

Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells

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Abstract

Invasion and metastasis of aggressive breast cancer cells is the final and fatal step during cancer progression, and is the least understood genetically. Clinically, there are still limited therapeutic interventions for aggressive and metastatic breast cancers available. Clearly, effective 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. Using a mouse model, we previously determined that metastatic breast cancer cells became significantly less invasive *in vitro* and less metastatic *in vivo* when Id-1 was down-regulated by stable transduction with antisense Id-1. It is not possible at this point, however, to use antisense technology to reduce Id-1 expression in patients with metastatic breast cancer. Here, we report that cannabidiol (CBD), a cannabinoid with a low-toxicity profile, could down-regulate Id-1 expression in aggressive human breast cancer cells. The CBD concentrations effective at inhibiting Id-1 expression correlated with those used to inhibit the proliferative and invasive phenotype of breast cancer cells. CBD was able to inhibit Id-1 expression at the mRNA and protein level in a concentration-dependent fashion. These effects seemed to occur as the result of an inhibition of the Id-1 gene at the promoter level. Importantly, CBD did not inhibit invasiveness in cells that ectopically expressed Id-1. In conclusion, CBD represents the first nontoxic exogenous agent that can significantly decrease Id-1

expression in metastatic breast cancer cells leading to the down-regulation of tumor aggressiveness. [Mol Cancer Ther 2007;6(11):2921–7]

Introduction

The development of breast cancer and its spread to other parts of the body requires several genotypic and phenotypic changes in the cells leading to de-differentiation, uncontrolled proliferation, and invasion. Invasion and metastasis to the 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 develops.

Id helix-loop-helix proteins are negative regulators of basic helix-loop-helix transcription factors (2). Strong evidence now suggests that the Id family of helix-loop-helix proteins control cellular processes related to tumor progression (3). We found that reducing Id-1 using antisense technology led to significant reductions in breast cancer cell proliferation and invasiveness *in vitro* and metastasis *in vivo* in mice (4). Furthermore, Id-1 overexpression in breast cancer cells was also found to be one of the most significant genes within a gene signature set that is correlated with the propensity of primary human breast cancer cells to metastasize to the lung (5).

Reducing Id-1 expression could provide a rational therapeutic strategy for the treatment of aggressive human breast cancers. It is not possible at this point, however, to use antisense technology to reduce Id-1 expression in humans with metastatic breast cancer. In our search for a nontoxic exogenous compound that could inhibit Id-1 expression, a potential candidate agent, cannabidiol (CBD), was discovered.

The endocannabinoid system was discovered through research focusing on the primary psychoactive component of *Cannabis sativa*, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), and other synthetic cannabinoids (6). Δ^9 -THC and additional cannabinoid agonists have been shown to interact with two G protein-coupled receptors named CB₁ and CB₂ (6). More recent studies have shown that CB₁ and CB₂ receptor agonists show promise as tumor inhibitors (7, 8). The psychotropic effects of Δ^9 -THC and additional cannabinoid agonists, mediated through the CB₁ receptor, limit their clinical utility. In addition to Δ^9 -THC, CBD is also present in significant quantities in *C. sativa* (9). CBD does not have appreciable affinity for CB₁ or CB₂ receptors and does not have psychotropic activities (10). CBD has been shown to inhibit breast cancer metastasis *in vivo* in mice (11). However, modulation of a distinct signaling pathway that would explain the inhibitory action of CBD on breast cancer metastasis has not been elucidated.

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Our data presented here show that CBD represents the first exogenous agent that can down-regulate Id-1 expression in aggressive hormone-independent breast cancer cells. We suggest that CBD down-regulation of Id-1 and corresponding inhibition of human breast cancer cell proliferation and invasiveness provides a potential mechanism for the antimetastatic activity of the compound.

