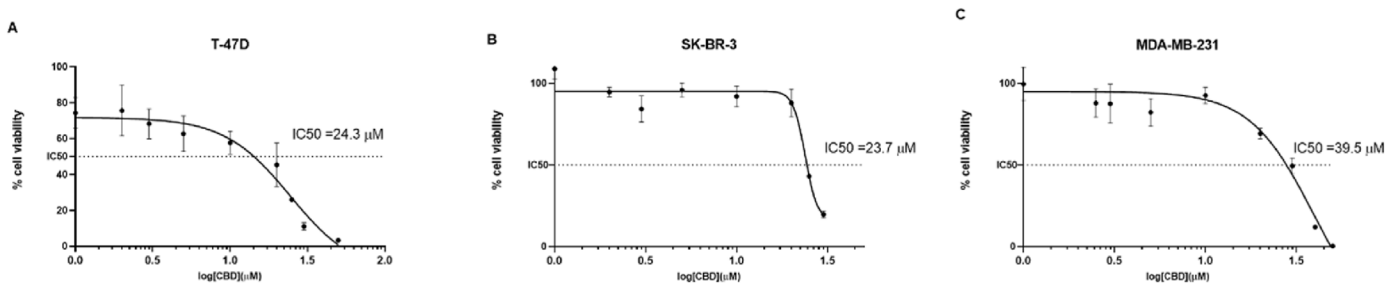

Fig. 2. The effect of CBD on BC cell viability. BC cell lines A) ER+ T-47D, B) HER2+ SK-BR-3, and C) TNBC MDA-MB 231 were exposed to increasing concentrations of CBD. Effects were determined by MTT assay after 48 h for T-47D and SK-BR-3 cell lines and after 72 h for MDA-MB 231 cell line. Values are expressed as percent survival of treated cells compared with the untreated control group (100%). Vehicle contained ≤0.4%, v/v DMSO. Error bars represent mean ± S.E.M. values. Effects were tested in 3 biologicals and 3 technical replicates. Data are expressed as IC50 values generated from dose-response curves using GraphPad Prism (San Diego, CA).

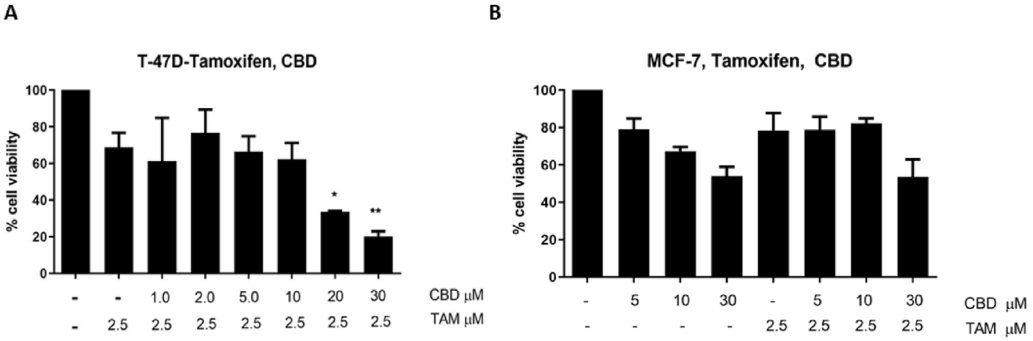

Fig. 3. Effect of cannabidiol alone or in combination with tamoxifen on ER+ cell lines A) T-47D and B) MCF-7. MTT assay was used to measure cell viability after 48 h. The percentage of cell viability was normalized to control cells without treatment. Each value is the mean ± SD (n = 3). *P < 0.05, **P < 0.01, compared with the control group (ANOVA) by GraphPad Prism (n = 3 biological experiments) (San Diego, CA). Legend: Cannabidiol (CBD), Tamoxifen (TAM).

effect was not observed in the MCF-7 BC cell line. Triple combinations of CBD, fulvestrant, and palbociclib were the most effective treatment for inhibiting the viability of T-47D and MCF-7 cells (Fig. 4C, F).

Overall, all estrogen receptor-positive and -negative BC cell lines respond to CBD, in a concentration range of 15–40 μM. Elevated CBD concentrations in combination with the endocrine therapeutic tamoxifen inhibited cell viability in an apparently additive manner or had no effect on tamoxifen treatment. CBD also did not attenuate the effects of treatments with fulvestrant (FUL) and palbociclib, which are used in combination with endocrine therapies in clinical practice (Fig. 4). In addition, we demonstrated that CBD did not attenuate the effects of standard therapy with trastuzumab and cisplatin in HER2+ and TNBC BC cell subtypes (Fig. 5).

