

Supporting Information – Experimental Results

Study 1 – Antitumor effect of cannabinoids on breast cancer

The effects of pure THC, versus a whole plant extract (WPE), versus cisplatin were compared using a MTT assay.

Several replicates of the MTT assay, a colorimetric assay, were performed. The following results are representative:

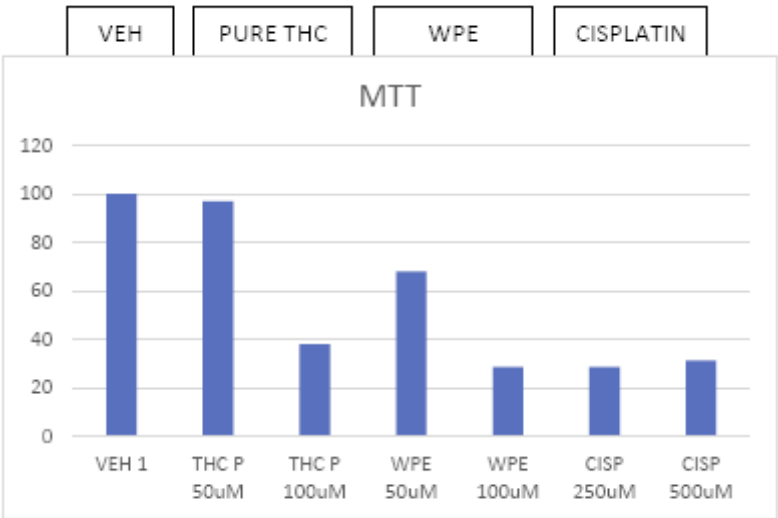


Figure 1

Study 2 – Antitumor action of a pure cannabinoid vs a botanical drug preparation in preclinical models of breast cancer

Materials and methods

Materials

Pure THC ($\geq 99\%$ HPLC) was from THC Pharm GmbH (Frankfurt, Germany). The cannabis drug preparation (CDP) was produced by Aunt Zelda's (Bodega Bay, CA). Briefly, organically-grown fresh cannabis flowers were frozen at -20°C for 48 h, and then macerated in ethanol for 24 h at the same temperature. The plant matter was vacuum filtered, scrubbed with charcoal, and re-filtered. Alcohol was evaporated with a rotary evaporator, followed by magnetic stirring on hot plate to achieve cannabinoid decarboxylation. The composition of the extract was determined as described below, as summarised in Tables 1a and 1b:

Table 1a – Cannabinoids in the cannabis drug preparation.

Cannabinoid	Concentration (mg/g)	Relative amount based on Cannabis extract (% w/w)
THCA	3.449	0.345
THC	551.308	55.1
THCV	ND	-
CBD	ND	-
CBDA	ND	-
CBG	3.667	0.367
CBN	ND	-
CBC	ND	-

Table 1b – Terpenes in the cannabis drug preparation.

Terpene	Concentration (mg/g)	Relative amount based on terpene fraction (% w/w)
α -Bisabolol	0.177	4.00
Camphene	BDL	-
3-Carene	BDL	-
β-Caryophyllene	1.948	44.05
Caryophyllene oxide	0.032	0.72
p-Cymene	0.178	4.03
Geraniol	ND	-
Guaiol	ND	-
α-Humulene	0.557	12.6
Isopulegol	0.023	0.52
D-Limonene	ND	-
Linalool	0.620	14.0
β -Myrcene	0.025	0.57
Nerolidol 1	0.357	8.07
Nerolidol 2	0.081	1.83
Ocimene	0.049	1.11
α -Pinene	0.015	0.34
β-Pinene	0.317	7.17
α -Terpinene	0.013	0.29
γ -Terpinene	0.013	0.29
Terpinolene	0.017	0.38
Total (terpenes)	4.422	

ND = not detected; BDL = below detectable limit

For experiments involving the five most abundant terpenes, β -caryophyllene, α -humulene and nerolidol 1 were purchased from Sigma-Aldrich (St. Louis, MO), and linalool and β -pinene from True Terpenes (Portland, OR). A terpene composition was prepared by mixing the mentioned terpenes in DMSO at the same concentrations present in the CDP. The CB₁R-selective antagonist SR141716 (SR1) was from NIMH (Bethesda, EEUU), the CB₂R-selective antagonist SR144528 (SR2) from Tocris Bioscience (Abingdon, UK), α -tocopherol (TOC) and tamoxifen from Sigma-Aldrich, cisplatin from Accord (Durham, NC), and paclitaxel from MedChem Express (Sollentuna, Sweden). Lapatinib was kindly donated by GlaxoSmithKline (Brentford, UK) and epirubicin by Dr. Gema Moreno-Bueno (MD Anderson Cancer Center, Madrid, Spain). For experiments in cell cultures, all drugs except epirubicin and cisplatin were dissolved in DMSO. Epirubicin was dissolved in H₂O, and cisplatin in PBS.