Materials and Methods

Cell Culture and Treatments

We used the human breast cancer cells lines MDA-MB231 and MDA-MB436 obtained from American Type Culture Collection. To prepare the MDA-MB231-Id-1 cells, cells were infected with a pLXSN-Id-1 sense expression vector. In all experiments, the different cell populations were first cultured in RPMI medium containing 10% fetal bovine serum. On the first day of treatment, the medium was replaced with vehicle control or drug in RPMI and 0.1% fetal bovine serum as previously reported (12). The media with the appropriate compounds were replaced every 24 h. Δ^9 -THC, CBN, CBD, CBG, and CP55,940 were obtained from the NIH through the National Institute of Drug Abuse. WIN55,212-2 was purchased from Sigma-RBI.

MTT Assay

To quantify cell proliferation, the MTT assay was used (Chemicon). 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 medium alone at 570 nm was subtracted, and percentage control was calculated as the absorbance of the treated cells/control cells $\times 100$.

Boyden Chamber Invasion Assay

Assays were done in modified Boyden chambers (BD Biosciences) as previously described (4). Cells at 1.5×10^4 per well were added to the upper chamber in 500 μ L of serum-free medium supplemented with insulin (5 μ g/mL). The lower chamber was filled with 500 μ L of conditioned medium from fibroblasts. After a 20-h incubation, cells were fixed and stained as previously described (4). Cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. Invasive breast cancer cells on the lower side of the filter were counted using a light microscope.

Quantitative Western Analysis

Proteins were separated by SDS-PAGE, blotted on Immobilon membrane, and probed with anti-Id-1 and the appropriate secondary antibody as previously described (4, 13). Band intensity values were obtained directly from the blot using AlphaEaseFC software or from film using Image-J (NIH). As a normalization control for loading, blots were stripped and reprobed with mouse alpha-tubulin (Abcam).

PCR

Total cellular RNA was isolated from breast cancer cells treated with vehicle control or with CBD. Transcripts for Id-1 and for β -actin were reverse-transcribed using

Superscript II Reverse Transcriptase II (Life Technologies), and PCR was done. The 5' and 3' PCR primers were AGGTGGTGCCTGTCTGTCT and TAATTCCTCTTGCC-CCCTGG for Id-1; and GCGGGAATCGTGCGTGACATT and GATGGAGTTGAAGGTAGTTTCGTG for β -actin. PCR was done in buffer containing 1 μ mol/L of each of the 5' and 3' PCR primers and 0.5 units of Taq polymerase using 25 cycles for amplification of Id-1 and β -actin cDNAs. The cycle conditions were 45 s denaturation at 94°C, 45 s annealing at 55°C, and 1 min extension at 72°C.

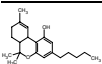
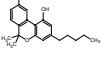
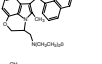
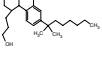
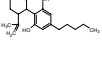
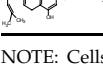
Id-1 Promoter Reporter Assays

A *SacI*-*Bsp*HI fragment of 2.2 kb corresponding to the 5' upstream region of human Id-1 gene and driving a luciferase gene in a PGL-3 vector (Promega) has already been described (Id-1-sbsluc; ref. 13). Cells were plated in six-well dishes in medium supplemented with 10% fetal bovine serum and 5 μ g/mL insulin. After 24 h, cells were cotransfected with 6 μ g of luciferase reporter plasmids and 2 μ g of pCMV β (Clontech) using Superfect reagent (Qiagen). pCMV β contained bacterial β -galactosidase and served to control for variation in transfection efficiency. Three hours after transfection, the cells were rinsed twice with PBS and were cultured in the absence or presence of CBD for 48 to 72 h. Cell pellets were lysed in 80 μ L of reporter lysis buffer (Promega) for 10 min at room temperature. Lysed cells were centrifuged and supernatants harvested. Luciferase and β -gal assays were done using Luciferase assay system (Promega), β -Gal assay kit (Clontech), and a 2010 luminometer (PharMing).