4. Discussion

In this study, we confirmed that both ER+ and ER- breast cancer cells expressed different, albeit similar, levels of CBD-binding receptors levels of CBRs such as CB1 and CB2, as well as GPR55 in the ER+ T-47D cell line. Perez-Gomez et al. [51] analyzed a large series of human BC tissue sections. CB2 was expressed in ~75% of the samples regardless of subtype. CB2 expression was strongly associated with HER2+ tumors, whereas no association was found between CB2 expression and HR+ or TNBC. There was an association between higher expression of CB2 in HER2+ disease and lower overall survival, higher likelihood of local recurrence, and development of distant metastases. This association was not observed in HR+ patients [51]. In another study performed on 87 invasive ductal carcinomas, CB1 expression was detected in 14% of cases, whereas CB2 immunoreactivity was detected in 72% of patients [49]. Morin-Buote et al. reported even higher expression levels of CBRs (93.7% for CB1 and 91.0% for CB2) in BC tissues [64]. Andradas et al. found that expression of GPR55 in basal/TNBC was also associated with worse overall and metastasis-free survival.

In our study, CBD decreased the viability of ER+ T-47D, HER2+ SK-BR-3, and TNBC MDA-MB-231 cells in a dose-dependent manner (Fig. 2). We found that the IC50 values were in the range of 20–40 μMol CBD. This is slightly more than the IC50 values recently reported by Schoeman et al. [58] in the same lines TNBC MD-MD 231 and ER+ MCF-7, which were in the range of 12–21 μM, and may be explained by the different exposure and purity of the CBD preparations. Sultan et al. also showed that CBD inhibited the viability of ER+ T-47D BC cell line in a dose-dependent manner by inducing apoptosis in breast cancer cell lines [53]. CBD is known to have antitumor effects in TNBC cell lines [46,55,56,65].

Previous studies have investigated the relationship between cannabinoids and HR+ BC cell lines. They have shown that tamoxifen binds to CB1 and CB2 and acts as an inverse agonist [66]. The nonsteroidal SERMs nafoxidine and raloxifene bind nonselectively to CB1 and CB2. In addition, ospemifin binds selectively to CB1 and bazedoxifin binds to CB2. The above SERMs acted as inverse agonists/antagonists at CBRs [67]. Blasco-Benito et al. [68] exposed the ER+ T-47D cell line to pure THC or cannabis preparation in combination with tamoxifen. Both combinations decreased viability in an additive manner. The additive effects between tamoxifen and cannabinoids observed in cell cultures were not detectable in animal models. The cannabis preparation appeared to be more potent than pure THC in producing antitumor responses [68]. Takeda et al. [69] showed that THC-mediated BC cell growth was stimulated by concomitant treatment with AIs. It was therefore suggested that THC might act as an enhancing agent during concomitant treatment with estrogen-lowering drugs [69]. THC interferes with estrogen signaling by upregulating ERβ [70], and fulvestrant increases ERβ expression in MCF-7 cell lines and animal models [71]. In addition, THC induces cell cycle arrest and apoptosis by downregulating cyclin-dependent kinase 1 [72].