Analysis of the cannabis drug preparation

The CDP cannabinoid and terpene composition was determined by Sonoma Labworks (Santa Rosa, CA). The presence and concentration of the following cannabinoids was determined by high-pressure liquid chromatography (HPLC): Δ^9 -tetrahydrocannabinolic acid (THCA), Δ^9 -tetrahydrocannabinol (THC), Δ^9 -tetrahydrocannabivarin (THCV), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabigerol (CBG), cannabinol (CBN) and cannabichromene (CBC). Separation, identification and quantitation of terpenes and residual solvents was performed by gas chromatography with flame ionization detector (GC-FID). The following terpenes were analyzed: α -bisabolol, camphene, 3-carene, β -caryophyllene, caryophyllene oxide, p-cymene, geraniol, guaial, α -humulene, isopulegol, D-limonene, linalool, β -myrcene, nerolidol 1, nerolidol 2, ocimene, α -pinene, β -pinene, α -terpinene, γ -terpinene and terpinolene. The presence of microbial contaminants and pesticides was ruled out by quantitative PCR and liquid chromatography tandem mass spectrometry (LC-MS/MS), respectively. Results were counteranalyzed by Canna Foundation (Valencia, Spain) and Ananda Analytics Lab (Madrid, Spain).

Cell lines and cultures

All human breast adenocarcinoma cell lines were from ATCC-LGC (Barcelona, Spain): MCF-7 and T47D (ER+, PR+, HER2-); BT474 and HCC1954 (HER2+); MDA-MB-231 and SUM 159 (ER-, PR-, HER2-). All of them were authenticated by STR profiling (Genomics core facility at Alberto Sols Biomedical Research institute, Madrid, Spain). They were cultured in RPMI (HCC1954, BT474 and T47D), MEM (MCF7), DMEM (MDA-MB-231) or Ham's F12 (SUM 159), supplemented with 10% FBS, 1% penicillin / streptomycin. T47D, MCF7, SUM 159 and BT474 cells were also supplemented with 10 μ g/mL insulin, and SUM159 with 0.5 μ g/mL hydrocortisone. All cells were maintained at 37°C in an atmosphere of 5% CO₂.

Cell viability assays

Cells were seeded at a density of 5000 cells/cm² in 10% FBS-containing medium. Twenty-four hours later, they were serum-starved overnight, and then treated with the indicated compounds for 24 h or 48 h. Cells were subsequently fixed and stained with a crystal violet solution (0.1% crystal violet, 20% methanol in H₂O) for 20 minutes. After intensive washing with H₂O, the stained cells were solubilized in methanol, and absorbance measured at 570 nm. Data are expressed as the percentage of viable cells vs. vehicle-treated cells, set at 100%, and represented as mean $\pm$ SEM of at least 3 independent experiments.

In experiments aimed at comparing the potency of pure THC vs. that of the CDP, the same amount of THC was used. For example, if the effect of 3 μ M THC was under study, it was

compared with an amount of CDP that provided 3 μ M THC. The cannabinoid receptor antagonists (1 μ M) and tocopherol (10 μ M) were added to the cell cultures 1 hour prior to THC.

Animal studies

All procedures involving animals were performed with the approval of Complutense University Animal Experimentation Committee and Madrid Regional Government, according to the European Official Regulations.

Tumors were generated in 6 week-old female nude mice (Envigo, Barcelona, Spain) by subcutaneous injection in the right flank of 5×10^6 HCC1954 cells or 5×10^6 MDA-MB-231 cells in PBS. For ER+/PR+ tumor generation, a 17 β -estradiol pellet (Innovative Research of America, Sarasota, FL) was subcutaneously inserted with a precision trochar (Innovative Research of America) 1 day before T47D cell injection (10×10^6 cells). Tumor volume was routinely measured with an external caliper, and when it reached 200 mm³ (for BT474 and T47D) or 100 mm³ (for 8 MDA-MB-231), animals were randomly assigned to the different experimental groups and treatment started. Pure THC and CDP were administered at a dose of 45 mg/Kg, 3 times a week, in 100 μ L of sesame oil, by oral gavage (for CDP, 45 mg/Kg means a dose of the extract that contains 45 mg/Kg of THC). Tamoxifen (2.5 mg/kg in 100 μ L of sesame oil) and cisplatin (3 mg/kg in 100 μ L of PBS) were administered i.p. 3 times a week; and lapatinib (100 mg/Kg) daily by oral gavage in 200 μ L of 0.5% hydroxypropyl methylcellulose plus 0.1% Tween 80. Control animals received the corresponding vehicles with the same pattern and route of administration. Animals were sacrificed after one month of treatment.

