## SUPPLEMENTARY MATERIALS AND METHODS

### *Lentiviral Id1 knockdown*

The Id1 and control shRNA lentiviral constructs were obtained from Open Biosystems and viral particles were assembled according to the manufacture's recommendations (pLKO Lentiviral Packaging System). The pLKO vectors were transfected into HEK293 cells along with packaging constructs, and the supernatants containing lentiviruses were collected and concentrated. MDA-MB231 cells were infected by the lentiviruses, and cells that expressed shRNA were selected using 1 μg/ml of puromycin. As a control, GFP-shRNA was used.

### *Immunohistochemistry*

Frozen lung tissues, five samples from each group, were obtained from vehicle- and CBD-treated mice. They were cut into 5-μm thick sections, fixed with 100% methanol, and permeabilized with 0.2% Triton X-100. After washing in TBS and blocking endogenous peroxidase activity with 3% hydrogen peroxide, they were incubated in 10% nonfat dry milk for 30 min at room temperature, and then incubated with 1 μg/ml of anti-Id1 antibody (Santa Cruz) or 1:250 of anti-Ki67 antibody (Bethyl) overnight at 4°C. The slides were washed in TBS and incubated with 1:500 streptavidin-horseradish peroxidase for 30 min. After washing in TBS, peroxidase was visualized by incubating in 0.5 mg/ml diaminobenzidine-4-HCl and 0.03% hydrogen peroxide in TBS for 3 min. Normal rabbit IgG or Id1 blocking peptide were used as controls. All of the sections were briefly counterstained with Mayer's hematoxylin solution, rinsed, dehydrated in graded alcohols, transferred in xylene, and mounted.

All foci in whole slides derived from two vehicle- and CBD-treated mice were assessed for percentage of cells staining positively for Id1 in the nucleus or cytoplasm, or both. The percentage of cells staining positive was attributed to the three groups according to their overall scores: negative if <10%; weakly positive if 10-40%; strongly positive if >40%. Ki67 staining was assessed for percentage of cells showing positive signal in the nucleus.

**SUPPLEMENTARY FIGURE LEGENDS**

***Supplementary Figure 1. CBD produces a dose-dependent reduction of metastatic spread to the lung and increases survival.*** Lung metastases were generated in BALB/c mice by i.v. injection of $2x10^4$ mouse 4T1 cells. **A)** One day after the injection, the tumor bearing mice were injected i.p. once a day with vehicle or CBD (0.1 to 5 mg/kg) for 14 days and metastasis was evaluated. % metastasis = total tumor number of lung metastatic foci in drug-treated group/total number of lung metastatic foci in vehicle-treated group where the respective controls (vehicle-treated mice) were set as 100%. **B)** Lung metastases measured in mice treated with vehicle or CBD 1 mg/kg included those with metastatic foci <2 mm and ≥2mm. **C)** Mice treated with vehicle or 1 mg/kg CBD, starting one day after i.v. injection of $2x10^4$ 4T1 cells, were observed until they demonstrated signs of disease progression that necessitated euthanasia. Survival between groups was compared using a log-rank Mantel-Cox test. *$p<0.05$, **$p< 0.01$, and $^{\#}p<0.001$ indicate statistically significant differences from control.

***Supplementary Figure 2. CBD reduces metastasis but not primary tumor growth.*** Primary tumors and subsequent secondary tumors (metastases) were generated in BALB/c mice by injection of $5x10^4$ mouse 4T1 cells into the mammary fat pad between the second and third nipple. Treatment with CBD was initiated upon detection of the first palpable tumor (day 7). The tumor bearing mice were injected i.p. three times a week with vehicle or CBD (0.1 to 2.5 mg/kg) for three weeks. **A)** To determine the tumor size *in situ*, the perpendicular largest diameters of the tumors were measured in millimeters as $(L \times W^2)/2$ based on a modified ellipsoidal formula. **B)** Visible lung metastases were counted and measured using a dissecting

microscope and % metastasis (total metastastic foci in treated/vehicle x 100) was calculated. **C)** The number of metastatic foci ≥2 mm was also determined. *p<0.05 and **p<0.005 indicate statistically significant differences from control.

