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# Cannabidiolic acid, a major cannabinoid in fiber-type cannabis, is an inhibitor of MDA-MB-231 breast cancer cell migration

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## Abstract

Cannabidiol (CBD), a major non-psychotropic constituent of fiber-type cannabis plant, has been reported to possess diverse biological activities, including anti-proliferative effect on cancer cells. Although CBD is obtained from non-enzymatic decarboxylation of its parent molecule, cannabidiolic acid (CBDA), few studies have investigated whether CBDA itself is biologically active. Results of the current investigation revealed that CBDA inhibits migration of the highly invasive MDA-MB-231 human breast cancer cells, apparently through a mechanism involving inhibition of cAMP-dependent protein kinase A, coupled with an activation of the small GTPase, RhoA. It is established that activation of the RhoA signaling pathway leads to inhibition of the mobility of various cancer cells, including MDA-MB-231 cells. The data presented in this report suggest for the first time that as an active component in the cannabis plant, CBDA offers potential therapeutic modality in the abrogation of cancer cell migration, including aggressive breast cancers.

### Keywords

Cannabidiolic acid; RhoA; Fiber-type cannabis plant; Cannabidiol; MDA-MB-231 cells



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**Conflict of interest statement**

The authors declare that there are no conflicts of interest in this study

## 1. Introduction

It is well-established that Δ′-tetrahydrocannabinol (Δ′-THC), the major psychoactive constituent in the medicinal/drug-type variety of the cannabis plant, displays a number of biological activities including anti-atherosclerotic, anti-proliferative and endocrine disrupting properties (Steffens et al., 2005; Watanabe et al., 2005; Takeda et al., 2008a, 2009a, 2011a; Guindon and Hohmann, 2011). There is also growing experimental evidence suggesting that cannabidiol (CBD), found principally in the fiber-type of cannabis, also possesses biological activities. For example, CBD has been reported as an inhibitor of human glioma cell migration and an inducer of programmed cell death in tumor cells, including breast cancer cells (Vaccani et al., 2005; Ligresti et al., 2006; Shrivastava et al., 2011). In fresh plants, the concentrations of neutral cannabinoids, including CBD, are much lower than those of cannabinoid acids that contain a carboxyl group (–COOH) in the structures. CBD is produced largely through non-enzymatic decarboxylation of its acidic precursor, cannabidiolic acid (CBDA), during extraction from the leaf materials of the plant (*see* Fig. 1) (Yamauchi et al., 1967). Although studies on the biological efficacies of CBD are ongoing, there is a relative lack of information regarding the potential biological activities of CBDA. We have reported that CBDA is a selective cyclooxygenase-2 (COX-2) inhibitor (Takeda et al., 2008b, 2009a), and it has been reported by others that CBDA is a necrosis-inducing factor for the leaf cells of cannabis plant (Morimoto et al., 2007). However, no investigations have evaluated potential anti-migration effects of CBDA on human breast cancer cells. Among the available experimental human breast cancer cell lines, MDA-MB-231 cells have been established as a valuable mesenchymal breast cancer model (*see* Fig. 2A–b) for pre-clinical studies as they are highly aggressive, both *in vitro* and *in vivo* (Price et al., 1990; Rochefort et al., 2003; Takeda et al., 2011b).

It is well-recognized that Rho family small GTPases (~21 kDa) regulate actin cytoskeletal dynamics, thereby affecting multiple cellular functions including cell mobility and polarity (Jaffe and Hall, 2005; Yamazaki et al., 2005). In the GTP-bound state, Rho family small GTPases are active and able to interact with specific downstream effectors such as Rhotekin, which lead to the translocation and activation of the effectors, and induction of various intracellular responses. In these respects, altered Rho GTPase activity or expression is implicated in cancer progression (Ellenbroek and Collard, 2007). Among the Rho subfamily members that include the isoforms RhoA, RhoB, and RhoC, RhoA has specifically been identified as an inhibitor of cancer cell mobility, including breast cancer cells (Vial et al., 2003; Simpson et al., 2004; Vega et al., 2011). Post-translational regulation of Rho activity has been demonstrated for RhoA. This Rho protein is phosphorylated *in vitro* and *in vivo* by kinases such as a cAMP-dependent protein kinase (PKA) on serine 188 (Ser188) (Lang et al., 1996; Dong et al., 1998; Ellerbroek et al., 2003), and it is generally accepted that phosphorylation of this site is an important negative regulation of the RhoA activity, leading to termination of the signaling process.

In this study we demonstrate for the first time that (i) CBDA is an inhibitor of MDA-MB-231 breast cancer cell migration, and that (ii) the mechanism responsible for the inhibitory effects of CBDA likely involves activation of RhoA *via* inhibition of PKA.

## 2. Materials and methods

### 2.1. Reagents

CBD and CBDA were isolated from the fiber-type cannabis leaves according to the established methods (Watanabe et al., 2005; Takeda et al., 2008b). The purity of CBD and CBDA was determined as >98% by gas chromatography (Takeda et al., 2008b). SC-560 (purity: >98%), DuP-697 (purity: >96%), SR141716A (purity: >98%), and SR144528 (purity: >98%) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 2,4-Dihydroxybenzoic acid (β-resorcylic acid, purity: >95%) was purchased from Wako Pure Chemical (Osaka, Japan). Pertussis toxin was purchased from Tocris Bioscience (Ellisville, MO, USA). All other reagents were of analytical grade commercially available and used without further purification.

