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PRECLINICAL STUDY

Pathways mediating the effects of cannabidiol on the reduction of breast cancer cell proliferation, invasion, and metastasis

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Abstract Invasion and metastasis of aggressive breast cancer cells are the final and fatal steps during cancer progression. Clinically, there are still limited therapeutic interventions for aggressive and metastatic breast cancers available. Therefore, effective, targeted, and non-toxic therapies are urgently required. Id-1, an inhibitor of basic helix-loop-helix transcription factors, has recently been shown to be a key regulator of the metastatic potential of breast and additional cancers. We previously reported that cannabidiol (CBD), a cannabinoid with a low toxicity profile, down-regulated Id-1 gene expression in aggressive human breast cancer cells in culture. Using cell proliferation and invasion assays, cell flow cytometry to examine cell cycle and the formation of reactive oxygen species, and Western analysis, we determined pathways leading to the down-regulation of Id-1 expression by CBD and consequently to the inhibition of the proliferative and invasive phenotype of human breast cancer cells. Then, using the mouse 4T1 mammary tumor cell line and the ranksum test, two different syngeneic models of tumor metastasis to the lungs were chosen to determine whether treatment with CBD would reduce metastasis in vivo. We show that CBD inhibits human breast cancer cell proliferation and invasion through differential modulation of the extracellular signal-regulated kinase (ERK) and reactive oxygen species (ROS) pathways, and that both pathways lead to down-regulation of Id-1 expression. Moreover, we demonstrate that CBD up-regulates the pro-differentiation factor, Id-2. Using immune competent mice, we then show that treatment with CBD significantly reduces primary tumor mass as well as the size and number of lung metastatic foci in two models of metastasis. Our data demonstrate the efficacy of CBD in preclinical models of breast cancer. The results have the potential to lead to the development of novel non-toxic compounds for the treatment of breast cancer metastasis, and the information gained from these experiments broaden our knowledge of both Id-1 and cannabinoid biology as it pertains to cancer progression.

Keywords Id-1 · Id-2 · Helix-loop-helix · Cannabinoid · ERK · ROS · Lung metastasis

Abbreviations

CBD	Cannabidiol
Δ^9-THC	Δ^9-Tetrahydrocannabinol
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
Id	Inhibitor of DNA binding
ROS	Reactive oxygen species
TOC	α-Tocopherol

Introduction

The process of metastasis to other tissues of the body is the final and fatal step during cancer progression and is the least understood genetically [1]. Despite all currently available treatments, breast cancer is most often incurable once clinically apparent metastases develop. Clearly,

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effective and non-toxic therapies for the treatment of aggressive and metastatic breast cancers are urgently required.

Id proteins are inhibitors of basic helix-loop-helix transcription factors that control cell differentiation, development, and carcinogenesis [2]. Whereas Id-2 protein has been reported to maintain a differentiated phenotype in normal and cancerous breast cells in mouse and human, increase of Id-1 expression was shown to be associated with a proliferative and invasive phenotype in all these cells [3, 4]. Id-1 enhances breast cancer cell proliferation and invasion through modulation of specific cyclin-dependent kinase inhibitors and matrix metalloproteinases [5]. We found that Id-1 was constitutively expressed at a high level in aggressive breast cancer cells and human biopsies, and that aggressiveness was reverted in culture and in vivo when Id-1 expression was targeted using antisense technology [3]. Importantly, Id-1 was identified as the most active candidate gene in a non-biased in vivo selection, transcriptomic analysis and functional verification validation of a set of human genes that mark and mediate breast cancer tumorigenicity and metastasis to the lungs [4]. Functional studies have demonstrated that Id-1 is required for tumor initiating functions during metastatic colonization of the lung microenvironment [6]. Id-1 also cooperates with the oncogenic Ras to induce metastatic mammary carcinoma, and inactivation of conditional Id-1 expression in the tumor cells can lead to a dramatic reduction of pulmonary metastatic load [7]. Since Id-1 is not expressed in differentiated tissue, but in metastatic tumor cells, reducing Id-1 expression provides a rationale and targeted therapeutic strategy for the treatment of aggressive human breast cancers [3, 4].

The endocannabinoid system was discovered through research focusing on the primary psychoactive active component of *Cannabis sativa*, Δ^9-tetrahydrocannabinol (Δ^9-THC), and other synthetic alkaloids [8]. While there are more than 60 cannabinoids in *Cannabis sativa*, those present in appreciable quantities include Δ^9-THC and cannabidiol (CBD) [9]. Δ^9-THC and additional cannabinoid agonist have been shown to interact with two G-protein coupled receptors named CB_1 and CB_2 [10]. The psychotropic effects of Δ^9-THC, mediated through the CB_1 receptor, limit its clinical utility. CBD, however, does not bind to CB_1 and CB_2 receptors with appreciable affinity and does not have psychotropic activities [11–13]. CBD is well tolerated in vivo during acute and chronic systemic administration [14–18], and cannabinoids are already being used in clinical trials for purposes unrelated to their anti-tumor activity [19, 20]. We recently discovered that CBD was the first non-toxic plant-based agent that could down-regulate Id-1 expression in aggressive hormone-independent breast cancer cells [21]. CBD has also been shown to inhibit breast cancer metastasis in xenograft models [22]. However, the distinct signaling pathways that would explain the inhibitory action of CBD on breast cancer metastasis have not been elucidated. In addition, CBD has been shown to modulate immune system function [23, 24]; therefore, determination of CBD antitumor activity in immune competent animals is critical.