Statistical Analysis

The IC₅₀ values with corresponding 95% confidence limits were compared by analysis of logged data (Graph-Pad Prism). When only the confidence limits of the IC₅₀

Table 1. Antiproliferative potencies of cannabinoids during a 3-d treatment of MDA-MB231 and MDA-MB436 breast cancer cells

Compound	MDA-MB231	MDA-MB436
 Δ^9 -THC	1.2 (1.0–1.4)	2.5 (1.8–3.4)
 CBN	1.2 (0.9–1.5)	2.6 (1.8–3.7)
 WIN55,212-2	1.7 (1.5–2.2)	2.4 (1.6–3.4)
 CP55,940	2.5 (1.5–4.1)	1.3 (0.7–1.6)
 CBD	1.3 (1.0–1.9)	1.6 (1.1–2.2)
 CBG	2.3 (2.1–2.5)	2.1 (1.5–3.0)

NOTE: Cells were treated with cannabinoid compounds for 3 d and the IC₅₀ values for the antiproliferative effects of the compounds were calculated. Data are the means and corresponding 95% confidence limits of at least three experiments. IC₅₀ values are reported in μ mol/L.

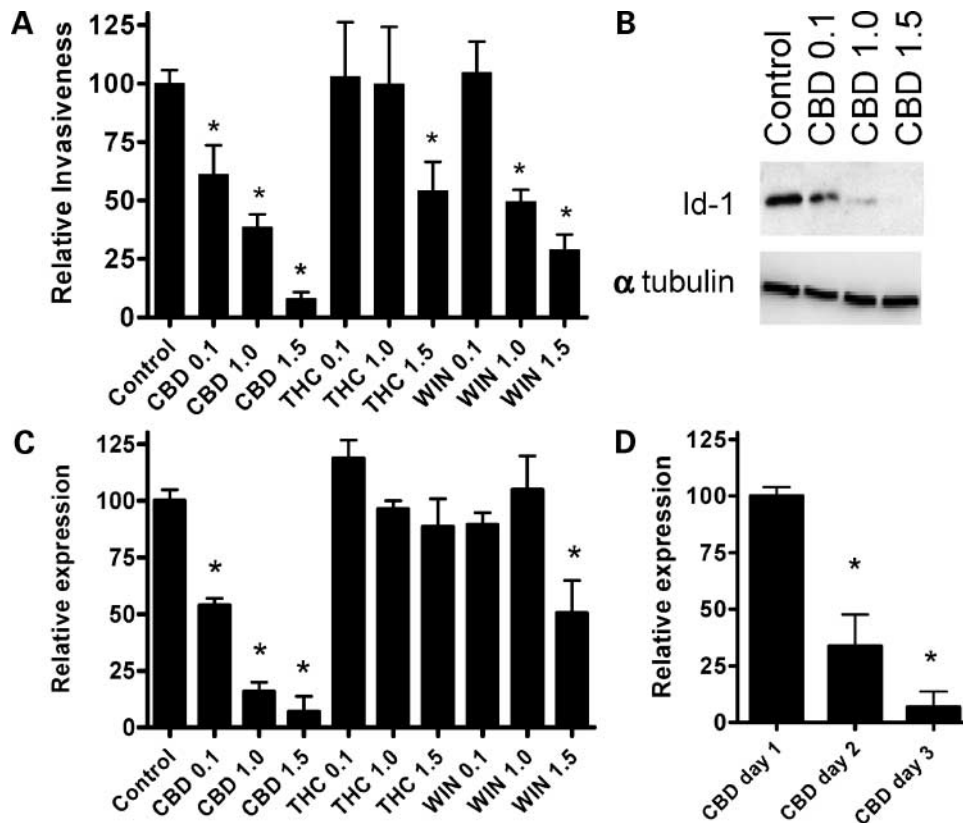


Figure 1. CBD is the most effective inhibitor of invasiveness and Id-1 expression in MDA-MB231 cells. **A**, the Boyden chamber invasion assay was used to determine the effects of cannabinoids on the invasiveness of aggressive human breast cancer MDA-MB231 cells. Compounds were added at concentrations of 0.1, 1.0, or 1.5 $\mu\text{mol/L}$. Data are presented as relative invasiveness of the cells through the Matrigel, where the respective controls are set as 100%. **B**, proteins from MDA-MB231 cells treated with vehicle (control), 0.1, 1.0, or 1.5 $\mu\text{mol/L}$ of CBD for 3 d were extracted and analyzed for Id-1 by Western blot analysis as described in Materials and Methods. **C**, proteins from MDA-MB231 cells treated with additional cannabinoids for 3 d were extracted and analyzed for Id-1 by Western blot analysis. Normalization was carried out by stripping the blots and reprobing with a monoclonal antitubulin antibody. Densitometry readings of the blots were taken and the percentage of relative expression was calculated as the expression of Id-1 in the treated cells / vehicle cells \times 100. **D**, the inhibitory effect of 1.5 $\mu\text{mol/L}$ of CBD on Id-1 expression was compared over a time course of 1, 2, and 3 d. Columns, mean of at least three replicates; bars, SE. Data were compared using a one-way ANOVA with a corresponding Dunnett's post hoc test. *, $P < 0.05$, statistically significant differences from control.