### 2.2. Cell cultures and proliferation assays

Cell culture conditions and methods were based on procedures described previously (Takeda et al., 2008a, 2009a, 2011b). Briefly, the human breast cancer cell lines, MDA-MB-231 and MCF-7 (obtained from the American Type Culture Collection, Rockville, MD, USA), were routinely grown in phenol red-containing minimum essential medium alpha (Invitrogen, Carlsbad, CA, USA), supplemented with 10 mM HEPES, 5% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, at 37 °C in a 5% $CO_2$–95% air-humidified incubator. Before chemical treatments, the medium was changed to phenol red-free minimum essential medium alpha (Invitrogen, Carlsbad, CA, USA) supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated serum (DCC-serum), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cultures of approximately 60% confluence in a 100-mm Petri dish were used to seed for the proliferation experiments. In the proliferation studies, the cells were seeded into 96-well plates at a density of ~5000 cells/well, and test substances were introduced 4 h after plating. After the indicated periods of incubation (*see* Results), cell proliferation was analyzed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS reagent; Promega, Madison, WI, USA), according to the manufacturer's instructions. Test chemicals were prepared in appropriate organic solvents including DMSO or ethanol. Control incubations contained equivalent additions of solvents with no measurable influence of vehicle observed on cell viability at the final concentrations used.

### 2.3. Cell morphology studies

For morphological examination of the breast cancer cells, MDA-MB-231 and MCF-7, images were obtained using a Leica DMIL inverted microscope (Leica Microsystems, Wetzlar, Germany), and captured with a Pixera® Penguin 600CL Cooled CCD digital camera (Pixera Co., Los Gatos, CA, USA). Data were processed using Pixera Viewfinder 3.0 software (Pixera Co., Los Gatos, CA, USA). The breast cancer cells were plated in 6-well plates. Three areas with approximately equal cell densities were identified in each well and images of each of these areas were captured.

### 2.4. Cell migration assays

The cancer cell wound-healing assay was performed according to established methods (Takahashi et al., 2008). Briefly, MDA-MB-231 cells were seeded into 24-well plates at a density of approximately $1 \times 10^5$ cells (1 mL/well) and incubated for 24 h. The formed monolayer was wounded by scratching lines with plastic tip. The wells were then washed one with PBS to remove cell debris, and photographed (*i.e.*, designated as time 0). CBD, CBDA, DuP-697, or SC-560 were then added to test their respective effects on MDA-MB-231 cell migration. In these assays, the cells were incubated in the presence of 5% DCC-serum (chemoattractant) at 37 °C under 5% $CO_2$ for 12 h, 24 h, or 48 h, after which the cells were imaged with photomicrosopy (*i.e.*, designated as time 12 h, 24 h, or 48 h) and the migration distance measured (Ohta et al., 2006). The percent wounded area filled was calculated as follows; [(mean wound breadth − mean remained breadth)/mean wound breadth] × 100 (%). The cancer cell Transwell (Boyden Chamber) migration assay (*i.e.*, vertical migration) was performed utilizing the CytoSelect™ 24-well cell migration and invasion assay in a colorimetric format (8 μm pore size) (Cell Biolabs, Inc., San Diego, CA, USA). The assay was performed exactly as specified by the manufacturer's protocol. In brief, MDA-MB-231 cells ($1.2 \times 10^5$ cells) suspended in serum free medium containing endotoxin-free bovine serum albumin (5 mg/mL) were added to the upper chamber of an insert, and the insert was placed in a 24-well plate containing medium with 5% DCC-serum (chemoattractant). When used, CBDA was added to the upper chamber. Migration assays were carried out for 12 h.

### 2.5. RhoA pull-down assay

To determine whether CBDA can modulate RhoA activities in MDA-MB-231 cells, the cells were treated with 25 μM CBDA or vehicle alone for 24 h or 48 h. RhoA pull-down assays with glutathione *S*-transferase-tagged Rho-binding domain of Rhotekin on glutathione-agarose beads was performed using a RhoA Activation Assay Biochem Kit (Cytoskeleton Inc., Denver, Co, USA). Active RhoA was normalized to the total RhoA by Western blot analysis (RhoA monoclonal antibody, Abcam, Cambridge, MA, USA; anti-mouse secondary antibody, Sigma–Aldrich, St. Louis, Mo, USA). Western blot analysis was performed based on procedures described previously (Takeda et al., 2009b). MDA-MB-231 cell extract loaded with GTPγS was used as a positive control, and the same extract was used to determine the phosphorylation status of RhoA using an anti-RhoA antibody specific to RhoA phosphorylated on Ser188 (Abcam, Cambridge, MA, USA). In addition, equivalence of protein loading was assessed by performing Western blot analysis using an anti-β-actin antibody (Sigma–Aldrich, St. Louis, Mo, USA). Quantification of band intensity was performed using NIH Image 1 .61 software (http://rsb.info.nih.gov/nih-image/).

### 2.6. Determination of protein kinase A activity

cAMP-dependent PKA activities were assayed using the PepTag® Assay for Non-Radioactive Detection of PKA (Promega, Madison, WI, USA) which quantifies the phosphorylation of fluorescent-tagged PKA-specific peptides. MDA-MB-231 cell lysate for PKA protein was prepared using a hypotonic extraction buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 mg/mL aprotinin, and 1 mg/mL

leupeptin). Aliquots of the PKA preparation were incubated for 30 min at 30 °C in PepTag® PKA reaction buffer (100 mM Tris–HCl, pH 7.4, 50 mM $MgCl_2$, and 5 mM ATP) and 0.4 mg/μL of the PKA-specific peptide substrate PepTag® A1 (L–R–R–A–S–L–G; Kemptide). For positive and negative controls, the reactions were performed in the presence or absence of PKA catalytic subunit, respectively. Reactions was stopped by heating for 10 min at 95 °C. Phosphorylated and non-phosphorylated PepTag® peptides were separated electrophoretically using a 0.8% agarose gel prepared in 50 mM Tris–HCl (pH 8.0). For the PKA assays, 20 μg of protein was applied. The phosphorylated peptides migrate toward the anode (+) and non-phosphorylated peptides migrate toward the cathode (−). The gel was then visualized and photographed. The band intensity was analyzed using NIH Image 1.61 software. The intensities of the phosphorylated peptide bands are proportional to the PKA activities.

