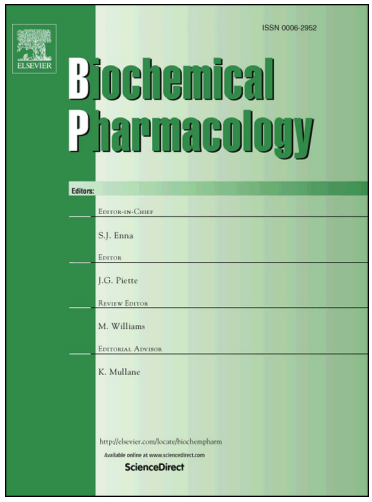

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Appraising the "entourage effect": antitumor action of a pure cannabinoid *versus* a botanical drug preparation in preclinical models of breast cancer

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Abstract

Breast cancer is the second leading cause of death among women. Although early diagnosis and development of new treatments have improved their prognosis, many patients present innate or acquired resistance to current therapies. New therapeutic approaches are therefore warranted for the management of this disease. Extensive preclinical research has demonstrated that cannabinoids, the active ingredients of *Cannabis sativa*, trigger antitumor responses in different models of cancer. Most of these studies have been conducted with pure compounds, mainly Δ^9-tetrahydrocannabinol (THC). The cannabis plant, however, produces hundreds of other compounds with their own therapeutic potential and the capability to induce synergic responses when combined, the so-called "entourage effect". Here, we compared the antitumor efficacy of pure THC with that of a botanical drug preparation (BDP). The BDP was more potent than pure THC in producing antitumor responses in cell culture and animal models of ER+/PR+, HER2+ and triple-negative breast cancer. This increased potency was not due to the presence of the 5 most abundant terpenes in the preparation. While pure THC acted by activating cannabinoid CB_2 receptors and generating reactive oxygen species, the BDP modulated different targets and mechanisms of action. The combination of cannabinoids with estrogen receptor- or HER2-targeted therapies (tamoxifen and lapatinib, respectively) or with cisplatin, produced additive antiproliferative responses in cell cultures. Combinations of these treatments *in vivo* showed no interactions, either positive or negative. Together, our results suggest that standardized cannabis drug preparations, rather than pure cannabinoids, could be considered as part of the therapeutic armamentarium to manage breast cancer.

Keywords: Cannabinoid, breast cancer, THC, botanical drug preparation, chemotherapy, targeted therapy

1. Introduction

It is estimated that 12% women will develop breast cancer at some time during their lives (1). Although the mortality rates associated to this disease are globally decreasing due to improvement in therapies and early diagnosis, there is an urgent need for new treatments. First, some patients show innate resistance to standard therapies, and others acquire resistance with time despite initial responsiveness. In addition, breast cancer is a very heterogeneous disease in terms of molecular features, prognosis, and treatments, and some specific subgroups present very poor outcomes and response to current therapies. Although sub-classification of breast cancer is a field in constant growth (2, 3), treatment decisions are presently made based on the presence of a very limited number of predictive markers, namely estrogen and progesterone receptors (ER and PR, respectively), and the HER2 oncogene. Thus, tumors with ER/PR expression, which represent roughly 75% of all breast cancer cases, are treated with therapies aimed at switching off the estrogenic signaling, either by targeting the receptors themselves [with selective estrogen receptor modulators (SERMs) as tamoxifen, a partial agonist of ER] or the endogenous synthesis of these hormones (with aromatase inhibitors) (1, 3). Tumors with overexpression of HER2, a member of the epidermal growth factor (EGF) receptor tyrosine kinase family, represent approximately 17% of all diagnosed breast cancer cases, and are treated with therapies aimed at hampering HER2 pro-oncogenic signaling (4). Targeted therapies include monoclonal antibodies against different extracellular domains of HER2 (as trastuzumab and pertuzumab), and small molecule tyrosine kinase inhibitors (as lapatinib and neratinib) that block the kinase activity of the intracellular domain of HER2, which is essential for receptor activation (4, 5). Although introduction of these treatments have greatly improved the outcome of these patients, most with advanced disease eventually relapse after treatment, suggesting that tumors acquire or intrinsically possess mechanisms to escape from HER2 inhibition (6). Finally, there is a breast

cancer subgroup of tumors that do not express either hormone receptors or HER2. It is therefore called triple-negative, and is the one with the worst prognosis as a whole, due to the highly aggressive features of their cancer cells, their heterogeneous nature, and to the lack of targeted therapies (7). These patients are treated with classical chemotherapies, which indiscriminately target cells undergoing proliferation, either tumoral or not (7).