Statistical analyses

All data were analyzed using Prism 6 (GraphPad), and are presented as mean $\pm$ SEM of at least 3 independent experiments. Unpaired 2-tailed Student's *t* test was used to assess 2 independent groups. One-way ANOVA was used to test multi-group comparisons with Tukey's post-hoc test. The groups with 2 independent variables were tested by 2-way ANOVA (*in vivo* experiments). Significance level was below 0.05 in all cases. IC₅₀ values were determined with CompuSyn software.

Results

Effects on preclinical models of hormone-sensitive breast cancer

Although ER+/PR+ breast cancer is associated with high rates of response to targeted treatments, innate and acquired resistance also occurs, which constitutes a clinical challenge because, like in other breast cancer subgroups, recurrences and disease dissemination are usually very difficult to treat. To analyze whether this subtype of breast cancer may be sensitive to cannabinoid treatment, T47D cells (a human ER+ and PR+ breast cancer cell line) were challenged with either pure THC or the CDP whose precise composition is detailed in Tables 1a and 1b. As shown in Fig. 1A, both THC and the CDP decreased the viability of T47D cells in a concentration dependent manner.

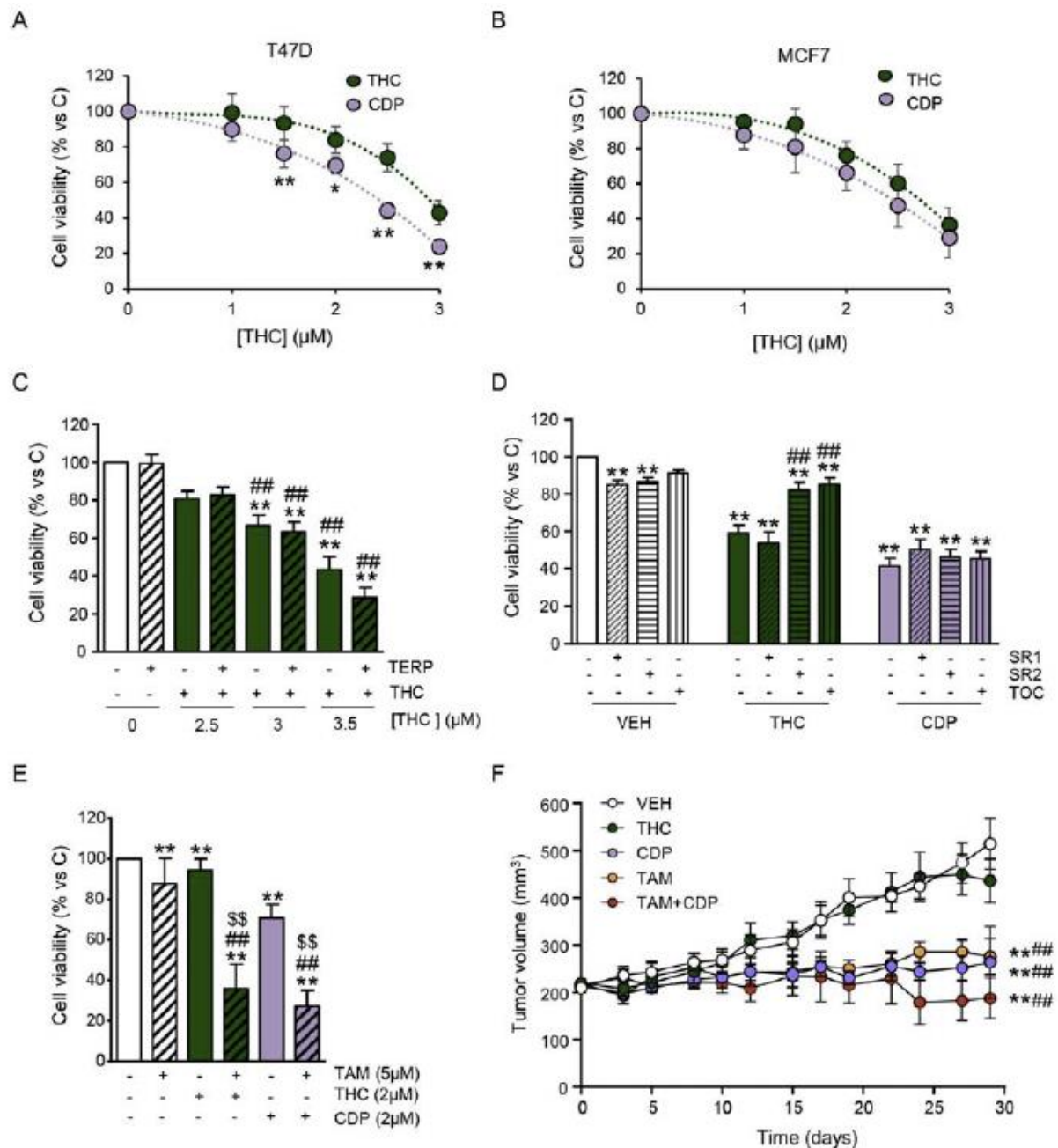


Fig. 1. Effects on preclinical models of ER+/PR+ breast cancer. (A-E) Cell viability, as determined by crystal violet staining, in response to the indicated treatments for 24 h. Results are expressed as % versus vehicle-treated cells, set at 100%. (A) Viability of T47D (A) and MCF7 (B) human breast ER+/PR+ adenocarcinoma cells in response to increasing concentrations of THC, administered either as a pure compound (THC) or as part of a cannabis drug preparation (CDP). (C-E) Viability of T47D cells upon treatment with pure THC, a terpene cocktail containing β-caryophyllene, linalool, α-humulene, nerolidol 1 and β-pinene (TERP), or the combination in the same proportion as in the CDP (C); in response to THC or CDP and the CB₁R-selective antagonist SR141716 (SR1, 1 μM), the CB₂R-selective antagonist SR144528 (SR2, 1 μM) or the antioxidant agent α-tocopherol (TOC, 10 μM) (D); or in response to tamoxifen (TAM), alone or in combination with THC or CDP. (F) Growth of ectopic tumors generated in nude mice by subcutaneous injection of T47D cells. Animals were treated with vehicle, THC (45 mg/Kg), CDP (containing 45 mg/Kg THC), TAM (2.5 mg/Kg), or the combination of TAM and CDP. *, p < 0.05

and **, $p < 0.01$ vs. vehicle-treated cells/animals; ###, $p < 0.01$ vs. TERP (C), THC or CDP (D), TAM (E) or THC (F); \$\$, $p < 0.01$ vs. THC or CDP (E).