### 2.7. Data analysis

$IC_{50}$ values were determined using SigmaPlot 11® software (Systat Software, Inc., San Jose, CA, USA), according to analyses described previously (Takeda et al., 2011b). Differences were considered significant when the *p* value was calculated as <0.05. Statistical differences between two groups were calculated by Student's *t* test. Other statistical analyses were performed by Scheffe's *F* test, a *post hoc* test for analyzing results of ANOVA testing. These calculations were performed using Statview 5.0 J software (SAS Institute Inc., Cary, NC, USA).

## 3. Results and discussion

### 3.1. Effects of CBDA on the migration of MDA-MB-231 cells

When compared with the epithelial-like morphology of MCF-7 cells that are capable of forming domes, with close contact between cells (*collective*, Fig. 2A–a), MDA-MB-231 cells exhibit a mesenchymal-like morphology (*mesenchymal*, Fig. 2A–b), a hallmark feature of tumor aggressiveness (Price et al., 1990; Rochefort et al., 2003; Jaffe and Hall, 2005; Yamazaki et al., 2005; Takeda et al., 2011b). Thus, we selected the use of MDA-MB-231 cells to investigate whether CBDA can inhibit the cell migration under the incubation period and its concentration that do not affect overall MDA-MB-231 cell growth (Fig. 2B, *see* also Fig. 6B). In this study we could not directly compare the efficacy of CBDA and CBD-mediated inhibition of the migration of MDA-MB-231 cells because of CBD's strong cytostatic effect (*see* also Ligresti et al., 2006); 10 μM and 25 μM CBD treatment for 12 h significantly reduced MDA-MB-231 cell viability (Fig. 2B). Thus, we compared 5 μM or 25 μM CBDA treatments with that of 5 μM CBD. As shown in Figs. 2C, 3A–a/b and B, CBDA inhibited migration of MDA-MB-231 cells in a concentration-dependent manner, whereas 5 μM CBD exhibited no significant inhibitory effects on migration. Futher, CBDA's significant abrogation of cell migration was detected even 48 h after its exposure (Fig. 3B). It is noteworthy that CBDA-mediated attenuation of MDA-MB-231 cell migration was more pronounced when assessing vertical migration *versus* horizontal migration. Although in this study we could not detect CBD-mediated inhibition of the migration of MDA-MB-231 cells, it has been reported that low concentrations of CBD (*i.e.*, 0.1–1.5 μM), treated once a day up to 72 h, suppress cell migration (McAllister et al., 2007). The discrepancy between the latter

report and the result presented here may be attributed to the difference in the experimental conditions employed. In short, our data strongly suggest that CBDA is an effective inhibitor of breast cancer cell migration *in vitro*.

### 3.2. COX-2 inhibitory activity of CBDA is not critical in the attenuation of MDA-MB-231 cell migration

It has been reported that selective COX-2 inhibitors interfere with the migration of certain cancer cell types (Banu et al., 2007; Kim et al., 2010). Previously, we reported that CBDA is an effective COX-2 inhibitor (Takeda et al., 2008b, 2009a). Therefore, we initially hypothesized that CBDA may exert its inhibitory effects on MDA-MB-231 cell migration *via* COX-2 inhibition. However, as demonstrated by the data shown in Fig. 4, vertical migration of MDA-MB-231 cells was not influenced by 25 μM DuP-697 (an established selective COX-2 inhibitor), nor SC-560 (an established selective COX-1 inhibitor), although the same concentration of CBDA completely inhibited cell migration. In addition, horizontal migration was not affected by DuP-697 (data not shown). These results indicate that COX-2 activity is not an essential factor for the migration of MDA-MB-231 cells, and that other pathway(s) are likely to be involved in the anti-migration effects of CBDA.

### 3.3. CBDA-mediated stimulation of RhoA activity

In an effort to investigate CBDA's mode of action, we focused on the Rho family of small GTPases, in particular the isoform RhoA, since active RhoA has been reported to inhibit breast cancer cell mobility *in vitro* (Vial et al., 2003; Simpson et al., 2004; Vega et al., 2011). We discovered that the active RhoA levels were remarkably stimulated by 25 μM CBDA, especially at 48 h post-treatments (Fig. 5A and B). In accordance with the RhoA activation time-course, horizontal migration of MDA-MB-231 cells was significantly inhibited by 25 μM CBDA at 48 h (*see* Fig. 3B). GTPγS, a hydrolysis-resistant GTP analog, was used as positive control for the experiment (Fig. 5A, left portion). Because RhoA can be regulated by post-transcriptional modification *via* phosphorylation on Ser188, leading to inactivation of the protein (Lang et al., 1996; Dong et al., 1998; Ellerbroek et al., 2003), we also assessed the phosphorylation status of RhoA following 25 μM CBDA exposure. Although total RhoA levels were not changed, the phosphorylation levels at Ser188 on RhoA were reduced by CBDA in a time-dependent manner, as compared with vehicle-treated groups (Fig. 5A and B). Thus, there exists an apparently negative relationship between the levels of active RhoA and phosphorylated RhoA, suggesting that CBDA activates RhoA *via* an inhibition of its phosphorylation.