values overlapped, significant differences were determined using unpaired Student's *t* test. Significant differences were also determined (Prism) using ANOVA or the unpaired Student's *t* test, where suitable. Bonferroni-Dunn post hoc analyses were conducted when appropriate. $P < 0.05$ values defined statistical significance.

Results

Cannabinoids Reduce the Growth of Aggressive Human Breast Cancer Cells

In order to test their antiproliferative activities, three groups of cannabinoid compounds were chosen: (a) natural cannabis constituents that have affinity for CB_1 and CB_2 receptors, Δ^9 -THC and CBN; (b) synthetic cannabinoid analogues that have high affinity for CB_1 and CB_2 receptors, WIN55,212-2 and CP55,940; and (c) natural cannabis constituents that do not have appreciable affinity for CB_1 and CB_2 receptors, CBD and CBG. Breast cancer cells were treated for 3 days and IC_{50}

values were calculated (Table 1). The rank order of potencies for the antiproliferative effects of the cannabinoids in MDA-MB231 cells was: CBD = Δ^9 -THC = CBN > WIN55,212-2 > CBG = CP55,940. The rank order of potencies for the antiproliferative effects of the cannabinoids in MDA-MB436 cells was: CBD = CP55,940 > CBG = WIN55,212-2 = Δ^9 -THC = CBN. Overall, the data showed that CBD was the most effective inhibitor of human breast cancer cell proliferation.

Cannabinoids Reduce Breast Cancer Cell Invasiveness

Invasion is an important step towards breast cancer cell metastasis. Therefore, we next determined the effects of several cannabinoids on the ability of the most aggressive human breast cancer cell line, MDA-MB231, to migrate and invade a reconstituted basement membrane in a Boyden chamber. All three compounds tested, i.e., CBD, Δ^9 -THC, and WIN55,212-2, significantly reduced the invasion of MDA-MB231 cells (Fig. 1A). Again, as was observed with the cell proliferation experiments, the most potent inhibitor of invasion was CBD.