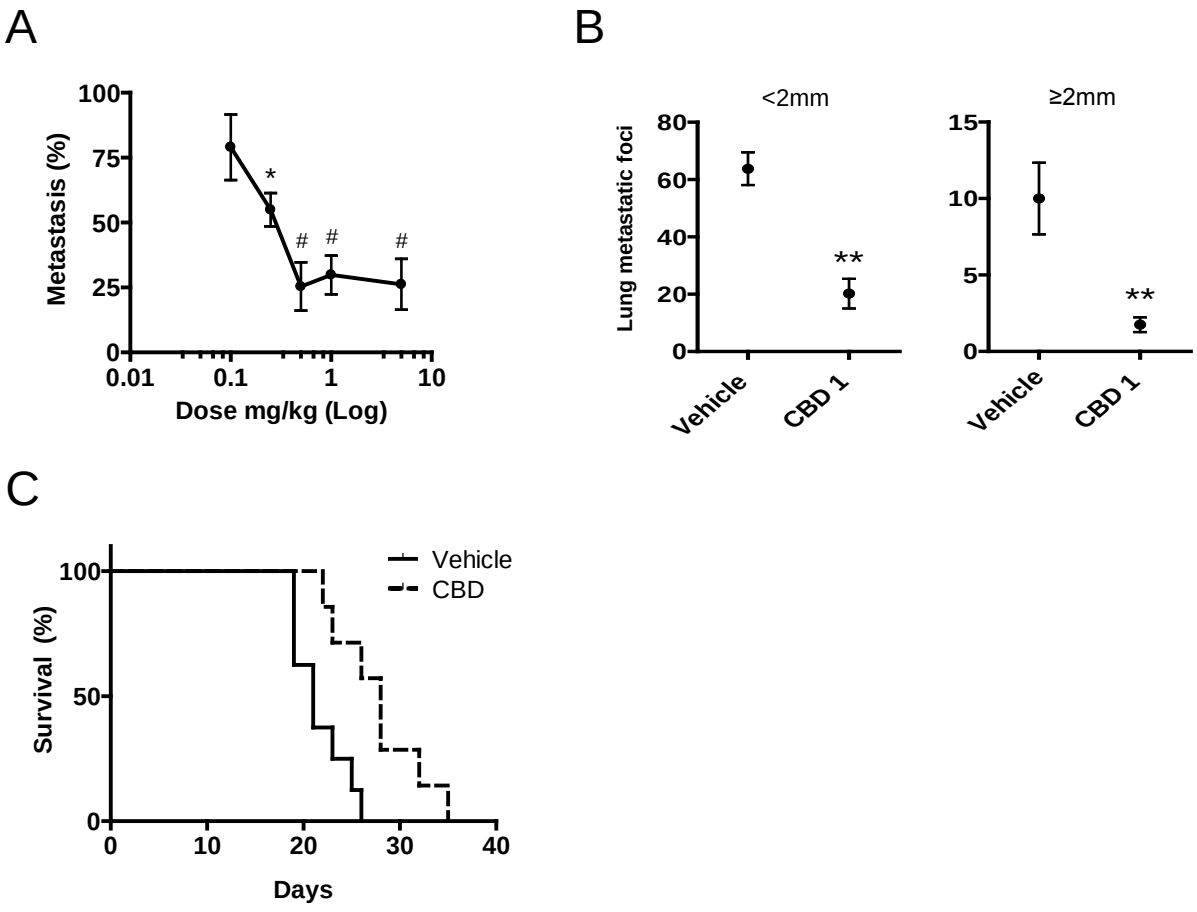
***Supplementary Figure 3. CBD or Id1 knockdown produce comparable anti-metastatic activity against human breast cancer cells.*** **A)** To confirm the correlation between the effects of CBD and inhibition of Id1 expression *in vivo*, we established stable pooled populations of MDA-MB231 cells expressing Id1 shRNA that showed a down-regulation of Id1 protein levels (lower panel) comparable to parental cells treated with 2 μM CBD for two days (upper panel). **B)** In culture, cell proliferation/viability (left panels) and invasion (right panels) rates were significantly reduced in parental MDA-MB231 cells treated with CBD or in MDA-MB231 cells expressing Id1 shRNA. **C)** Left panel: lung metastases were generated in athymic *nu/nu* mice after i.v. injection of $5x10^5$ MDA-MB231. One day after the injection, the tumor bearing mice were injected i.p. once a day with vehicle or 0.5 to 1mg/kg CBD for six weeks. The percentage of metastatic foci (total metastatic foci in treated/vehicle x 100) was compared between vehicle and CBD treated groups. Right panel: the percentage of metastatic foci was compared between athymic *nu/nu* mice injected i.v. with $5x10^5$ MDA-MB231 cells stably expressing Ctl shRNA or Id1 shRNA. **D)** Left panel: the number of lung metastatic foci ≥1 mm was compared between vehicle and CBD treated groups. Right panel: the number of lung metastatic foci ≥1 mm was compared between group injected with cells stably expressing Ctl shRNA or Id1 shRNA. *p< 0.05 and **p< 0.01 indicate statistically significant differences from control.