### 3.4. CBDA-mediated inhibition of PKA activity and implications for its anti-migration mechanism

It has been reported that the phosphorylation status of RhoA is modulated by cAMP-dependent PKA (Lang et al., 1996; Dong et al., 1998; Ellerbroek et al., 2003). Therefore, we hypothesized that CBDA may interfere with PKA expressed in MDA-MB-231 cells, that in turn would result in activation of RhoA (*see* a model described in Fig. 6C). First, to confirm the efficacy of the PKA assay used in the present study, experiments were performed in the absence or presence of purified PKA catalytic subunit (Fig. 6A–a; lanes 1 and 2). A

phosphorylated band that migrated toward the anode (+) was detected only in the presence of purified PKA (lane 2). As shown in Fig. 6A–a, PKA activity was substantially reduced by 25 μM CBDA at 48 h when compared with the vehicle-treated group (Fig. 5A–a; lanes 4 *vs.* 5), although PKA activity in the MDA-MB-231 cells was up-regulated following 48 h incubation (lane 4). Densitometric analysis of the phosphorylated band in the CBDA-treated sample indicated its decrease to ~50% of the levels detected in the vehicle-treated sample (Fig. 6A–b). Taken together with the results described in Fig. 5, the data suggest that CBDA-mediated anti-migration effects are primed by PKA inhibition, which in turn leads to decreased levels of phosphorylated RhoA. However, it is not yet clear mechanistically as to how CBDA inhibits PKA activity in MDA-MB-231 cells. Studies are ongoing to investigate these parameters with a possibility that some PKA specific inhibitors contain a β-resorcylic acid moiety in the structures as the moiety is present in CBDA (*see* Fig. 1A) (Schirmer et al., 2006; Winssinger and Barluenga, 2007).

Recent experimental evidence, for example as expressed in prostate carcinoma cells, suggests the involvement of cannabinoid receptor CB receptors (CBs), and especially CB1, as specific agonist targets whose activation results in the abrogation of cell migration (Nithipatikom et al., 2012). Further, it is known that CB1 activation results in inhibition of adenylate cyclase, coupled with inactivation of PKA. Thus, one anti-migration strategy for preventing cancer cell migration is an activation of CB1. However, efforts to develop therapies using CB1 agonists are hampered by associated adverse side effects, such as psychoactive effects, due to agonism at receptors within the central nervous system (Pertwee, 2009). In these respects, it should be noted that non-psychoactive cannabinoid, CBD, does not exhibit affinity for either CB1 or CB2. Although at present we have not assessed whether CBDA directly interacts with these receptor subtypes, it was reported previously that CBDA exhibits no affinity for either CB1 or CB2 receptors (Bisogno et al., 2001). Consistent with this finding, the results obtained here indicated that the anti-migration effects of CBDA were not affected by co-treatments with the established CB1/CB2 receptor antagonists, SR141717A and SR144528, respectively, or with pertussis toxin, a blocker of the coupling between CB receptors and G proteins (data not shown). Thus, it is suggested that CBDA does not require CBs to exert its anti-migration activity in MDA-MB-231 cells.

Based on the high consistency observed between time-course profiles of CBDA-mediated RhoA activation kinetics and inhibition of MDA-MB-231 cell migration, these data suggest that CBDA's RhoA activation specifically mediates the inhibition of horizontal cell migration rather than that of vertical migration. Since a number of cancer cells, including the mesenchymal-like MDA-MB-231 cells, exhibit low RhoA activity, this condition likely facilitates a higher migration potential in the cells (Jaffe and Hall, 2005; Yamazaki et al., 2005; Ellenbroek and Collard, 2007). Cancer cell metastasis is triggered when migration of cancer cells is abnormally up-regulated (Jaffe and Hall, 2005; Yamazaki et al., 2005; Ellenbroek and Collard, 2007). Therefore, RhoA activators may represent useful pharmaceutical approaches for inhibiting cancer cell migration in cell types represented by the mesenchymal-like MDA-MB-231 cells. The data presented in this report support the

view that CBDA is a biologically active component of the fiber-type cannabis plant with potential utility as an effective anti-migration agent.

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## Abbreviations

| | |
|---|---|
| **CBDA** | cannabidiolic acid |
| **CBD** | cannabidiol |
| **COX-2** | cyclooxygenase-2 |
| **PKA** | cAMP-dependent protein kinase |
| **Δ′-THC** | Δ′-tetrahydrocannabinol |

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## HIGHLIGHTS

- Cannabidiolic acid (CBDA) exists as a major component in the fiber-type cannabis.
- CBDA is identified as an “active component”.
- CBDA is an inhibitor of highly aggressive human breast cancer cell migration.
- The mechanism responsible for the effects of CBDA involves activation of RhoA.
- RhoA is an inhibitor of cancer cell mobility.