Surprisingly, the botanical preparation was more potent than the pure cannabinoid. Thus, the IC_{50} value was $2.2 \mu M$ for CDP and $2.9 \mu M$ for THC. To determine whether this response was T47D-specific or could be extrapolated to other hormone-sensitive breast cancer cells, similar experiments were conducted in MCF7 cells (another human ER+ and PR+ breast cancer cell line). As for T47D, both pure THC and the CDP decreased the viability of MCF7 cells, an effect that was concentration dependent (Fig. 1B). In this case, the extract tended to be more potent than the pure cannabinoid as well (IC_{50} THC = $2.8 \mu M$; IC_{50} CDP = $2.4 \mu M$).

It is important to highlight that, in these experiments, the effect of concentration of THC was controlled for, which was administered either as pure compound or as part of the CDP. Therefore, the observed differences in potency are due to the THC-accompanying compounds present in the botanical drug preparation. To determine if the most abundant terpenes in the CDP were responsible for that effect, a terpene cocktail was prepared containing the same concentrations of β -caryophyllene, linalool, α -humulene, nerolidol 1 and β -pinene that are present in the extract (Table 1b), and used this composition alone or in combination with THC, at the same proportion as in the CDP as well. Results on cell viability are shown in Fig. 1C.

The primary targets of cannabinoid antiproliferative action in ER+/PR+ breast cancer cells were examined next. As shown in Fig. 1D, the effect of pure THC was not affected by pre-incubation with the CB_1R -selective antagonist SR141716 (SR1). On the contrary, THC action was partially prevented by the CB_2R -selective antagonist SR144528 (SR2), and by the antioxidant compound alpha-tocopherol (TOC) (Fig. 1D). These results indicate that the effect of THC on ER+/PR+ breast cancer cell viability is produced by activation of CB_2 receptors, and the generation of reactive oxygen species (ROS). Of interest, neither blockade of CB_2R nor preincubation with TOC prevented the antiproliferative effect of the CDP, suggesting that, in this case, different/complementary targets and mechanisms of action are activated.

The impact of cannabinoid-based therapies, on the efficacy of current standard antitumor therapies for hormone-sensitive breast cancer, was analysed next. Specifically, THC or the CDP were combined with tamoxifen. When submaximal concentrations of this SERM and pure THC were applied together to the cell cultures, the viability of T47D cells decreased in an additive manner (Fig. 1E). Similar effects were observed when tamoxifen and the CDP were combined (Fig. 1E).