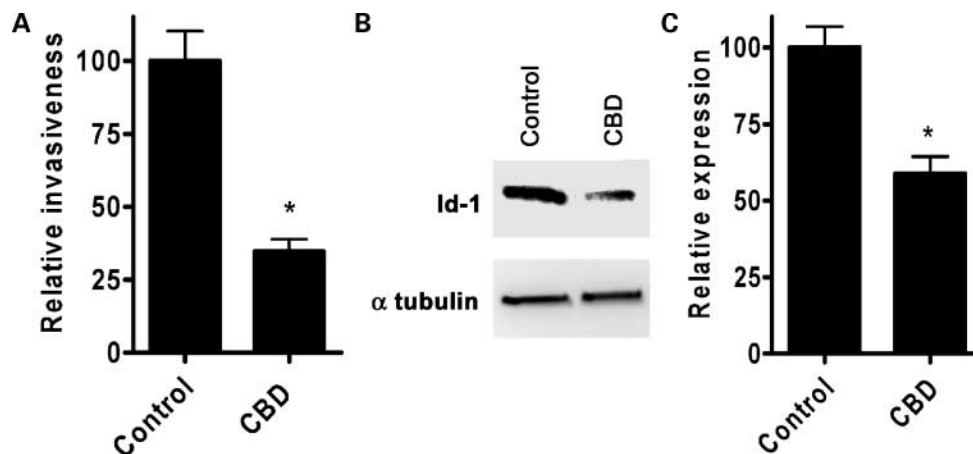


Figure 2. CBD reduces invasion as well as Id-1 expression in MDA-MB436 cells. **A**, the Boyden chamber invasion assay was used to determine the effects of CBD on the invasiveness of human breast cancer MDA-MB436 cells. Data are presented as relative invasiveness of the cells through the Matrigel, where the respective controls are set as 100%. **B**, proteins from MDA-MB436 cells treated with vehicle (*control*) or 2.0 $\mu\text{mol/L}$ of CBD for 3 d were extracted and analyzed for Id-1 by Western blot analysis. Normalization was carried out by stripping the blots and reprobing with a monoclonal antitubulin antibody. **C**, densitometry readings of the blots were taken from three independent experiments and the percentage of relative expression was calculated as the expression of Id-1 in the treated cells / vehicle cells \times 100. *, $P < 0.05$, statistically significant differences from control.

CBD Down-regulates Id-1 Expression

We predicted that CBD, the most potent inhibitor of breast cancer cell proliferation and invasion tested, would regulate the expression of key genes that control breast cancer cell proliferation and invasiveness. A potential candidate protein that could mediate the effects of CBD on both phenotypes was the helix-loop-helix protein Id-1. We determined that treatment of MDA-MB231 cells with CBD led to a concentration-dependent inhibition of Id-1 protein expression (Fig. 1B and C). The inhibitory effect of CBD on Id-1 expression occurred at concentrations as low as 100 nmol/L. CBD was significantly more effective at reducing Id-1 protein expression compared with other cannabinoid compounds (Fig. 1C). The CBD concentrations effective at inhibiting Id-1 expression correlated with those used to inhibit the proliferative and invasive phenotype of MDA-MB231 cells. Furthermore, the down-regulation of Id-1 protein in the presence of CBD seemed to precede, and not follow, the inhibitory effects of CBD on the proliferation and invasiveness of MDA-MB231 cells (Fig. 1D), suggesting that Id-1 down-regulation represents a cause rather than a consequence of a decrease in breast cancer cell aggressiveness.

The Effects of CBD on Invasion and Id-1 Protein Expression Can Be Reproduced in an Additional Breast Cancer Cell Line

Based on the data presented in Table 1, CBD could also decrease cell proliferation in another breast cancer cell line other than MDA-MB231, the MDA-MB436 cells. The metastatic cell line MDA-MB436 is able to invade through the peritoneum and colonize visceral organs when injected in athymic nude mice (14). However, these cells are less metastatic than the MDA-MB231 cell line. Using the MDA-MB436 cells, we confirmed the effects of CBD on a decrease of cell invasion (Fig. 2A) associated with a down-regulation of Id-1 protein expression

(Fig. 2B and C). These data suggest that the effects of CBD on breast cancer cell phenotypes, potentially through a decrease in Id-1 expression, are not restricted to one particular cell line but could represent a more general phenomenon.

CBD Inhibits the Transcription of the Id-1 Gene

In order to determine if CBD modulated Id-1 at the gene expression level, we investigated if Id-1 mRNA was down-regulated by CBD. As shown in Fig. 3A using reverse transcription-PCR, Id-1 mRNA expression was significantly reduced upon treatment with CBD in MDA-MB231 cells. To determine if this effect was due to the inhibition of transcription, a construct was used that contained the Id-1 promoter fused to a luciferase reporter in a PGL-3 basic vector. This construct was transiently transfected, and 24 h after transfection, MDA-MB231-Id-1-luc cells were treated with CBD for 2 or 3 days and luciferase activity was measured. Transfection efficiency and analysis of equal amounts of total protein were controlled by cotransfection of the cells with pCMVB containing β -galactosidase. Treatment with CBD resulted in a significant inhibition of luciferase activity, with the greatest inhibition occurring on day 3 (Fig. 3B and C). The effect on the down-regulation of Id-1 mRNA and promoter expression could also be reproduced in the MDA-MB436 cell line (Fig. 3D and E). Overall, all these findings correlated with the inhibition of Id-1 expression as assessed by Western analysis.