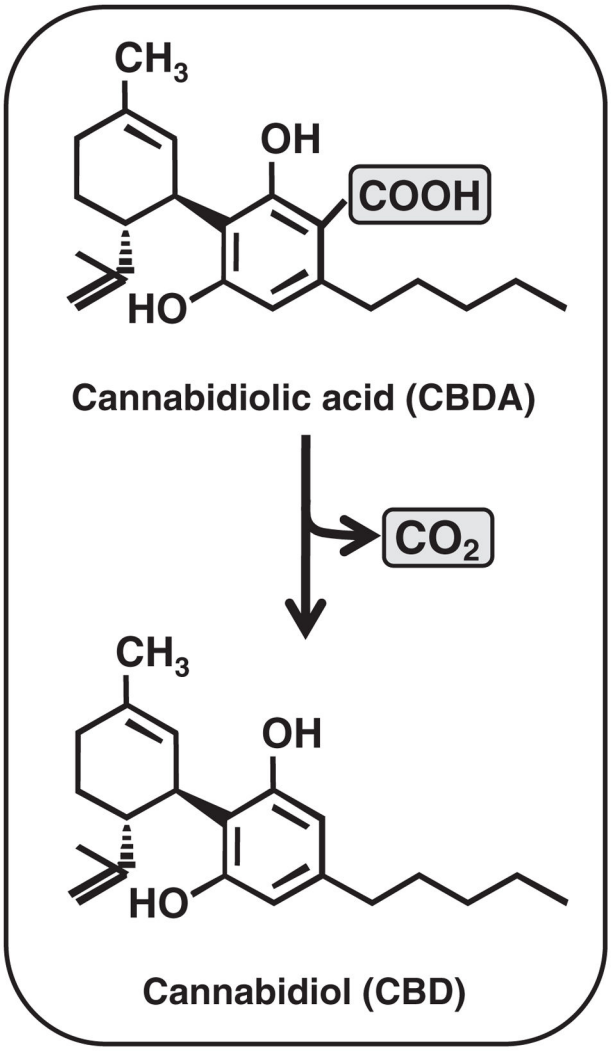


**Fig. 1.**

Chemical structures of CBDA and CBD. In the fiber-type cannabis plant, the concentration of CBD is much lower than that of its precursor CBDA. CBD is formed artificially from CBDA by non-enzymatic decarboxylation during extraction step (Yamauchi et al., 1967).

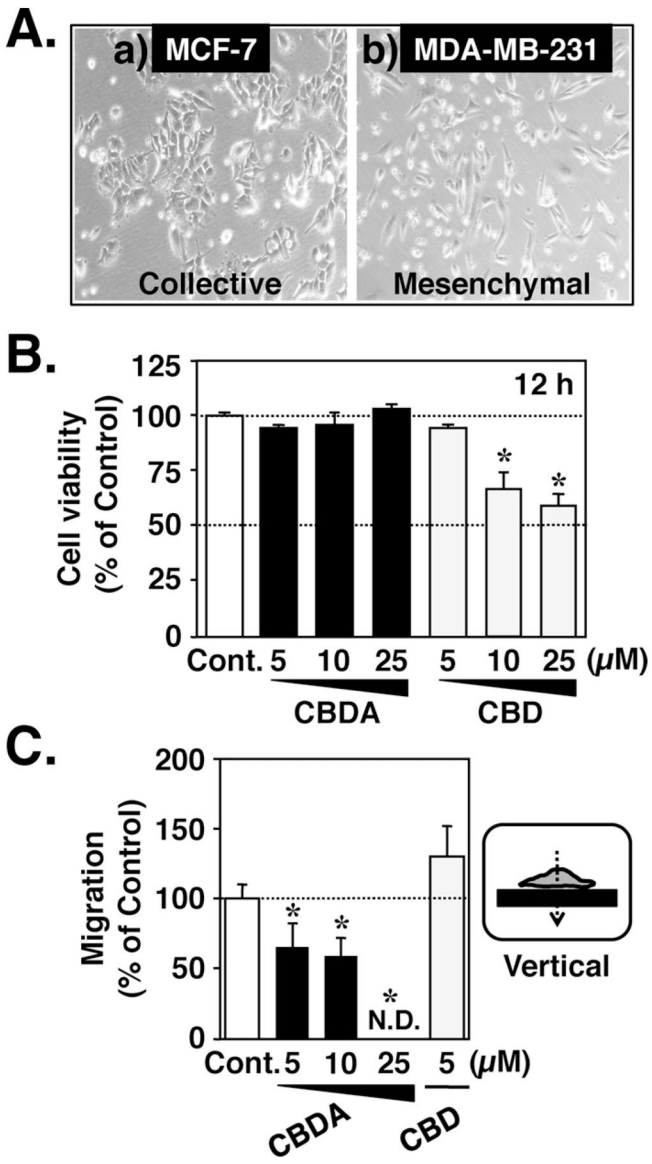


**Fig. 2.**

Effect of CBDA on the vertical migration of highly aggressive human breast cancer MDA-MB-231 cells. (A) Morphologies of two human breast cancer cell lines; MCF-7 cells (a) and MDA-MB-231 cells (b). MCF-7 cells display epithelial morphology (*collective*) and MDA-MB-231 cells display single elongated morphology (*mesenchymal*). (B) MDA-MB-231 cells were exposed for 12 h to CBDA (5, 10, 25 μM) and CBD (5, 10, 25 μM). After the treatments, cell viability was measured according to the methods described in Section 2. Data are expressed as the percent of vehicle-treated group (indicated as Cont.), as mean ± S.D. ($n = 6$). *Significantly different ($p < 0.05$) from the vehicle-treated control. (C) Transwell migration assays (vertical migration) were performed to determine MDA-MB-231 cell migration 12 h after treatments with 5 μM, 10 μM, or 25 μM CBDA and 5 μM CBD. Data are expressed as the percent of vehicle-treated group (indicated as Cont.), as mean ± S.D. ($n = 8$). *Significantly different ($p < 0.05$) from the vehicle-treated control. N.D., not detectable due to complete inhibition of the migration.