We sought to investigate the signal transduction pathways leading to CBD-induced down-regulation of Id-1. We found that CBD up-regulates the active isoform of the extracellular signal-regulated kinase (ERK) and production of reactive oxygen species (ROS), and that interfering with either of these two pathways can prevent the inhibition of Id-1 expression, cell proliferation, and/or invasion by CBD treatments in human breast cancer cells. Furthermore, we show that treatment with CBD leads to the inhibition of Id-1 gene expression, proliferation, and invasion in mouse mammary cancer cells, and a reduction of primary tumor volume and number of lung metastatic foci in vivo. CBD, therefore, represents a potential non-toxic exogenous agent for the treatment of patients with metastatic breast cancer.

Materials and methods

Ethics statement

All procedures used in this study were in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and incorporated the 1985 U.S. Government Principle. Studies were approved by the California Pacific Medical Center Research Institute's Institutional Animal Care and Use Committee under the protocol #07.05.005. Animals were maintained using the highest possible standard care, and priority was given to their welfare above experimental demands at all times.

Cell culture and treatments

Human breast cancer MDA-MB231 cells were obtained from ATCC and cultured in RPMI media containing 10% fetal bovine serum (FBS). Mouse 4T1 cells were originally obtained from Dr. Fred Miller of the Karmanos Cancer Institute (Detroit, MI) and were cultured in Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, and insulin (0.5 HSP units/ml). On the first day of treatments, media were replaced with vehicle control or 1.5 μM CBD in 0.1% FBS-containing media as reported [25]. The media with the appropriate compounds were replaced every 24 h. CBD was obtained from NIH through the National Institute of Drug Abuse.

Western analysis

Proteins were separated by SDS/PAGE, blotted on Immobilon membrane, and probed with anti-Id-1, anti-Id-2, anti-phospho-p38, anti-phospho-ERK1/2, anti-ERK1/2, anti-NFkB, and the appropriate secondary antibody as we previously described [3, 26]. As a normalization control for loading, blots were stripped and re-probed with mouse anti-α-tubulin or anti-β-actin (Abcam, Cambridge, MA).

Cell cycle analysis

Cells were grown in Petri dishes and received drug treatments for 2 days. The cells were then harvested and centrifuged at 1200 rpm for 5 min. The pellet was washed with PBS + 1% BSA, and centrifuged again. The pellet was resuspended in 0.5 ml of 2% paraformaldehyde (diluted in PBS) and fixed overnight at room temperature. The next day, cells were pelleted and resuspended in 0.5 ml 0.3% Triton in PBS and incubated for 5 min at room temperature. Cells were then washed twice with PBS + 1% BSA. Cells were finally suspended in PBS (0.1% BSA) with 10 μg/ml Propidium Iodide and 100 μg/ml RNAse. Cells were then incubated for 30 min at room temperature before being stored at 4°C. Cell cycle was measured using a FACS Calibur, and Cell Quest Pro and Modfit software.

MTT assay

To quantify cell viability, the MTT assay was used (Chemicon, Temecula, CA). Cells were seeded in 96-well plates. Upon completion of the drug treatments, cells were incubated at 37°C with MTT for 4 h, and then isopropanol with 0.04 N HCl was added and the absorbance was read after 1 h in a plate reader with a test wavelength of 570 nm. The absorbance of the media alone at 570 nm was subtracted, and % control was calculated as the absorbance of the treated cells/control cells $\times 100$.

Boyden chamber invasion assay

Assays were performed in modified Boyden Chambers (BD Biosciences, San Diego, CA) as previously described [3]. Cells at 1.5×10^4 per well were added to the upper chamber in 500 μl of serum-free medium supplemented with insulin (5 μg/ml). The lower chamber was filled with 500 μl of conditioned medium from fibroblasts. After a 20-h incubation, cells were fixed and stained as previously described [3]. Cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. All the invasive breast cancer cells on the lower side of the filter were counted using a light microscope.

Reactive oxygen species measurements

The production of cellular reactive oxygen species (ROS)/H_2O_2 was measured using 2′-7′Dichlorodihydrofluorescein (DCFH-DA, Sigma-Aldrich). Cells were plated onto 6-well dishes and received drug treatments for 3 days. On the second day, 10 μM DCFH-DA was added to the media (DMEM with 0.1% FBS) and the cells were incubated with DCFH-DA for 12 h. The next day, the cells were trypsinized, washed with PBS, and the fluorescent intensity was measured using a FACS Calibur and Cell Quest Pro software.