CBD Does Not Inhibit Cell Invasiveness in Cells that Ectopically Express Id-1

To determine if Id-1 represented a key mediator of CBD effects in highly aggressive breast cancer cells, Id-1 was constitutively expressed into MDA-MB231 cells (+Id-1 as described in Fig. 4). The ectopic Id-1 gene, which is not under the control of the endogenous promoter, was introduced in the cells using the pLXSN retroviral vector.

As a control, cells were infected with an empty pLXSN vector (–Id-1). Ectopic Id-1 expression increased invasion in MDA-MB231 cells in agreement with our previous studies (13, 15). However, the difference in invasion between cells that ectopically expressed Id-1, or the control vector lacking Id-1, was not reflected in Fig. 3A because the data was represented as relative invasiveness (with all the control cells set at 100%). In cells expressing the control vector, treatment with CBD led to a significant reduction in cell invasiveness (Fig. 4A). Western blotting confirmed the down-regulation of Id-1 expression in this cell population (Fig. 4B). Importantly, and in contrast with the results in control cells, CBD did not inhibit cell invasiveness (Fig. 4A) or Id-1 expression (Fig. 4B) in MDA-MB231+Id-1 cells that ectopically expressed Id-1.

Discussion

Metastasis is the final and fatal step in the progression of breast cancer. Currently available therapeutic strategies at this stage of cancer progression are often nonspecific, have

only marginal efficacy, and are highly toxic. This is in part due to the lack of knowledge about the molecular mechanisms regulating the development of aggressive cancers. Therapeutic approaches targeting only specific mechanisms involved in the development of aggressive cancers are urgently need. The expectation would be that this strategy would reduce unwanted toxicities associated with the therapy itself.

We previously showed that the helix-loop-helix protein Id-1, an inhibitor of basic helix-loop-helix transcription factors, plays a crucial role during breast cancer progression (4). Id-1 stimulated proliferation, migration, and invasion in breast cancer cells (13, 16). Moreover, targeting Id-1 expression partially in breast cancer cells reduced invasion *in vitro* and breast cancer metastasis in preclinical animal models (4, 5). Based on these data, we hypothesized that Id-1 could be a promising candidate for future therapy approaches, and that inhibiting Id-1 expression and/or activity might be of benefit for patients with breast cancer. This approach may be highly effective and safe in advanced breast cancer patients, given (a) the relationship between high Id-1 expression levels and aggressive breast