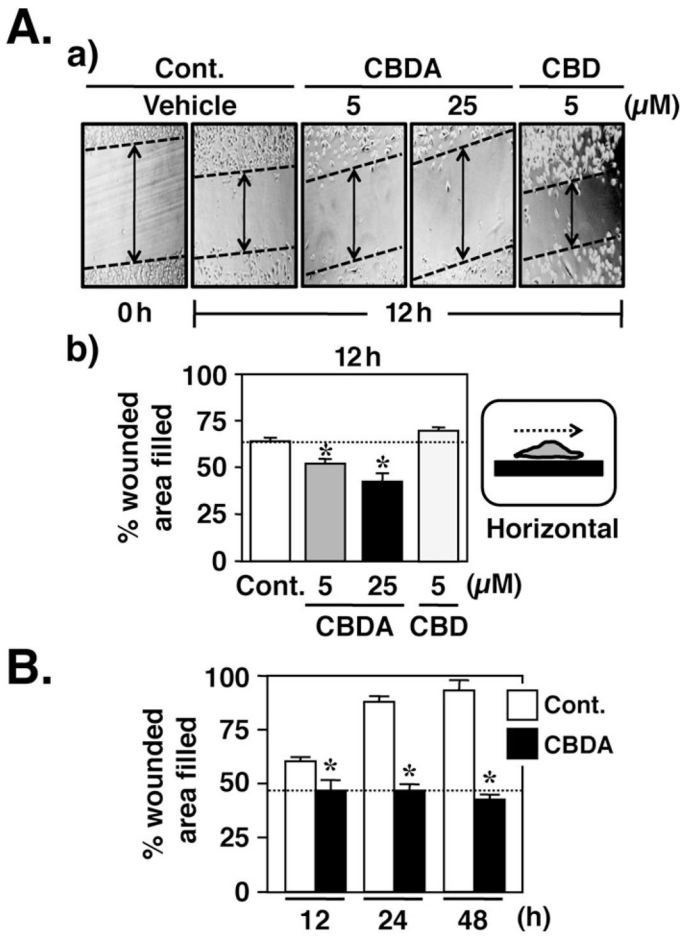


**Fig. 3.**

Effect of CBDA on the horizontal migration of MDA-MB-231 cells. (A) Wound-healing assays (horizontal migration) were performed to determine the effects of CBDA or CBD on MDA-MB-231 cell migration. (a) Representative images of the migrating cells were captured 12 h after vehicle (indicated as Cont.), 5 μM or 25 μM CBDA and 5 μM CBD treatments. (b) Migration data presented in panel (a) was assessed on the basis of percent wounded area filled in. Data are expressed as the percent of vehicle-treated group (indicated as Cont.), as mean ± S.D. ($n$ = 8). (B) Migration data was assessed on the basis of percent wounded area filled in 12, 24, or 48 h after treatments with 25 μM CBDA. Data are expressed as the percent of vehicle-treated group (indicated as Cont.), as mean ± S.D. ($n$ = 8) *Significantly different ($p < 0.05$) from the respective vehicle-treated controls.