***Supplementary Figure 4. The activity of various cannabinoids was compared for the down-regulation of Id1 expression.*** **A)** Structures of CBD, CP55940, THC, abnormal (Abn)-CBD, O-1918, and O-1663. **B)** Proteins from MDA-MB231 cells treated with 1.5 μM of multiple cannabinoids for three days were extracted and analyzed for Id1 by Western blot analysis. Normalization was carried out by stripping the blots and re-probing with a monoclonal anti-tubulin antibody. Densitometry readings of the blots were taken and the percentage relative expression was calculated as the expression of Id1 in the treated cells/vehicle cells x 100.

***Supplementary Figure 5. $CB_2$ receptor expression and CBD-dependent generation of ROS in breast cancer cells.*** **A)** Protein lysate from mouse spleen, mouse 4T1 cells or human MDA-MB231 breast cancer cells was analyzed for $CB_2$ receptor expression. **B)** Mouse 4T1 cells (upper panel) were treated with 20 μM α-tocopherol (TOC), 1 μM the $CB_1$ receptor antagonist (SR141716A - SR1), or 1μM the $CB_2$ receptor antagonist (SR144528 - SR2). Human MDA-MB231 cells (lower panel) were treated with 20 μM α-tocopherol (TOC), 4 μM the $CB_1$ receptor antagonist (SR141716A - SR1), or 4 μM the $CB_2$ receptor antagonist (SR144528 - SR2). Cell proliferation/viability was then evaluated using the MTT assay. **C)** Human MDA-MB231 cells were treated with vehicle or CBD (μM) for two days and the production of ROS was then measured using 2'-7'dichloro-dihydrofluorescein and cell flow cytometry.

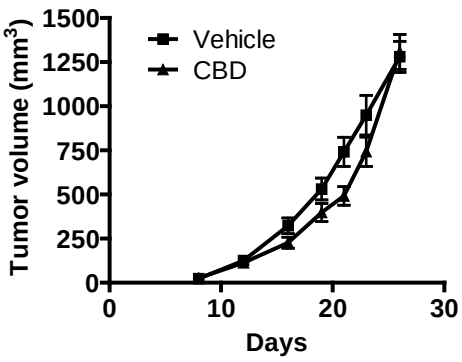
***Supplementary Figure 6. O-1663 produces a significant inhibition of advanced stage breast metastasis.*** **A)** Lung metastases were generated in BALB/c mice by i.v. injection of $2x10^4$ 4T1. One week after the injection of the cells, the tumor bearing mice were injected i.p. once a day with vehicle or O-1663 for 14 days. In the treated group, 50% of the mice were still alive and