Finally, experiments were conducted to determine whether all the observations made in cell cultures were also evident in a more physiological setting. Specifically, ER+/PR+ tumors in immune-deficient mice were generated by subcutaneous injection of T47D cells. At the THC doses used in this study, the pure compound did not trigger any significant antitumor response (Fig. 1F – green dots). On the contrary, and administered at the same dose of THC, the CDP produced a remarkable decrease in tumor growth (Fig. 1F – purple dots), confirming the higher potency of the botanical preparation vs. the pure cannabinoid as observed *in vitro*. Tamoxifen, and the combination of tamoxifen and CDP, also impaired tumor growth, as shown in Fig. 1F (see orange and red dots, respectively).

Effects on preclinical models of HER2+ breast cancer

Although with worse general prognosis than those with ER+/PR+ cancer, patients with HER2-overexpressing tumors have significantly better clinical outcomes since the incorporation of HER2-targeted therapies to the medical practice. However, local and distant recurrences are not unusual, and their therapeutic management is mostly palliative. However, as for most of the preclinical research conducted so far, those studies were carried out with pure cannabinoids. Here, it was observed that both pure THC and the CDP decreased the viability of HCC1954 human HER2+ breast cancer cells (Fig. 2A).

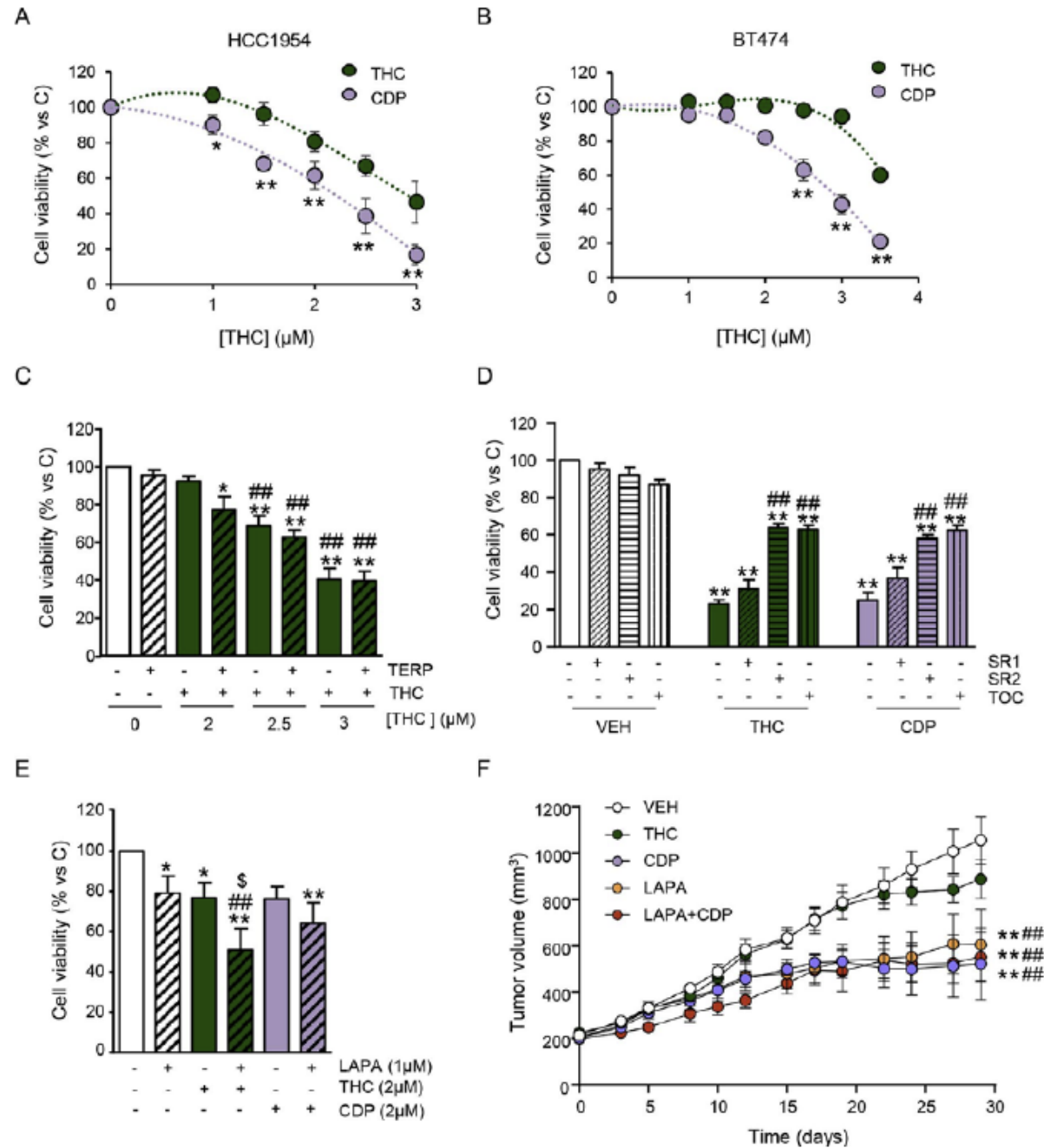


Fig. 2. Effects on preclinical models of HER2+ breast cancer. (A-E) Cell viability, as determined by crystal violet staining, in response to the indicated treatments for 24 h. Results are expressed as % versus vehicle-treated cells, set at 100%. (A) Viability of HCC1954 (A) and BT474 (B) human breast HER2+ adenocarcinoma cells in response to increasing concentrations of THC, administered either as a pure compound (THC) or as part of a cannabis drug preparation (CDP). (C-E) Viability of HCC1954 cells upon treatment with pure