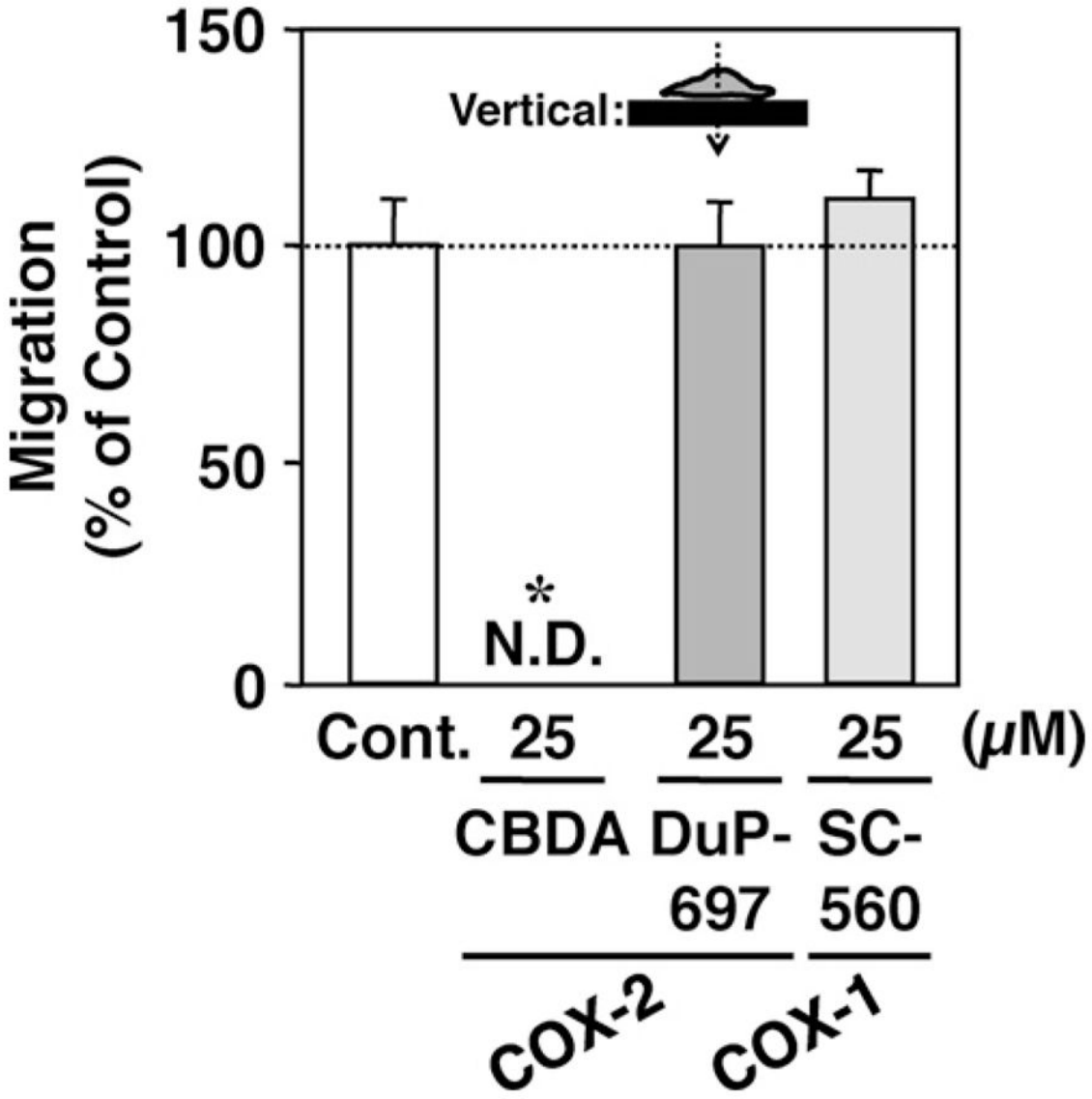


**Fig. 4.**

COX-2 inhibitory activity of CBDA is not essential to attenuate MDA-MB-231 cell migration. Transwell migration assays were performed to determine the effects of COX-2 selective inhibitors (25 μM CBDA or 25 DuP-697) and COX-1selective inhibitor (25 μM SC-560) on MDA-MB-231 cell migration 12 h after their respective treatments. Data are expressed as the percent of vehicle-treated group (indicated as Cont.), as mean ± S.D. ($n$ = 8). *Significantly different ($p < 0.05$) from the vehicle-treated control. N.D., not detectable due to complete inhibition of the migration.

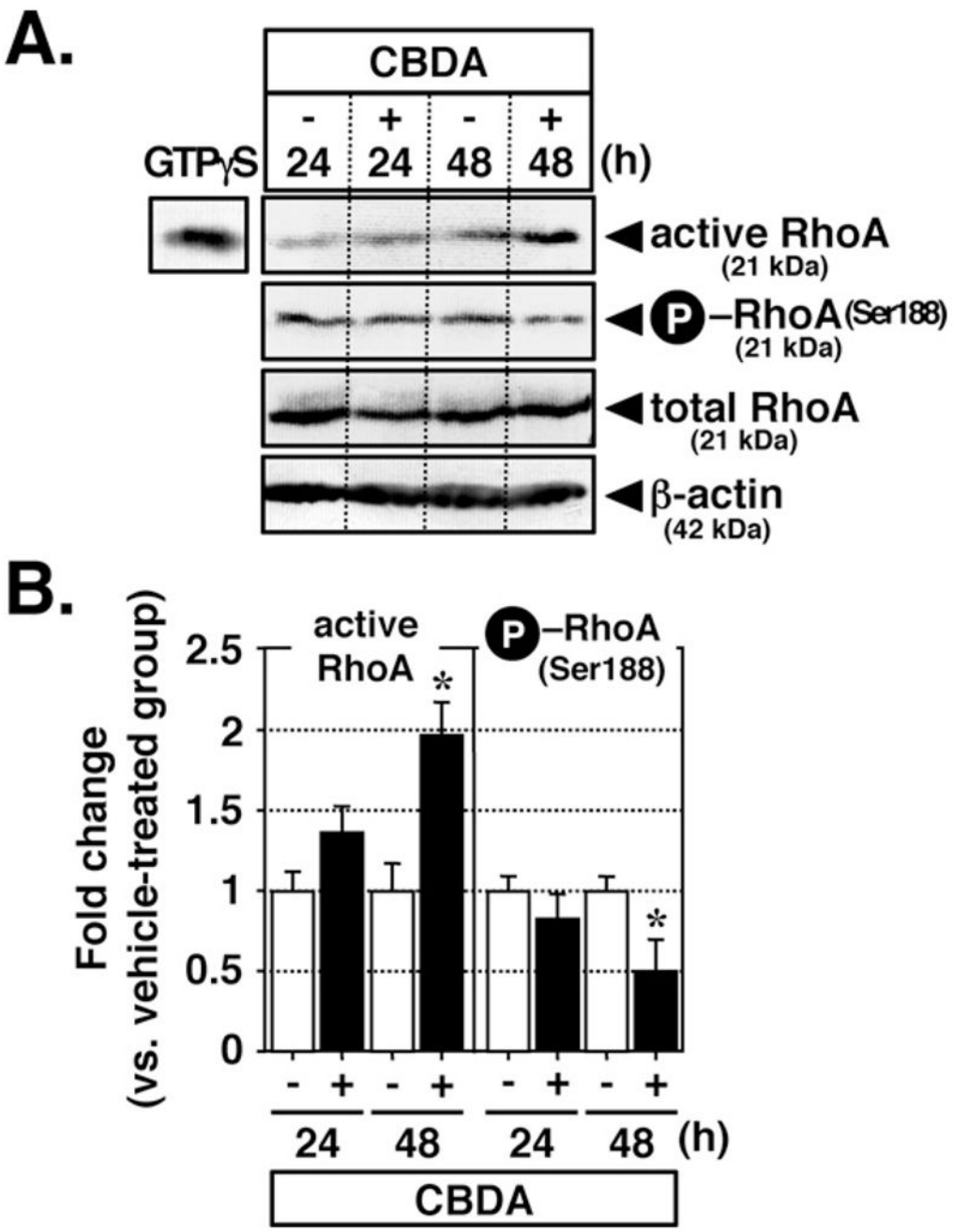


**Fig. 5.**

CBDA stimulates RhoA activity. (A) RhoA affinity pull-down assays were used to determine the level of active RhoA according to the methods described in Section 2. Resulted pull-down samples were subjected to Western blot analyses using an anti-RhoA antibody. Active RhoA was increased by 25 μM CBDA (indicated as +) in a time-dependent manner. Western blot analyses were also performed using an anti-RhoA antibody specific to RhoA phosphorylated at Ser188 and a β-actin antibody. (B) Results are expressed as the ratio of active RhoA to total RhoA protein in each cell lysate. Data are expressed as the fold change *vs.* vehicle-treated group (indicated as −), as mean ± S.D. ($n = 3$). *Significantly different ($p < 0.05$) from the vehicle-treated control.