demonstrated no signs of disease progression at time of euthanasia (2 months), few metastatic foci were observed when lungs were stained with India ink and visualized using a dissecting microscope. **B)** Athymic *nu/nu* mice were treated with vehicle or O-1663 (1 mg/kg) starting 18 days after i.v. injection of human MDA-MB231-luc-D3H2LN breast cancer cells, a time point where the presence of lung tumors was confirmed using BLI. Fifteen minutes before imaging, mice were injected i.p. with 150 mg/kg of luciferin. Mice were evenly distributed between vehicle and O-1663 treated groups before the initiation of treatment. Comparison of tumor progression over time was analyzed between studies by comparing the absolute unit of radiance in photons(p)/sec(s)/cm2/steridian(sr). **C)** All athymic *nu/nu* mice where then imaged one week (left panel) and ten days (right panel) later after the initiation of the study until animals in the vehicle group demonstrated signs of disease and began to be euthanized. **D)** Regression of tumor burden, beyond the initial size the tumor when the treatment started, was observed in 25% of the athymic *nu/nu* mice. One mouse, where the tumor was regressing in the lung, was removed from the study to confirm the imaging results by visualizing the lung using an India ink stain and a dissecting microscope. **E)** Athymic *nu/nu* mice treated with vehicle or CBD (1 mg/kg) starting two days after i.v. injection of MDA-MB231-luc-D3H2LN cells were observed until they demonstrated signs of disease progression that necessitated euthanasia.




Supplementary Figure 1

A

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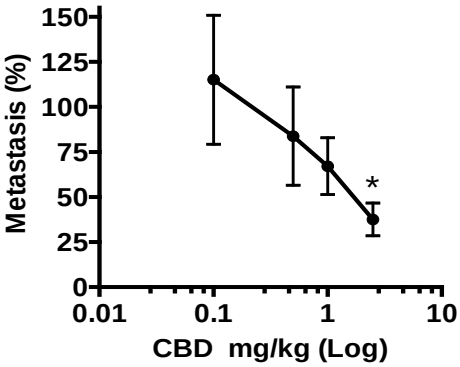