Generation of primary breast tumors and metastases

Mouse 4T1 cells grown in DMEM with 10% FBS were harvested from dishes while in their exponential growth phase in culture with 0.1% trypsin/EDTA, and washed twice with serum-free DMEM. Breast primary tumors and metastases were generated in female BALB/c mice by the subcutaneous injection of 1×10^5 cells of the mouse mammary tumor cell line 4T1 under the fourth major nipple. Treatment with cannabidiol was initiated upon first detection of the primary tumors (approximately 1 mm^3 at day 7). To determine the tumor size in situ, the perpendicular largest diameters of the tumors were measured in millimeters and tumor volume was calculated as $(L \times W^2)/2$ based on a modified ellipsoidal formula. Approximately 30 days after injection of the tumor cell line, the mice were euthanized. The weight and size of primary tumors were determined, and the lungs were dissected out, infused with 15% India ink intratracheally, and fixed in Fekete's solution. Visible lung metastases were measured and counted by using a dissecting microscope. For the tail vein experiments, mice were injected i.v. with 5×10^4 4T1 cells. Two days after the injection, the tumor-bearing mice were injected i.p. once a day with vehicle (control) or 1 mg/kg CBD for 15 days. After mice were euthanized, the lungs were dissected and the foci counted as described above.

Statistical analysis

The IC_{50} values with corresponding 95% confidence limits were compared by analysis of logged data (GraphPad Prism, San Diego, CA). Significant differences were also determined using ANOVA or the unpaired Student's *t*-test, where suitable. Bonferroni-Dunn post hoc analyses were conducted when appropriate. Pairwise differences in G_0/G_1, S and G_2/G_M in treated vs. control groups were made using a Wilcoxon ranked-sign test. P values < 0.05 defined statistical significance.

To measure secondary tumor growth rates in the orthotopic mouse model, we calculated at each time point the total tumor burden per mouse by summing the product of the number of metastatic foci in a size category times the midpoint size for that category (e.g., for category 0–1, the midpoint is 0.5; for 1–2, the midpoint is 1.5). We then calculated the average tumor burden per metastatic focus by dividing the total tumor burden by the number of metastatic foci. For these summaries we compared (dose > 0 vs. dose 0) at each time point using the Wilcoxon ranksum test since the distributions of these measures were skewed. Pairwise differences in the area between the primary tumor growth curves were also compared using a ranksum test. Our range of measurements of secondary lung metastases size included metastatic foci <1 mm, 1–2 mm, and >2 mm. From these data, an average volume per metastatic focus was calculated. For example, mouse 1 in the control group had 28 metastatic foci <1 mm, 29 foci 1–2 mm, and 11 foci >2 mm. The total volume was $(28 \times 0.5) + (29 \times 1.5) + (11 \times 2.5) = 85$. Therefore, the average volume per metastatic foci was calculated to be 1.25, where the total volume was divided by the number of metastases ($87/68 = 1.25$). Differences in the average volume per metastatic foci were compared using a ranksum test. P values < 0.05 defined statistical significance.

Results

CBD up-regulates extracellular signal-regulated kinase phosphorylation

We have recently shown in culture that cannabidiol effectively down-regulates Id-1 gene expression in breast cancer cells through the inhibition of the endogenous Id-1 promoter and its corresponding mRNA and protein levels [21]. However, the signal transduction mechanisms leading to CBD-induced down-regulation of Id-1 have not been discovered. The ability of CB_1 and CB_2 agonists to inhibit cell growth and invasion has been linked to the modulation of ERK and p38 MAPK activity [27–29]. We determined in MDA-MB231 cells that when inhibition of Id-1 was first observed (48 h), there was a corresponding increase in the active isoform of ERK with no significant change in total ERK (Fig. 1A). In contrast, no modulation of p38 MAPK activity was observed at 24 h as well as 48 h.

We and others previously reported that another member of the Id family, Id-2, was specifically and highly expressed in non-invasive breast cancer cells and represented a marker of good prognosis in breast cancer patients [30, 31]. We therefore determined whether Id-2 expression was up-regulated in human metastatic breast cancer cells upon CBD treatment. After 72 h in the presence of CBD, Id-1 expression was almost undetectable whereas the expression of the pro-differentiation factor Id-2 was significantly increased (Fig. 1B). In order to determine whether the modulation of transcription factor expression was a general phenomenon produced during treatment with CBD, we also assessed the expression of NFkB. We did not detect any difference in the overall level of expression between control and CBD-treated cells.

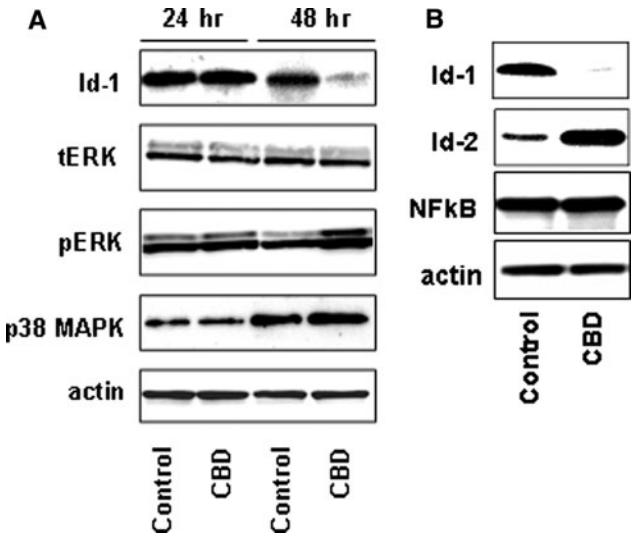

Fig. 1 CBD up-regulates ERK phosphorylation and Id-2 expression. (**A**) Proteins from MDA-MB231 cells treated with 1.5 μM CBD (as previously described [21]) for 1 or 2 days were extracted and analyzed for Id-1, total ERK, pERK, or p38 by Western blot analysis. (**B**) Proteins from MDA-MB231 cells treated with CBD for 3 days were extracted and analyzed for Id-1, Id-2, or NFkappaB by Western blot analysis