THC, a terpene cocktail containing β -caryophyllene, linalool, α -humulene, nerolidol 1 and β -pinene (TERP), or the combination in the same proportion as in the CDP (C); in response to THC or CDP and the CB₁R-selective antagonist SR141716 (SR1, 1 μ M), the CB₂R-selective antagonist SR144528 (SR2, 1 μ M) or the antioxidant agent α -tocopherol (TOC, 10 μ M) (D); or in response to lapatinib (LAPA), alone or in combination with THC or CDP. (F) Growth of ectopic tumors generated in nude mice by subcutaneous injection of BT474 cells. Animals were treated with vehicle, THC (45 mg/Kg), CDP (containing 45 mg/Kg THC), LAPA (100 mg/Kg), or the combination of LAPA and CDP. *, $p < 0.05$ and **, $p < 0.01$ vs. vehicle-treated cells/animals; ##, $p < 0.01$ vs. TERP (C), THC or CDP (D), LAPA (E) or THC (F); \$, $p < 0.05$ vs. THC (E).

As for hormone-sensitive cells, the botanical preparation was more potent than the pure compound (IC_{50} THC = 2.7 μ M; IC_{50} CDP = 2.0 μ M). Surprisingly, this response was not HCC1954-specific, as it was also observed in another HER2+ human breast cancer cell line (BT474) (Fig. 2B). The IC_{50} values were 3.7 μ M for pure THC and 2.7 μ M for the CDP. Results on cell viability for the cocktail containing the 5 most abundant terpenes in the botanical extract, or the combination of this cocktail with pure THC, are shown in Fig. 2C, which again points to different compounds or a combination of compounds as responsible for the increased potency of the CDP, compared to pure THC. Regarding mechanism of action, and as in ER+/PR+ cells, THC decreased cell viability by activating CB₂R and generating ROS, as indicated by the observation that this effect was prevented by SR2 and TOC, and not by SR1 (Fig. 2D). In this case, however, and unlike hormone-sensitive cells, the effect of the CDP was partially prevented by CB₂R blockade and scavenging of ROS (Fig. 2D), which suggests that additional targets and mechanisms of actions are involved in cannabinoid action.