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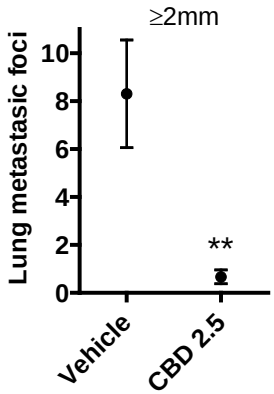
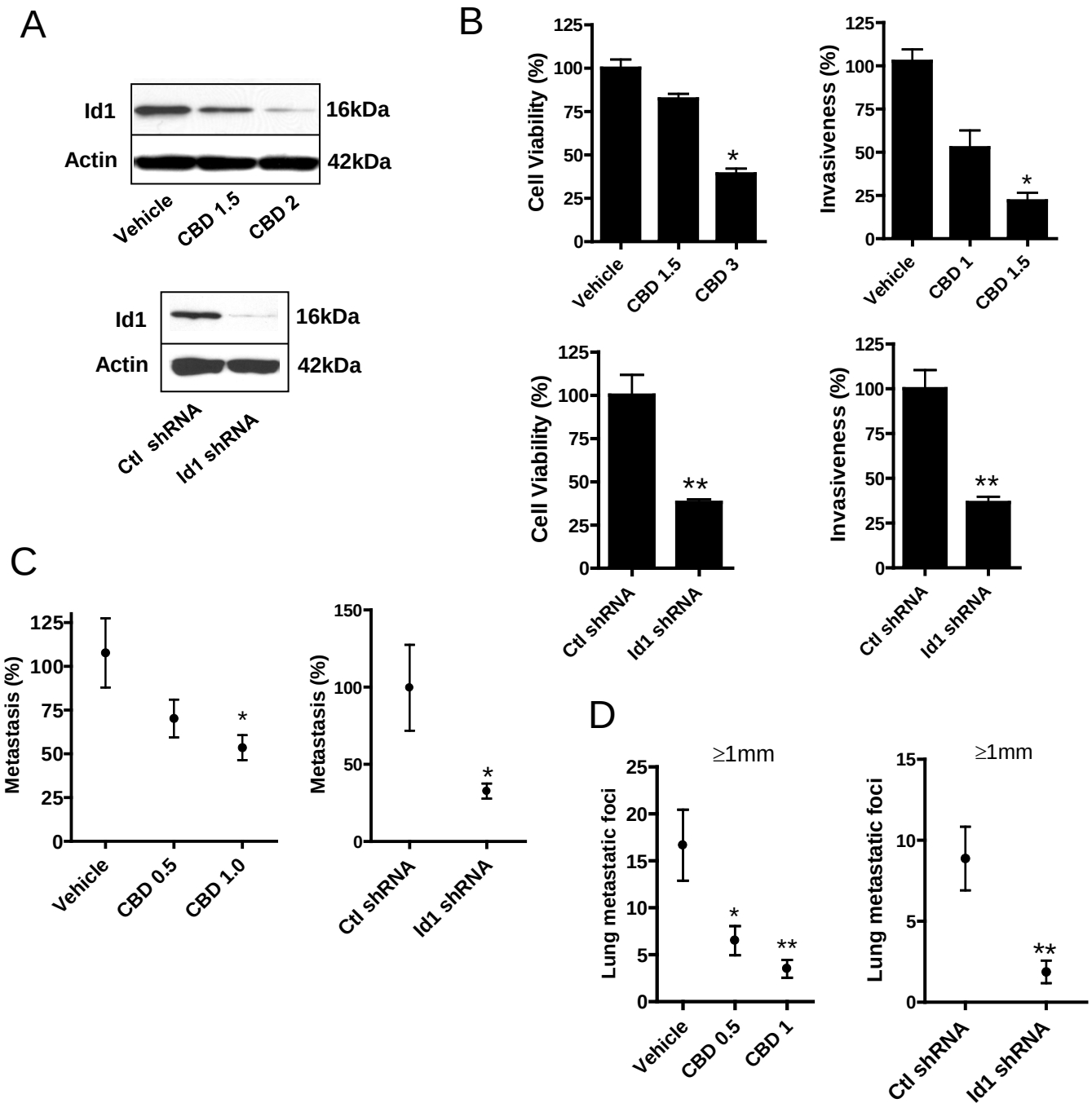
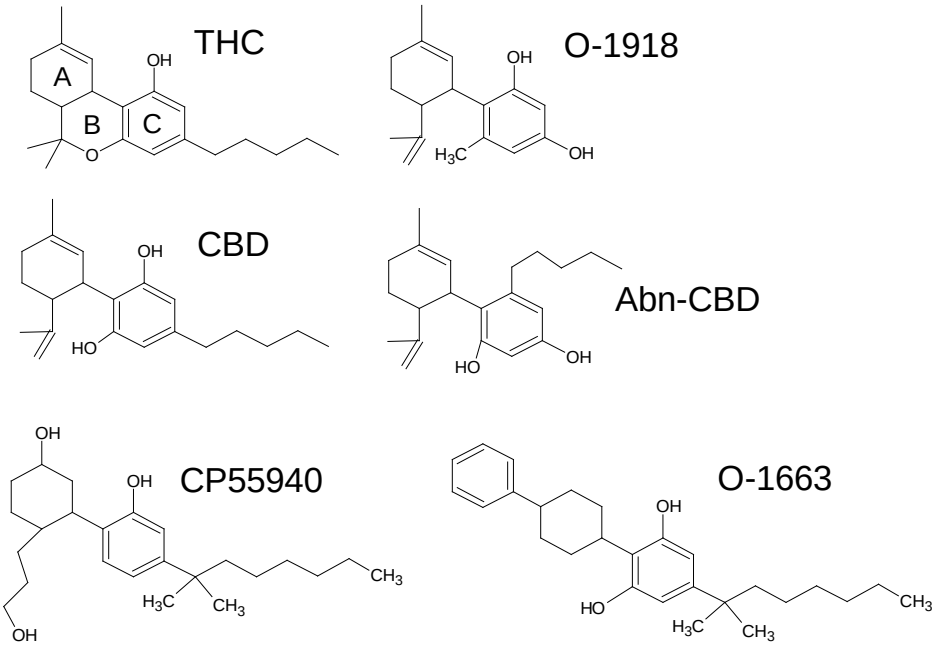