CBD inhibits Id-1 expression and corresponding human breast cancer cell proliferation and invasiveness through ERK

We next determined whether CBD inhibition of Id-1 and corresponding breast cancer proliferation and invasion was directly linked to the upregulation of ERK activity. We found that the ERK inhibitor, U0126, could partially reverse the ability of CBD to inhibit the proliferation of MDA-MB231 cells (Fig. 2A). We also found that U0126 could partially reverse the ability of CBD to inhibit the invasion of MDA-MB231 cells (Fig. 2B). These data suggest that activation of ERK by CBD leads to the inhibition of breast cancer cell aggressiveness. To further confirm the involvement of ERK, the expression of Id-1 in MDA-MB231 cells treated with CBD in the presence and absence of U0126 was assessed using Western analysis (Fig. 2C). The ERK inhibitor was able to attenuate the ability of CBD to inhibit Id-1 expression. Taken as a whole, these data suggest that CBD-induced up-regulation

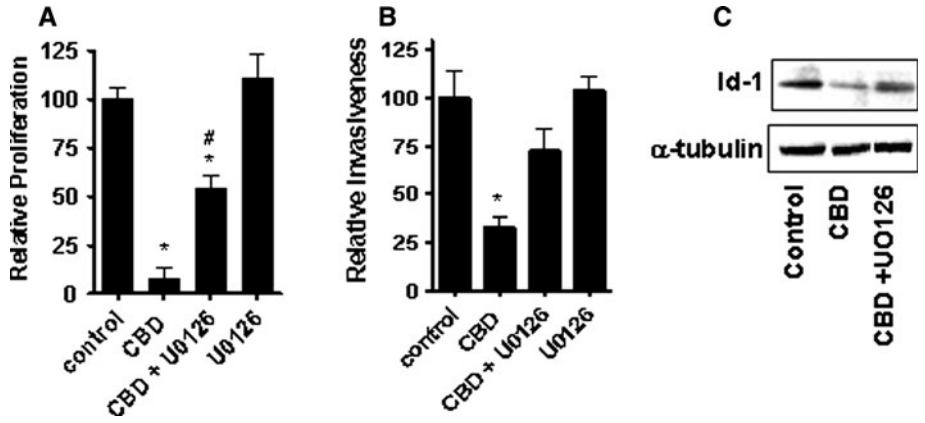

Fig. 2 ERK partly mediates the inhibitory activity of CBD on cell growth and invasion. MDA-MB231 cells were treated for 3 days with vehicle (Control) or 1.5 μM CBD in the presence and absence of 0.1–0.5 μM U0126. Cell proliferation (**A**) and invasion (**B**) were measured using the MTT and Boyden chamber assays, respectively. Data are presented as relative proliferation or invasiveness of the cells, where the respective controls are set as 100%. (**C**) Proteins from MDA-MB231 cells treated with vehicle (control) or 1.5 μM of CBD for 3 days in the absence or presence of U0126 were extracted and analyzed for Id-1 by Western blot analysis. (*) indicates statistically significant difference from control ($P < 0.05$). (#) indicates statistically significant difference from CBD ($P < 0.05$)

of the active isoform of ERK leads to inhibition of both breast cancer cell proliferation and invasion, in part, through the down-regulation of Id-1 expression.

TOC reduces the ability of CBD to inhibit Id-1 expression and corresponding breast cancer cell proliferation and invasion

The ability of CBD to inhibit cancer cell proliferation has also been associated with mitochondrial damage and the increase in production of ROS [28, 32], whereas, the effects of ROS induction by CBD on invasion have not been reported. We determined that the ROS scavenger, α-tocopherol (TOC), could reverse the ability of CBD to inhibit the proliferation of MDA-MB231 cells (Fig. 3A). Moreover, we found that TOC could also significantly reverse the ability of CBD to inhibit the invasion of MDA-MB231 cells (Fig. 3B) and to down-regulate Id-1 protein expression (Fig. 3C). In agreement with the experiments utilizing TOC, CBD led to a direct increase in ROS formation (Fig. 3D), an increase that was reverted upon co-treatment with TOC. These data suggest that production of ROS by CBD can lead to the inhibition of both human breast cancer cell proliferation and invasion, in part, through the down-regulation of Id-1 gene expression.

CBD down-regulates Id-1 expression, proliferation, and invasion in mouse metastatic breast cancer cells

We next determined that CBD treatment of mouse metastatic breast cancer 4T1 cells, an aggressive cell line that over-expresses Id-1 [3], led to an inhibition of Id-1 protein expression (Fig. 4A). Using the MTT assay, we determined that the IC_{50} value for CBD inhibition of 4T1 cell