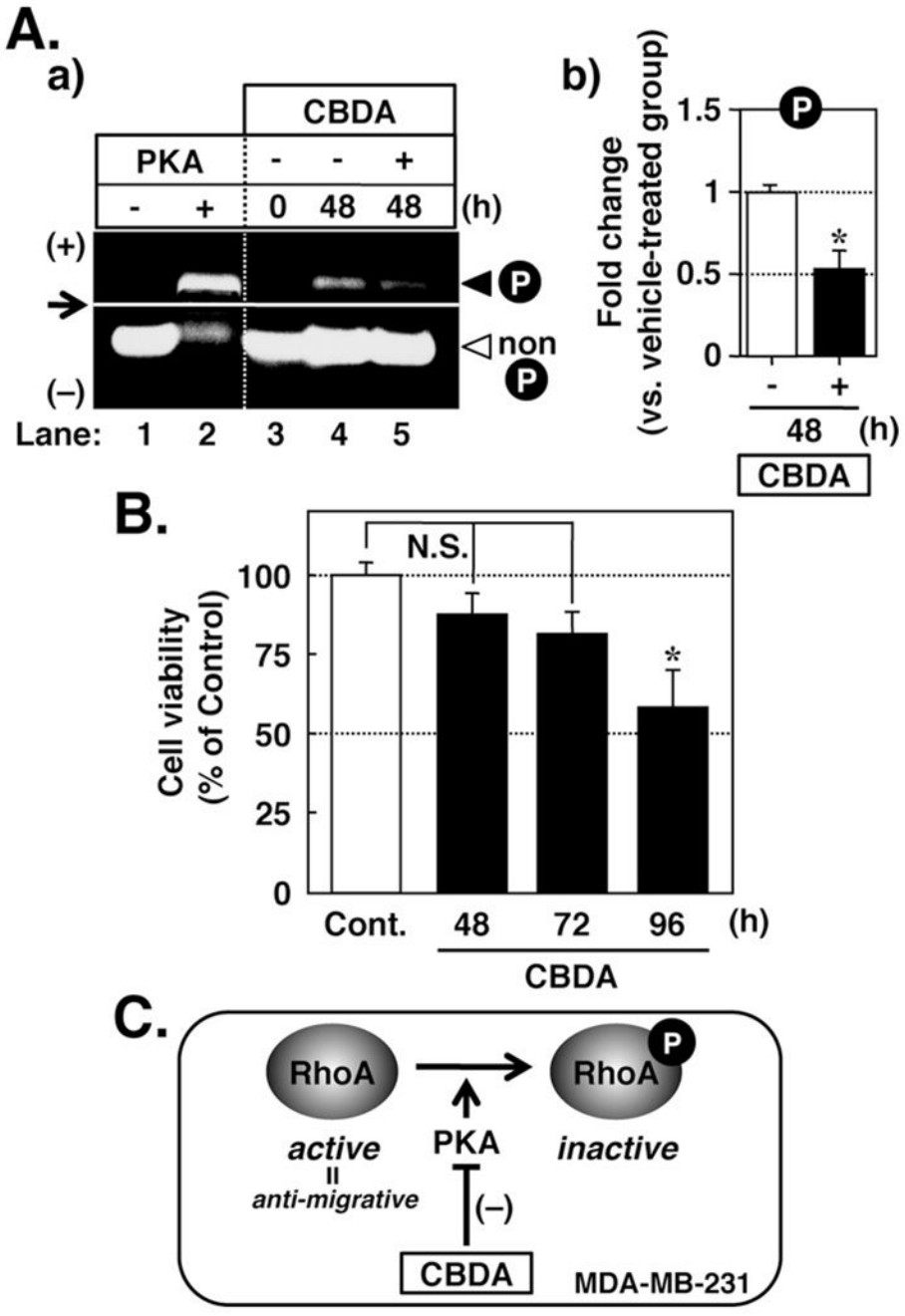


**Fig. 6.**

CBDA inhibits PKA activity. (A) (a) A representative photograph of phosphorylated bands and non-phosphorylated bands of PKA-specific substrate peptide in MDA-MB-231 cells incubated with vehicle (time 0, lane 3), vehicle (48 h, lane 4), and 25 μM CBDA (48 h, lane 5) was shown. For positive and negative controls, the reactions were performed in the presence (lane 1) or absence (lanes 2) of PKA catalytic subunit, respectively. An arrow in the image indicates wells that samples are applied. (b) The relative intensity of phosphorylated bands in MDA-MB-231 cells is shown. Data are expressed as fold change *vs.* non-CBDA-treated group (left panel; lanes 4 *vs.* 5), as mean ± S.D. ($n = 3$). *Significantly different ($p < 0.05$) from the vehicle-treated control. (B) MDA-MB-231 cells were treated with vehicle (indicated as Cont.) or 25 μM CBDA for 48, 72, or 96 h. After the treatments, cell viability was measured according to the methods described in Section 2. Data are expressed as the percent of

vehicle-treated group, as mean ± S.D. ($n$ = 6). *Significantly different ($p < 0.05$) from the vehicle-treated control. N.S., not significant. (C) A model of CBDA-mediated RhoA activation through PKA inhibition.