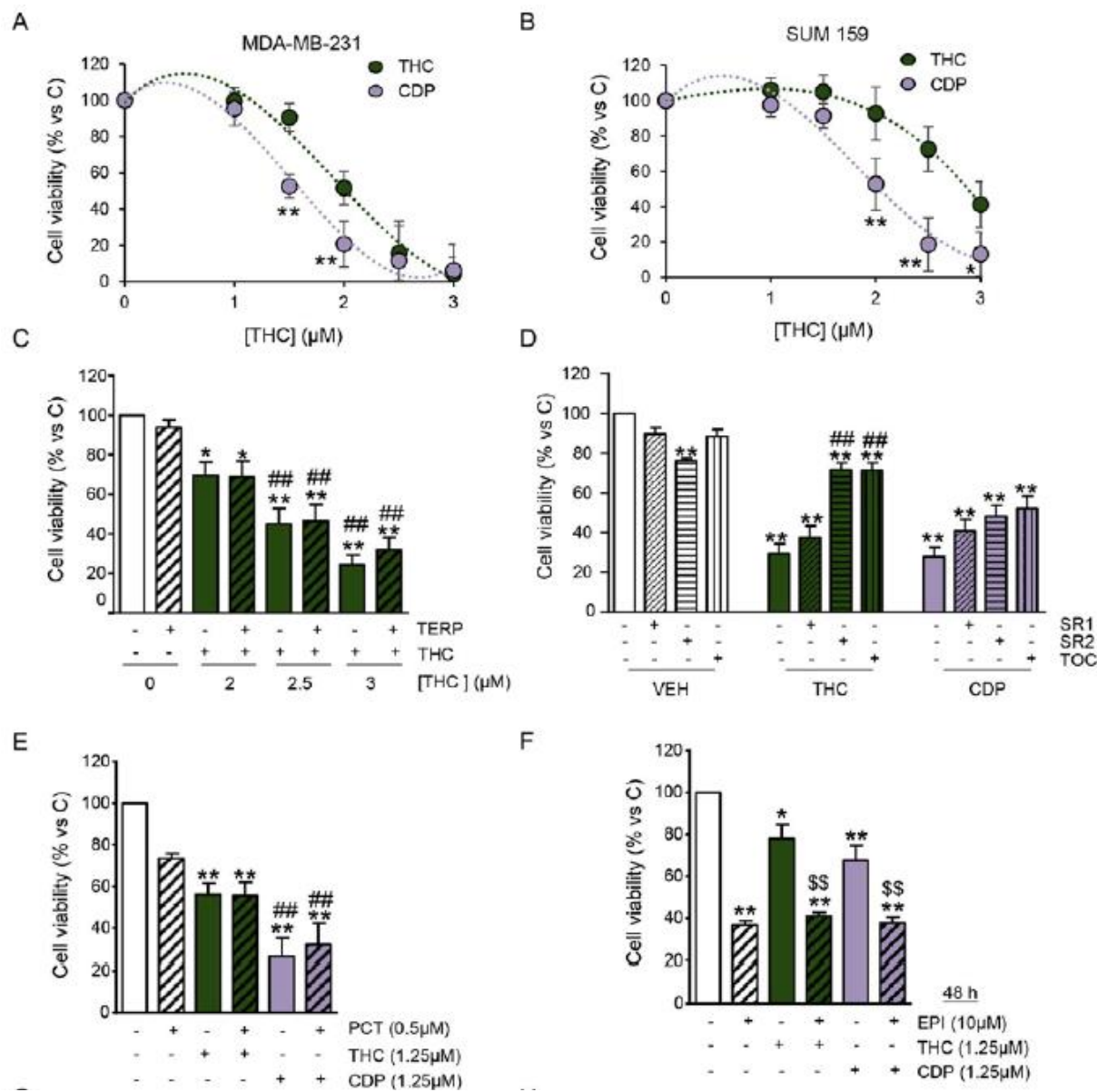
For the combination therapy experiments, lapatinib, a small molecule tyrosine kinase inhibitor that targets both HER2 and HER1 (EGFR) was used. Simultaneous addition of submaximal concentrations of lapatinib and pure THC produced an additive decrease in cancer cell viability (Fig. 2E), an effect that was also observed when combining the kinase inhibitor and a submaximal concentration of the CDP (Fig. 2E).

The effect of the cannabinoid-based therapies alone, or in combination with lapatinib in an animal model of HER2+ breast cancer, was then tested. Specifically, subcutaneous tumors were generated by injection of BT474 human HER2+ breast cancer cells into immune-compromised mice. As in cultured cells, and using the same doses of THC in the pure-compound group as in the CDP group, the botanical extract was significantly more potent than the pure cannabinoid in impairing tumor growth (Fig. 2F). As in the case of tamoxifen, lapatinib did not have any impact, either positive or negative, on the effect of the CDP (Fig. 2F).

Effects on preclinical models of triple-negative breast cancer

Triple-negative is the breast cancer subtype with the worst prognosis. These cancer cells are very aggressive in terms of proliferation, migration and invasion features. In addition, they lack specific molecular markers that today can be therapeutically targetable, which makes indiscriminate chemotherapy the only recommended treatment for these patients. Although a small percentage of them respond very well to chemotherapy, the vast majority does not, and the key aim of therapies implies increasing disease-free survival. To study the effect of

cannabinoids on this breast cancer subtype, human MDA-MB-231 cells were used as a model. Both pure THC and the CDP decreased the viability of these cells in a concentration-dependent manner (Fig. 3A).