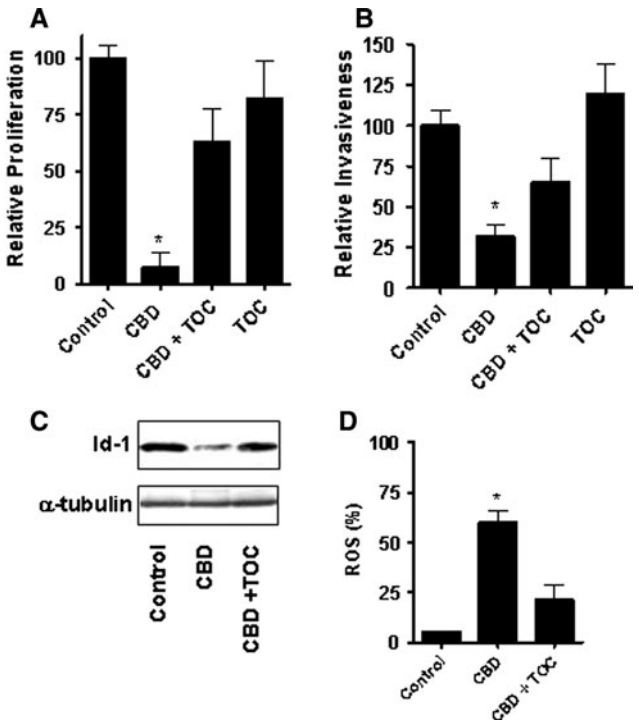

Fig. 3 Production of ROS represents another factor involved in the inhibitory activity of CBD. MDA-MB231 cells were treated for 3 days with vehicle (Control) or 1.5 μM CBD in the presence and absence of 20 μM TOC. Cell proliferation (**A**) and invasion (**B**) were measured using the MTT and Boyden chamber assay, respectively. (**C**) Proteins from cells treated with vehicle (control) or 1.5 μM of CBD for 3 days in the absence or presence of TOC were extracted and analyzed for Id-1 by Western blot analysis. (**D**) The production of ROS was measured using 2′-7′Dichloro-dihydrofluorescein (Sigma-Aldrich). (*) indicates statistically significant differences from control ($P < 0.05$)

proliferation was 1.5 μM (1.3–1.7). The significant reduction in 4T1 cell proliferation observed in the presence of CBD led us to hypothesize that there would be a

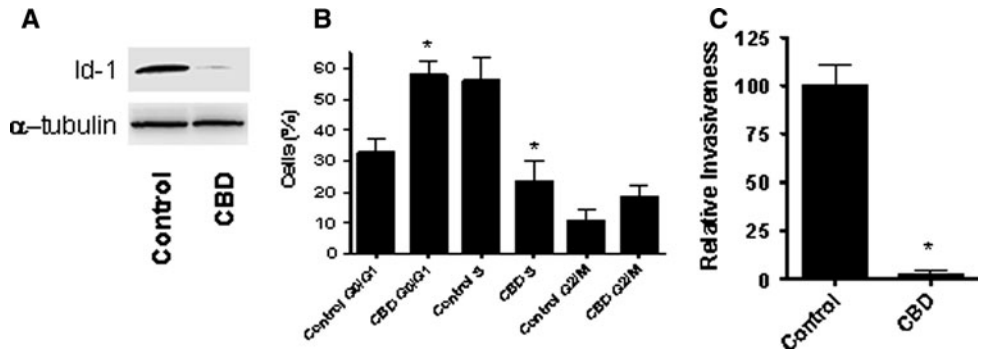

Fig. 4 CBD inhibits the expression of Id-1 and corresponding breast cancer proliferation and invasion in mouse 4T1 cells. (**A**) 4T1 cells were treated for 3 days with 1.5 μM CBD, proteins were extracted and analyzed for Id-1 expression. (**B**) 4T1 cells were collected and cell cycle analyzed using a desktop FACS Calibur with Cell Quest Pro software (BD Bioscience, CA). The distribution of cells in different cell cycle stages was determined according to their DNA content. (**C**) Invasion assays were carried out using the Boyden chamber assay. (*) indicates statistically significant differences from control ($P < 0.05$)

corresponding modulation of the cell cycle. Therefore, 4T1 cells were treated with vehicle (Control) or 1.5 μM CBD, and the cell cycle was analyzed using propidium iodide staining and cell flow cytometry (Fig. 4B). Pairwise differences in G_0/G_1, S, and G_2/G_M of control and treated (CBD 1.5 μM) cells were compared using a Wilcoxon ranked-sign test. CBD produced a significant increase in the population of cells in the G_0/G_1 phase (33% ± 4 for the Control and 58% ± 4 for 1.5 μM CBD) and a marked decrease in cells in the S phase (56% ± 7 for the Control and 23% ± 7 for 1.5 μM CBD). The differences for G1 and S were statistically significant (2-sided, $P = 0.03$). However, the difference for the number of cells in G_2/G_M was not significant ($P = 0.06$) (11% ± 3 for the Control and 19% ± 3 for CBD 1.5). Similar changes were observed in MDA-MB231 cells, albeit, the overall magnitude of effect produced by CBD was reduced compared to 4T1 cells (data not shown). In agreement with our previous findings in human metastatic breast cancer cells [21], we observed that the down-regulation of Id-1 in 4T1 cells led to a corresponding inhibition of cell invasiveness (Fig. 4C).