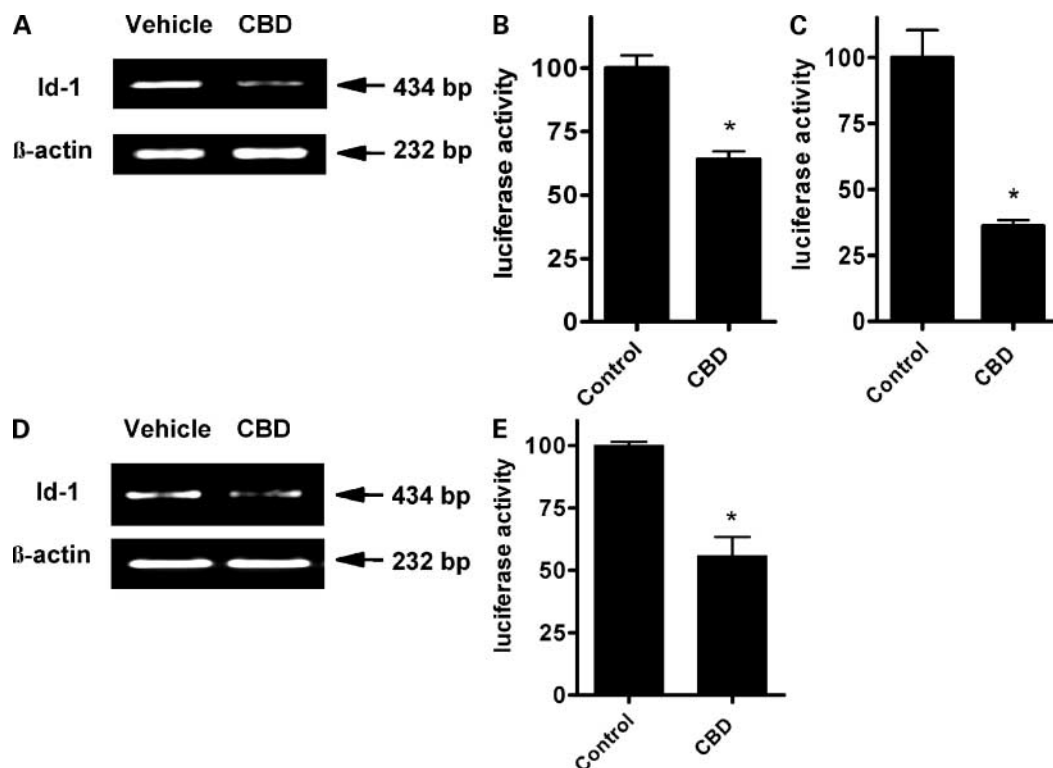


Figure 3. CBD inhibits the expression of Id-1 gene at the mRNA and promoter levels in MDA-MB231 and MDA-MB436 cells. **A**, the inhibition of the Id-1 gene product (434 bp) by CBD was investigated in MDA-MB231 cells using reverse transcription-PCR. Expression of the β -actin gene product (232 bp) was used as a control. **B**, luciferase activity in MDA-MB231 cells transiently transfected with Id-1-sb-luc was determined in the presence of vehicle (control) or 1.5 μ mol/L of CBD. Cells were treated for 2 d and luciferase activity was measured. **C**, cells were treated for 3 d. For both **B** and **C**, all values were normalized for the amount of β -gal activity present in the cell extracts. **Columns**, mean of at least three replicates; **bars**, SE. The data are represented as percentage of activity of the treated cells / vehicle cells \times 100. Data were compared using the unpaired Student's *t* test. *, $P < 0.05$, statistically significant differences from control. **D**, the inhibition of the Id-1 gene product by CBD was investigated in MDA-MB436 cells using reverse transcription-PCR. Expression of the β -actin gene product was used as a control. **E**, luciferase activity in MDA-MB436 cells transiently transfected with Id-1-sb-luc was determined in the presence of vehicle (control) or 2 μ mol/L of CBD. Cells were treated for 2 d and luciferase activity was measured.

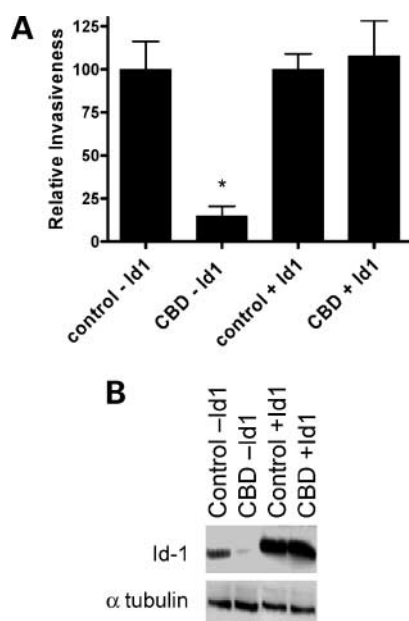


Figure 4. Ectopic expression of Id-1 blocks the effect of CBD on MDA-MB231 invasiveness. **A**, data are presented as relative invasiveness of control MDA-MB231 cells (*-Id-1*) and of MDA-MB231 cells that ectopically expressed Id-1 (*+Id-1*) after a 2-d treatment with vehicle (*control*) or 1.5 μ mol/L of CBD (*CBD*), and then an overnight invasion assay. The respective controls are set as 100%; *columns*, mean of at least three replicates; *bars*, SE. Data were compared using the unpaired Student's *t* test. *, *P* < 0.05, statistically significant differences from control. **B**, the inhibitory effect of CBD on Id-1 expression in *-Id-1* and *+Id-1* MDA-MB231 cells was compared using Western analysis. Equal loading was confirmed by stripping the blots and reprobing with a monoclonal antitubulin antibody.

cancer cell behaviors; (b) partial reduction in Id-1 activity can achieve significant outcomes; and (c) Id-1 expression is low in normal adult tissues, thereby eliminating unwanted toxicities generally associated with currently available therapeutic modalities.