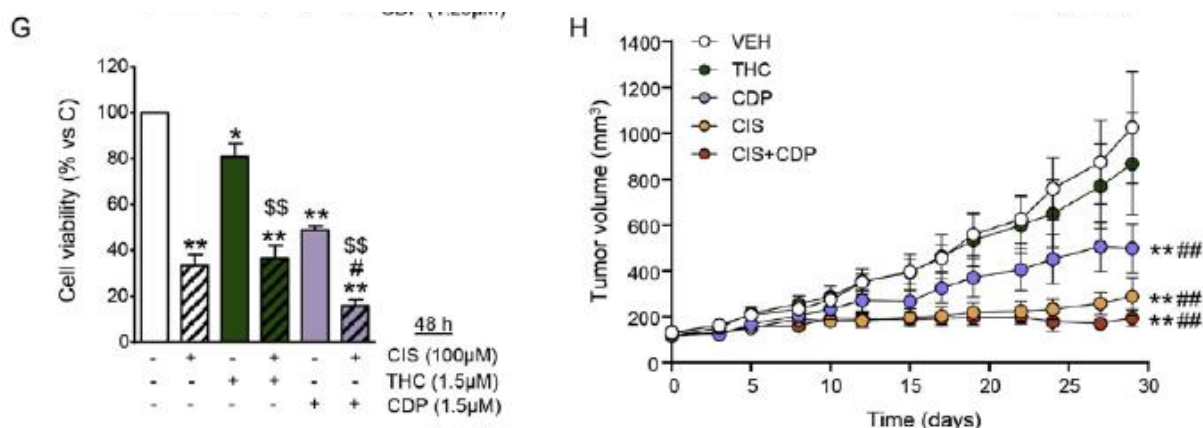


Fig. 3. Effects on preclinical models of triple-negative breast cancer. (A-G) Cell viability, as determined by crystal violet staining, in response to the indicated treatments for 24 h unless otherwise stated. Results are expressed as % versus vehicle-treated cells, set at 100%. (A) Viability of MDA-MB-231 cells (A) and SUM159 (B) human breast triple-negative adenocarcinoma cells in response to increasing concentrations of THC, administered either as a pure compound (THC) or as part of a cannabis drug preparation (CDP). (C-E) Viability of MDA-MB-231 cells upon treatment with pure THC, a terpene cocktail containing β -caryophyllene, linalool, α -humulene, nerolidol 1 and β -pinene (TERP), or the combination in the same proportion as in the CDP (C); in response to THC or CDP and the CB₁R-selective antagonist SR141716 (SR1, 1 μ M), the CB₂R-selective antagonist SR144528 (SR2, 1 μ M) or the antioxidant agent α -tocopherol (TOC, 10 μ M) (D); or in response to paclitaxel (PCT) (E), epirubicin (EPI) for 48 h (F), or cisplatin (CIS) (G) for 48 h, alone or in combination with THC or CDP. (H) Growth of ectopic tumors generated in nude mice by subcutaneous injection of MDA-MB-231 cells. Animals were treated with vehicle, THC (45 mg/Kg), CDP (containing 45 mg/Kg THC), CIS (3 mg/Kg), or the combination of CIS and CDP. *, $p < 0.05$ and **, $p < 0.01$ vs. vehicle-treated cells/animals; #, $p < 0.05$ and ##, $p < 0.01$ vs. TERP (C), THC (D), PCT (E), THC (F) or CIS (G); \$, $p < 0.05$ and \$\$, $p < 0.01$ vs. THC or CDP.

As in cells expressing ER or PR, or overexpressing HER2, the CDP was more potent than the pure cannabinoid (Fig. 3A), with IC₅₀ values of 1.6 μ M and 1.9 μ M, respectively. This effect was not cell line-specific, as it was observed in another human triple-negative breast cancer cell line (SUM159) (Fig. 3B). The corresponding IC₅₀ values were 2.8 μ M for THC, and 2.1 μ M for the CDP. Results on cell viability for the terpene cocktail, and its combination with pure THC are shown in Fig. 3C. The effect of THC on the viability of MDA-MB-231 cells, when administered as a pure compound, was due, at least partially, to CB₂R activation and ROS generation. Thus, SR2 and TOC were able to prevent THC-induced decrease in cancer cell viability, while blockade of CB₁R with SR1 was not (Fig. 3D). In contrast, the antiproliferative effect of the CDP was not prevented by any of the pharmacological tools used in this study (Fig. 3D), suggesting once again that different or additional mechanisms of action are activated.

Finally, the cannabinoid treatments, alone and in combination with cisplatin, were tested in an *in vivo* setting. Triple-negative tumors in immune-deficient mice were generated by subcutaneous injection of MDA-MB-231 cells. As in the other two subtypes of breast cancer, the CDP was significantly more potent than the pure cannabinoid when the same dose of THC was administered (Fig. 3H). Similarly, the combination of cannabinoids with cisplatin did not affect, either positively or negatively, the antitumor action of the latter (Fig. 3H).

In summary, the above data supports the use of specific terpenes, in combination with specific cannabinoids, in specific relative ratios, for the treatment of cancers as set out in the claims of the patent application.