CBD reduces breast cancer metastasis in vivo using syngeneic models

CBD has been shown to reduce breast cancer metastasis in vivo in a xenograft model of breast cancer using the MDA-MB231 cells [22]. However, it is known that CBD can modulate specific functions of the immune system [23, 24]. Since the immune system has an important role in the inhibition of cancer progression [33], it was essential to determine whether CBD demonstrates antitumor activity in an immune competent mouse model using syngeneic animals. In addition, we wanted to carry out a more detailed analysis on secondary metastatic foci formation in the presence of CBD. As our in vivo model, we used the 4T1 murine metastatic breast cancer cells described above, which primarily metastasize to the lung of syngeneic BALB/c mice. As presented above, CBD was effective at down-regulating Id-1 and corresponding 4T1 cell proliferation and invasiveness (Fig. 4). We therefore determined whether CBD would inhibit tumor growth and metastasis of 4T1 cells to the lung in vivo.

To investigate the activity of CBD in vivo, 4T1 cells were first grown orthotopically in syngeneic BALB/c mice. In this model, the activity of CBD can be assessed on the growth of the primary tumor as well as on the metastatic spread to the lung. Mice were treated daily by intra-peritoneal injection with vehicle (used as a control) or either 1 or 5 mg/kg of CBD. We found that both doses of CBD significantly reduced the growth of the primary tumor in vivo (Fig. 5A). Pairwise differences in the area between the primary tumor growth curves were compared using a ranksum test. A significant reduction in the primary tumor growth was observed in both CBD-treated groups beginning at day 18 and continued throughout the study ($P < 0.01$). The peak inhibitory activity of CBD was observed from day 22–24 ($P < 0.0001$). As demonstrated in Fig. 5A, the primary tumor acquired resistance to the inhibitory properties of CBD by approximately day 25, and by the end of the study (day 30) there was no significant difference between treatment groups when the weight of the tumors were assessed (Fig. 5B).

We next determined that 1 and 5 mg/kg CBD reduced metastasis of 4T1 breast cancer cells in vivo. The average number of metastatic foci was 26 in the control group, 17 in the group treated with 1 mg/kg CBD, and 10 in the group treated with 5 mg/kg CBD (Fig. 5C). In each of these groups secondary lung metastases were measured to include metastatic foci <1 mm, 1–2 mm, and >2 mm. From these data, an average volume per metastatic foci was

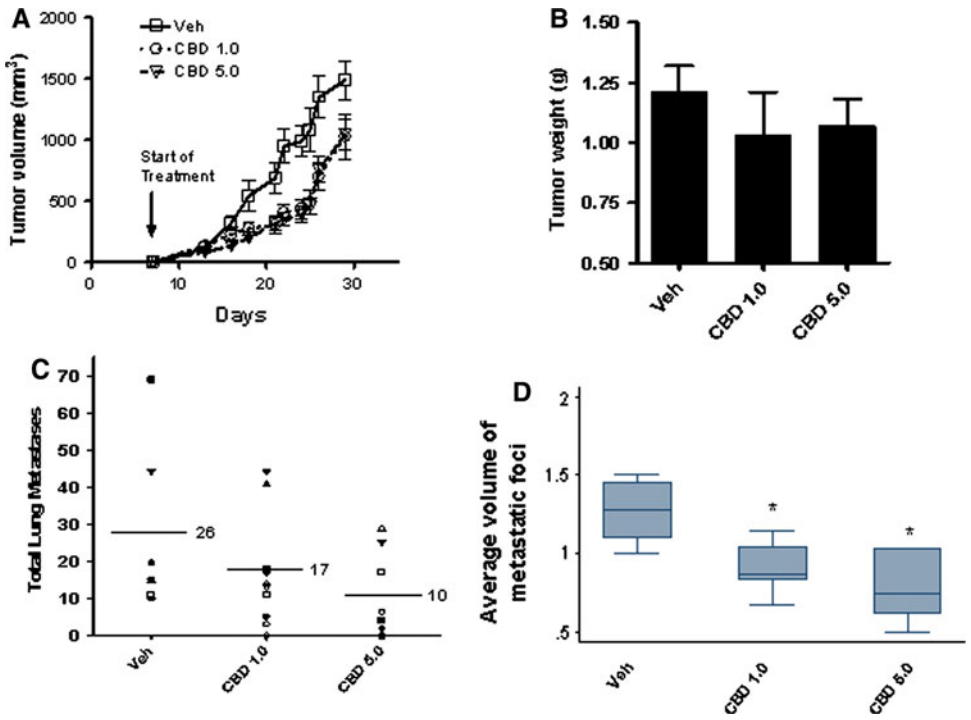

Fig. 5 CBD reduces primary tumor growth and metastasis of 4T1 cancer cells in an orthotopic mouse model. Primary tumors and subsequent secondary tumors (metastases) were generated in BALB/c mice by subcutaneous injection of 1×10^5 4T1 cells under the fourth major nipple. Treatment with CBD was initiated upon detection of the first palpable tumor (approximately 7 days). (**A**) The primary tumor volume was calculated by measuring the perpendicular largest diameters of the tumor with a caliper. (**B**) The weight of the tumors was also measured. (**C**) The visible lung metastases were measured using a dissecting microscope. (**D**) The average volume per metastatic foci was calculated as described in the methods. (*) indicates statistically significant differences from vehicle ($P < 0.05$)

calculated as described in the methods. Differences in the average volume per metastatic foci were compared using a ranksum test. We determined that 1 and 5 mg/kg of CBD significantly decreased the average volume per metastases in a dose-dependent manner ($P < 0.05$) as shown in Fig. 5D. In addition, a visual analysis of the lungs using a dissecting microscope revealed no metastases in one of the eight mice in the 1 mg/kg group and in two of the eight mice in the 5 mg/kg group (Fig. 5C). This is in contrast to the control group, where metastases could be visualized in all the lungs analyzed. The data also showed that CBD selectively inhibited the formation of metastatic foci >1 mm (Supplementary Figure 1).