Supplementary Figure 2




Supplementary Figure 3

A

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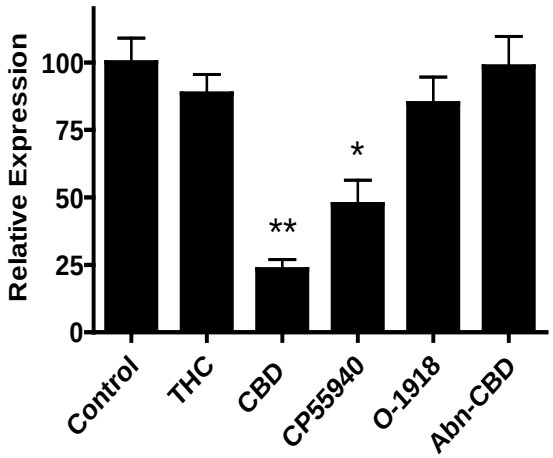


Supplementary Figure 4

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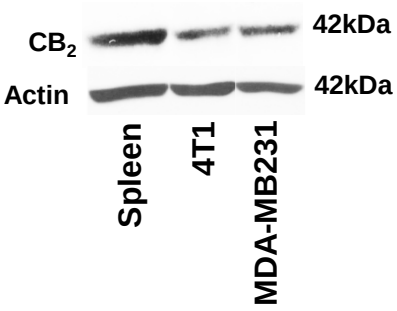


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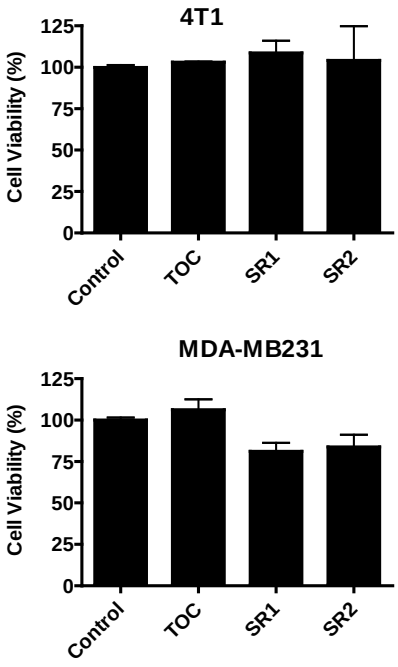