In our study, CBD did not attenuate the effect of the treatments tested,

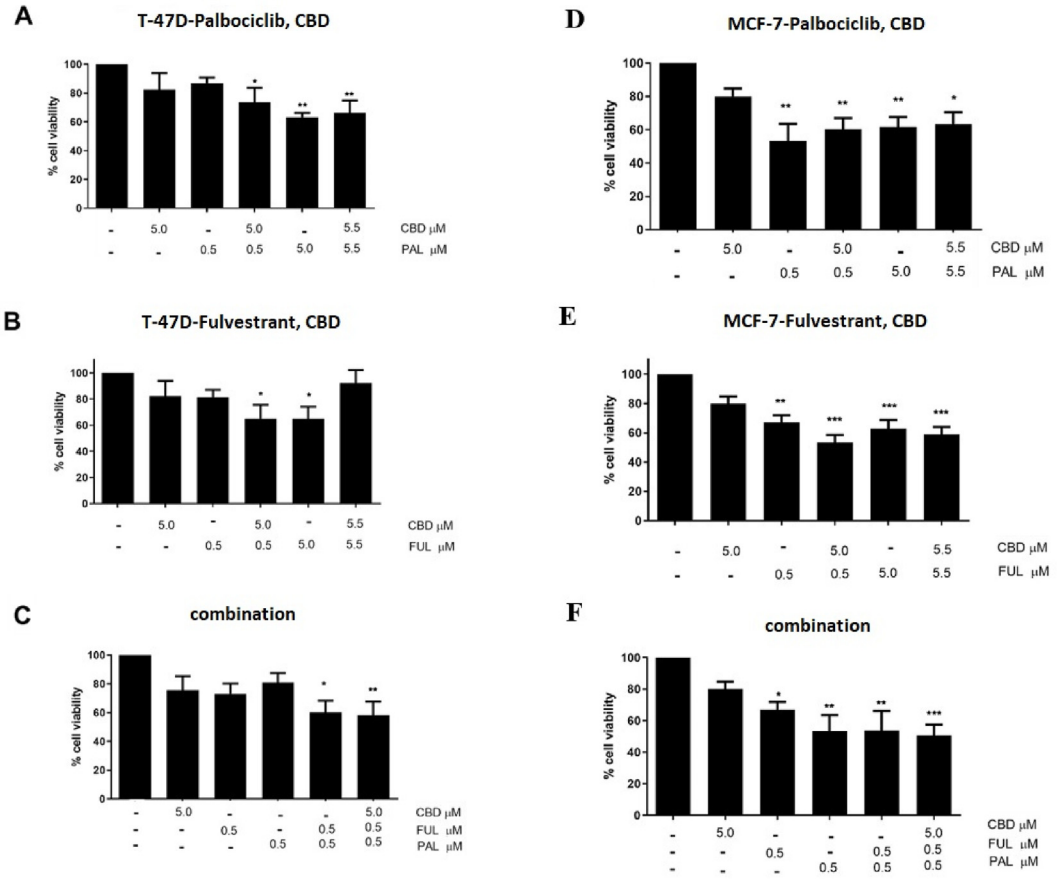

Fig. 4. Effect of cannabidiol, palbociclib, and fulvestrant alone or in combination on ER+ T-47D and MCF-7 cell line. MTT assay was used to measure cell viability at 72 h. The percentage of cell viability was normalized to control cells without treatment. Each value is the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs control group (ANOVA) by GraphPad Prism (n = 3 biological experiments) (San Diego, CA). Legend: Cannabidiol (CBD), fulvestrant (FUL), palbociclib (PAL).

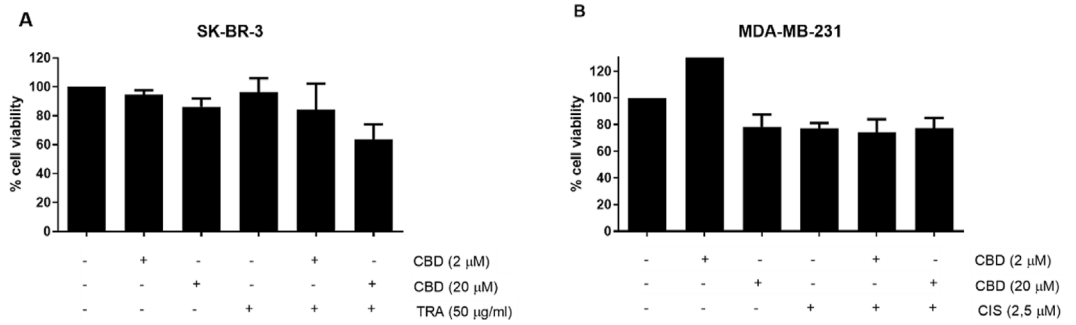

Fig. 5. Effect of cannabidiol alone or in combination with trastuzumab on A) HER2+ SK-BR-3 cell line and B) cisplatin on MDA-MB-231 cell line. Effects were determined by MTT assay at 48 h for SK-BR-3 and 72 h for MDA-MB-231. Results are expressed as percentage of treated cells compared with untreated control cells, with untreated cells set as 100%. Vehicle consisted of ≤0.4%, v/v DMSO. Error bars represent mean ± S.E.M. values. Effects were tested in 3 biological and 3 technical replicates. Legend: Cannabidiol (CBD), trastuzumab (TRA), cisplatin (CIS).

except when T-47D BC cells were exposed to a combination of 5 μM fulvestrant and 5 μM CBD (Fig. 4B). However, this effect was not observed in the MCF-7 BC cell line. The addition of CBD to tamoxifen had an additive negative effect on cell viability in the T-47D BC cell line (Fig. 3A), but the same effect was not observed in the MCF-7 cell line (Fig. 3B). Triple combinations of CBD, fulvestrant, and palbociclib were most effective in inhibiting the viability of T-47D and MCF-7 cells (Fig. 4C and F). CBD did not enhance or reduce the effect of fulvestrant in any of the ER+ BC lines (Fig. 4B and E). Fraguas-Sánchez et al. observed the antitumor activity of CBD-loaded microparticles in ER+ (MCF-7) and ER- cell lines and observed the antitumor activity of CBD-loaded microparticles as monotherapy and in combination with chemotherapy [73]. We demonstrated that CBD did not attenuate the effect of standard therapeutics (trastuzumab and cisplatin) in HER2+ and TNBC BC cell subtypes (Fig. 5).

5. Conclusion

Exposure of BC patients to cannabinoids may directly affect the therapeutic mechanism of endocrine treatment and kinase-targeting or cytotoxic drugs, either through the interaction of CBRs with other plasma membrane receptors on cancer cells, including hormone receptors,

growth factor receptors, or BC resistance proteins and multidrug-resistant proteins. In this study, we systematically investigated the potential effects of purified CBD on a group of ER+/-BC cells as well as HER2+ and TNBC cells. We used CBD for the first time in combination with tamoxifen, fulvestrant, and palbociclib. In addition, we tested the combination of CBD with trastuzumab and cisplatin. CBD did not attenuate the effect of the standard therapeutics. It should be noted that after *in vitro* research, studies in appropriate animal models are needed to determine the benefit and safety of using CBD in different BC subtypes before clinical trials can be conducted.

Author contributions

L.D. and M.N. conceived and wrote the article. S.B., N.D., M.N. conceived the study, and M.N. and F.K. performed IF analyzes, cell viability assays, and GraphPad analyzes. M.N. and F.K. performed all experiments with CBD. N.D. and T.L.T. revised and edited the article. All authors read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.adcanc.2022.100038.

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