To further determine whether CBD could effectively reduce the formation of metastases, we injected 4T1 cells directly into the tail vein of syngeneic BALB/c mice and then treated the animals (Fig. 6). In this model, cancer cells have direct access to the blood stream resulting in a significant enhancement of lung metastasis and reduced variability in the number of metastases formed. Two days after i.v. injection of 4T1 cells, the tumor-bearing mice were injected i.p. once a day with vehicle or 1 mg/kg CBD for 15 days. As described for the orthotopic model, treatment with CBD resulted in a reduction of the total amount of metastatic foci, specifically intermediate sized (1–2 mm) or macrometastases (>2 mm). In conclusion, the in vivo data demonstrate that CBD decreases primary tumor growth and significantly reduces metastasis in immune competent mouse models of breast cancer.

Discussion

The path of cancer progression is determined by alterations in the regulatory mechanisms of growth/invasion and differentiation. The expression of Id-1 protein (an inhibitor of basic helix-loop-helix transcription factors) has been reported to be dysregulated in over 20 types of cancer, and suggested as a key determinant of tumorigenesis and/or metastasis in a wide range of tissues, including the breast [34, 35]. Reducing Id-1 expression (a gene whose expression is absent in most of the healthy adult tissues) could therefore provide a rational therapeutic strategy for the treatment of aggressive cancers. Our previous data suggested that CBD represented a non-toxic plant derived compound that could reduce Id-1 expression and corresponding breast cancer metastasis [21]. A key piece of data that was needed in order to increase enthusiasm for the

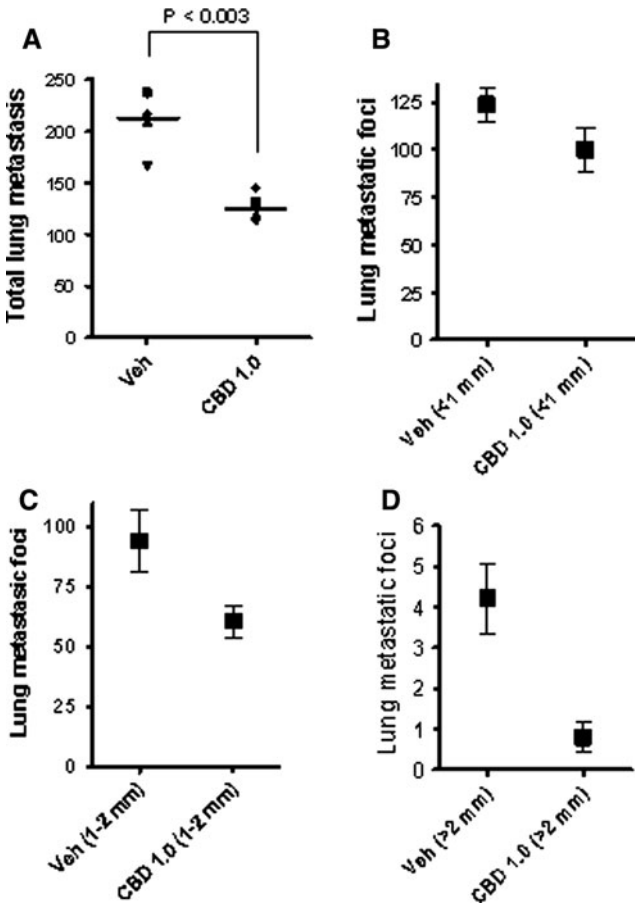

Fig. 6 CBD reduces the number of metastatic foci in the syngeneic model of tail vein injection. Lung metastases were generated in BALB/c mice after tail vein injection of 5×10^5 4T1 cells. One day after the injection, the tumor-bearing mice were injected i.p. once a day with vehicle or 1 mg/kg CBD for 15 days. Visible lung metastases were counted and measured by using a dissecting microscope (**A**). Lung metastases measured included those (**B**) <1 mm, (**C**) 1–2 mm, and (**D**) >2 mm

development of future clinical trials was the establishment of molecular pathways leading to reduction of Id-1 expression and corresponding breast cancer metastasis. In addition, CBD has been shown to be immune suppressive; therefore, it was essential to determine whether antimetastatic activity of the cannabinoid would be observed in immune competent mice.

Activation of the ERK is generally accepted to result in the stimulation of cell growth [36]. However, other studies show that in certain instances ERK activation can lead to the inhibition of cell growth [37–39]. The defining factor is the duration of the stimulus, i.e., more sustained up-regulation of ERK activity leads to inhibition of cell growth whereas short-term up-regulation leads to cell growth [37–39]. In cancer cell lines, CB_1 and CB_2 agonists have been shown to modulate ERK and p38 MAPK [28, 29, 40, 41]. However, there is a clear difference in the activity produced (sustained stimulation versus inhibition), and is dependent upon the agonist used and the cancer cell line studied. Sustained up-regulation of ERK activity by treatment with CB_1 and CB_2 agonists has been shown to be an essential component of receptor-mediated signal transduction leading to the inhibition of brain cancer cell growth [40, 42]. Although CBD has negligible affinity for CB_1 and CB_2 receptors, CBD has been shown to control cell migration through the activation of ERK [13, 43]. Our data demonstrated that treatment of breast cancer MDA-MB231 cells with CBD leads to the up-regulation of the active isoform of ERK, and this led to the inhibition of Id-1 gene expression, and consequently the down-regulation of cell growth and invasion.