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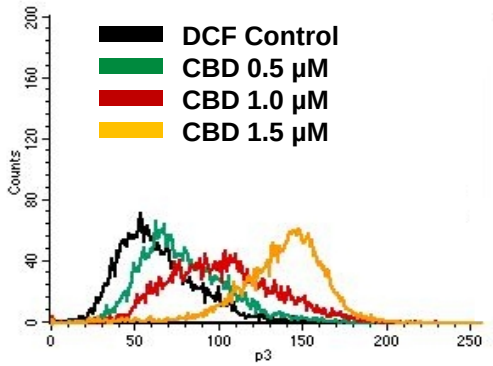
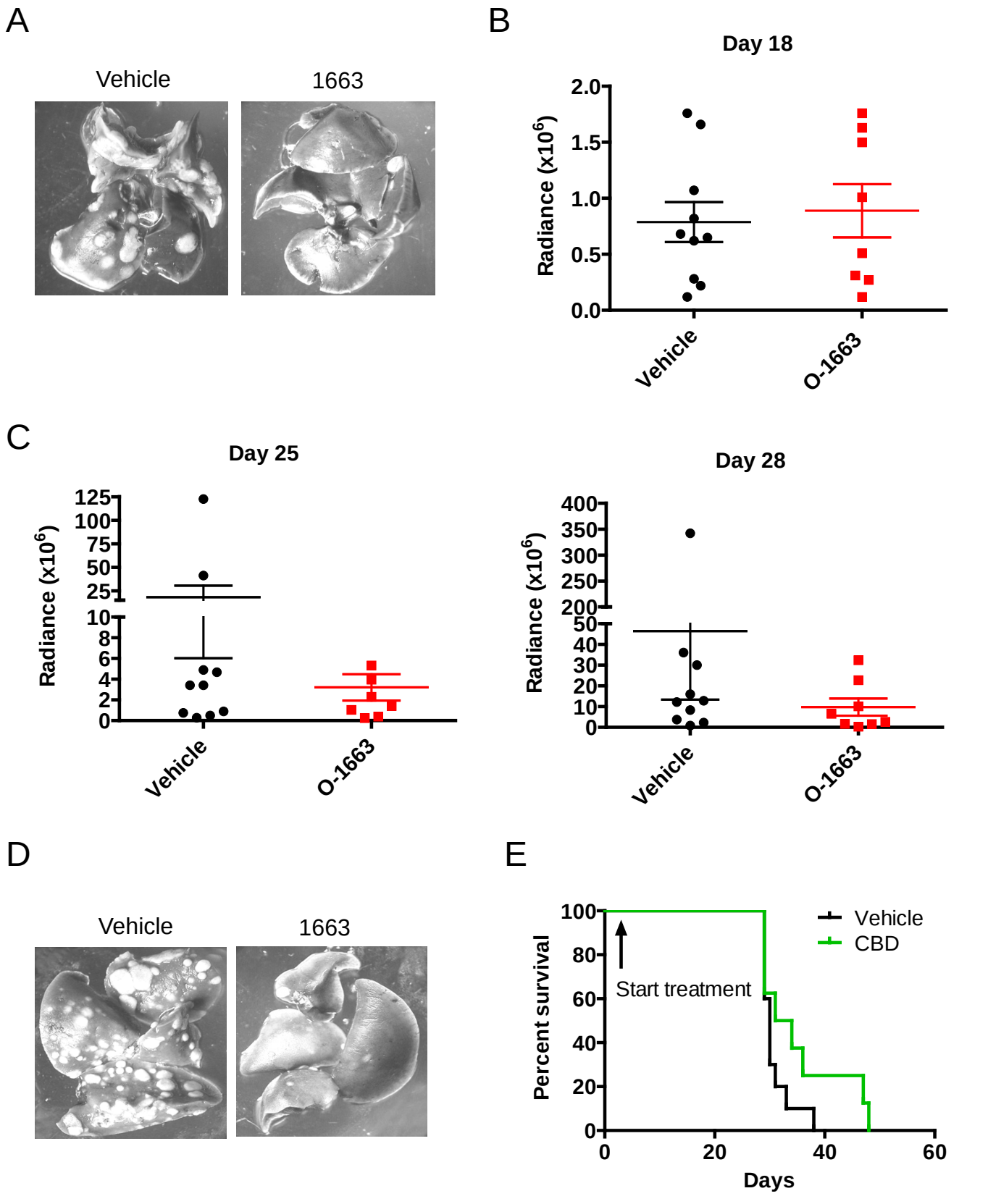



Supplementary Figure 5




Supplementary Figure 6

***Supplementary Table 1. CBD inhibits Id1 expression in lung metastatic foci.*** The data presented correspond to the number of metastatic foci in the lungs harvested from vehicle- and CBD-treated mice, where immunohistochemical detection of Id1 was either negative, weakly positive or strongly positive. P value <0.0004 was calculated using Chi-square test.

| | Negative | Weakly Positive | Strongly Positive | Total |
|---|---|---|---|---|
| **Vehicle** | **0** | **9** | **14** | **23** |
| **CBD** | **4** | **13** | **1** | **18** |
| **Total** | **4** | **22** | **15** | **41** |

***Supplementary Table 2. Inhibition of cAMP by O-1663 and WIN55,212-2 in $CB_2$-transfected CHO cells (CHO-$CB_2$).*** Experiments were carried out as previously described (Felder *et al.,* 1995). Data represent the mean with corresponding confidence limits (CL) for 3 independent determinations.

| Cell line | Compound | $IC_{50}$ (CL) | $E_{max}$ (CL) |
|---|---|---|---|
| CHO-$CB_2$ | WIN55,212-2 | 0.27 nM (0.04-1.8) | 102% (101-104) |
| | O-1663 | 260 nM (120-380) | 95% (81-108) |