However, approaches targeting Id-1 expression, including gene therapy using antisense oligonucleotide, short interfering RNA, and nonviral or viral plasmid-based strategies, are not yet routinely used in the clinic. Therefore, the development of new strategies to modulate Id-1 expression/functional activity is needed. A range of small molecules that target the molecular pathology of cancer are now being developed, and a significant number of them are being tested in ongoing human clinical trials (17). We propose that the use of CBD, as an inhibitor of Id-1, represents a novel strategy to treat breast cancer. A wide range of cannabinoid compounds were tested and CBD, a nonpsychoactive cannabinoid constituent, was the most potent inhibitor of human breast cancer cell aggressiveness through Id-1 mRNA and protein down-regulation.

Cannabinoid agonists working through CB₁ and CB₂ receptors have been shown to act as tumor inhibitors in a variety of cancer models (7, 8). Present evidence also shows that the cannabinoid constituent CBD, which has negligible affinity for CB₁ and CB₂ receptors, also has antitumor

activity (18–20). Specifically, Ligresti et al. have recently shown that CBD inhibits the metastasis of aggressive human breast cancer cancers *in vivo* (11). However, the primary molecular pathways involved in CBD inhibition of invasion and metastasis remain to be clarified. Overall, the IC₅₀ values, even being within the range observed by other laboratories (21, 22), were lower than those reported by Ligresti et al. (11). This difference is likely due to the fact that we did the experiments in lower serum concentrations, which have been shown to improve the antiproliferative activity of cannabinoids (23).

Here, we report that CBD acting as a potent Id-1 inhibitor might effectively inhibit genotypic and phenotypic changes that allow aggressive breast cancers to invade and metastasize. Most importantly, ectopic expression of Id-1 in MDA-MB231 breast cancer cells abolished the effects of CBD on cell invasion. Cells were infected with an Id-1 gene (pLXSN vector) that is not under the control of the endogenous Id-1 promoter. As presented in Fig. 3, CBD seems to act by down-regulating endogenous Id-1 gene expression at the promoter level, not as a result of mRNA and/or protein destabilization. Therefore, CBD should not have any effect on the Id-1 expression from the pLXSN vector. Indeed, ectopic expression of Id-1 in MDA-MB231 breast cancer cells was able to abolish the effects of CBD.

These data indicate that Id-1 is a key factor whose expression needs to be down-regulated in order to observe the beneficial effects of CBD on the reduction of breast cancer cell aggressiveness. Based on previous findings (reviewed in ref. 15), we suggest that a decrease in Id-1 protein upon CBD treatment might consequently lead to a down-regulation of growth-promoting genes such as *Zfp289* as well as to a down-regulation of invasion-promoting genes such as the membrane type matrix metalloproteinase (*MT1-MMP*).

Plant cannabinoids are stable compounds with low-toxicity profiles that are well tolerated by animals and humans during chronic administration (24, 25). A formulation including a 1:1 ratio of THC and CBD has recently been used in a clinical trial for the treatment of multiple sclerosis (26). The few side effects reported were related to the psychoactivity of Δ^9 -THC. If CBD shows efficacy for treatment of metastatic breast cancer in humans, the low toxicity of the compound would make it an ideal candidate for chronic administration.

Because CBD inhibits Id-1 expression in aggressive breast cancer cells, a rational drug design strategy could be used to potentially create more potent and efficacious analogues. Moreover, reducing Id-1 expression with cannabinoids could also provide a therapeutic strategy for the treatment of additional aggressive cancers because Id-1 expression was found to be up-regulated during the progression of almost all types of solid tumors investigated (27).

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