ROS can act in concert with intracellular signaling pathways to regulate the balance of cell proliferation versus cell cycle arrest [44]. The ability of CBD to inhibit cancer cell proliferation has been associated with mitochondrial damage and the increase in production of ROS potentially through alterations in NAD(P)H oxidases [28, 32]. We determined that CBD directly produced the formation of ROS and that the ROS scavenger, α-tocopherol (TOC), could reverse the ability of CBD to inhibit Id-1 expression as well as the proliferation and invasion of MDA-MB231 cells. These data provide evidence that the production of ROS by CBD leads to the inhibition of breast cancer cell aggressiveness. Overall, our results also suggest that the two independent pathways activated by CBD (ERK and ROS) can both decrease Id-1 gene expression, and consequently cell proliferation and invasion.

We found that systemic administration of CBD could lead to a significant reduction in primary tumor growth and metastasis in immune competent mouse models of breast cancer. The primary tumors acquired resistance to the inhibitory properties of CBD by approximately day 25. The data are suggestive of adaptive versus innate drug resistance and could involve a number of mechanisms such as the altered activity of key metabolic pathways [45]. Treatment of tumor-bearing mice with CBD produced a dose-dependent reduction in the total number and volume of secondary tumors formed, and preferentially reduced metastatic foci over 1 mm in size. In the highest dose group (5 mg/kg), a visual analysis of the lungs using a dissecting microscope revealed no metastases in two of the eight mice. The more effective inhibition of metastasis by CBD compared to its activity on primary tumor growth may be the result of its preferential effects on cell invasiveness, particularly in the in vivo model where metastasis occurs following primary tumor formation. Regarding the model of metastasis after tail vein injection, it has been previously reported that trapping and extravasation into lung tissue after tail vein injection, and before metastasis formation, could take up to 3–4 days [46]. Thus, since extravasation was only complete after 4 days, a treatment

with CBD starting one day after the injection of cells could have some effects on the escape of cancer cells from the circulation. The inhibition of 4T1 cell growth could also explain the differences in size of metastatic foci between control and treated groups in both in vivo models presented.

We analyzed the expression of the marker of proliferation Ki67 in 4T1-derived metastases in lung samples collected from vehicle- and CBD-treated groups (data not shown). Only a limited number of tumor-bearing lung samples could be obtained from mice that responded to CBD treatment because of the significant efficacy of the treatment. Moreover, the need to use a majority of the lungs for measurement of metastatic foci precluded the immunohistochemical analysis of these samples. In the limited number of tumor-bearing samples available, no differences in the proliferation of 4T1 cells between vehicle- and CBD-treated groups were observed. Although, this result suggests CBD does not inhibit 4T1 cell proliferation in the lung, we hypothesize that the formation of a limited number of tumors in the CBD-treated group could also be the result of the expansion of clones or populations of tumor cells resistant to the treatment. Therefore, it is expected that differences in Ki67 staining would not be observed. Future studies will be needed in order to understand the full spectrum of inhibitory effects that CBD treatment has on the formation of metastases in lung.

In summary, the effects of CBD may occur through reduction of tumor cell proliferation, a decrease in the intravasation and extravasation of tumor cells, and/or through tumor reinitiation after cells reach their target tissues. Moreover, new blood vessels are required for a tumor mass to progress beyond 2 mm [47]; therefore, CBD may also inhibit angiogenesis. Indeed, the most dramatic effect of CBD on secondary tumors was on the reduction of metastatic foci >2 mm. Since Id-1 is also expressed in endothelial cells during neoangiogenesis [2], treatment with CBD could also down-regulate Id-1 gene expression in both tumor cells and endothelial cells involved in neovascularization.

Conclusions

We expect that analogs of CBD could be created that are more active at inhibiting Id-1 and corresponding breast cancer cell aggressiveness compared to CBD. Therefore, our objective is to bring CBD to the clinic first, and then follow up with second-generation analogs. The development of a compound (and perhaps a family of compounds) that is non-toxic, efficacious, and specifically targets metastatic cancer cells would make a significant contribution to the eradication of breast cancer. There is a general consensus in the field of cancer research that targeting multiple pathways that control tumor progression is the best strategy for the eradication of aggressive cancers [48]. Since CBD has a low toxicity, it would be an ideal candidate for use in combination treatments with additional drugs already used in the clinic. Importantly, CBD appears to be interacting through a cellular system that regulates the expression of key transcriptional factors (e.g., Id-1) that control breast cancer cell proliferation, migration, and invasion. The experiments described in this manuscript not only define the pathways that CBD is working through to control breast cancer cell aggressiveness, but also demonstrate the efficacy of CBD in pre-clinical models. A greater understanding of this system may lead to future therapies for breast cancer patients, including the additional refinement of CBD analog synthesis.

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Conflict of interest The authors have declared that no competing interests exist.

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