It is well documented that cannabinoids, the active ingredients of the hemp plant *Cannabis sativa*, produce antitumor responses in preclinical models of cancer, by tackling different stages of cancer progression such as uncontrolled cancer cell proliferation and survival, angiogenesis and metastasis (8, 9). The vast majority of these studies has been performed with pure compounds, mainly Δ^9-tetrahydrocannabinol (THC). The cannabis plant, however, produces hundreds of additional compounds (other cannabinoids, terpenoids, flavonoids, polyphenols, etc.) that have been much less studied but show promising therapeutic properties (anti-proliferative, anti-inflammatory, immune-stimulant, etc.), and/or the potential capability of enhancing some THC actions (10, 11), the so-called "entourage effect". In this context, we aimed at comparing the antitumor activity of pure THC *versus* a cannabis drug preparation, and at determining whether cannabinoid-based therapies can improve or interfere with current standard treatments in breast cancer. We addressed these questions in both *in vitro* and *in vivo* preclinical models of the different subtypes of breast cancer.

2. Material and Methods

2.1. Materials

Pure THC (≥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 -20ºC for 48h, and then macerated in ethanol for 24h 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 resulting cannabinoid and terpene composition of the extract was determined as described below, and it is detailed in Table 1. 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 cocktail of terpenes was prepared by mixing the mentioned terpenes in DMSO at the same concentrations present in the CDP. The CB_1R-selective antagonist SR141716 (SR1) was from NIMH (Bethesda, EEUU), the CB_2R-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 H_2O, and cisplatin in PBS.

2.2. Analysis of the cannabis drug preparation.

The CDP cannabinoid and terpene composition was determined by Sonoma Labwoks (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, guaiol, 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 counter-analyzed by Canna Foundation (Valencia, Spain) and Ananda Analytics Lab (Madrid, Spain).

2.3. 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 10µg/mL insulin, and SUM159 with 0.5µg/mL hydrocortisone. All cells were maintained at 37 ºC in an atmosphere of 5% CO_2.

2.4. Cell viability assays.

Cells were seeded at a density of 5000 cells/cm^2 in 10% FBS-containing medium. Twenty-four hours later, they were serum-starved overnight, and then treated with the indicated compounds for 24 or 48h. Cells were subsequently fixed and stained with a crystal violet solution (0.1% crystal violet, 20% methanol in H_2O) for 20 minutes. After intensive washing with H_2O, the stained cells were solubilized in methanol, and absorbance measured at 570nm. Data are expressed as the percentage of viable cells *vs.* vehicle-treated cells, set at 100%, and represented as mean ± 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 μM THC was under study, it was compared with an amount of CDP that provided 3 μM THC. The cannabinoid receptor antagonists (1μM) and tocopherol (10μM) were added to the cell cultures 1 hour prior to THC.

2.5. 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 $5x10^6$ HCC1954 cells or $5x10^6$ MDA-MB-231 cells in PBS. For ER+/PR+ tumor generation, a 17β-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 ($10x10^6$ cells). Tumor volume was routinely measured with and external caliper, and when it reached $200mm^3$ (for BT474 and T47D) or $100mm^3$ (for

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 45mg/Kg, 3 times a week, in 100µL of sesame oil, by oral gavage (for CDP, 45mg/Kg means a dose of the extract that contains 45mg/Kg of THC). Tamoxifen (2.5mg/kg in 100µL of sesame oil) and cisplatin (3mg/kg in 100µL of PBS) were administered i.p. 3 times a week; and lapatinib (100mg/Kg) daily by oral gavage in 200µL 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.

2.6. Statistical analyses.

All data were analyzed using Prism 6 (GraphPad), and are presented as mean ± 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. IC_{50} values were determined with CompuSyn software.

3. Results

3.1. 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 (1). To analyze whether this subtype of breast cancer may be sensitive to cannabinoid treatment, we challenged T47D cells (a human ER+ and PR+ breast cancer cell line) with either pure THC or the CDP whose precise composition is detailed in Table 1. As shown in Fig. 1A, both THC and the CDP decreased the viability of T47D cells in a concentration-dependent manner. Of interest, the botanical preparation was more potent than the pure cannabinoid. Thus, the IC_{50} value was 2.2 μM for CDP and 2.9 μM for THC. To determine whether this response was T47D-specific or could be extrapolated to other hormone-sensitive breast cancer cells, we conducted similar experiments 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 μM; IC_{50} CDP = 2.4 μM).

It is important to highlight that, in these experiments, comparison between effects was conducted for the same concentrations of THC, administered either as pure compound or as part of the CDP. This would conceivably imply that 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, we generated a terpene cocktail containing the same concentrations of β-caryophyllene, linalool, α-humulene, nerolidol 1 and β-pinene that are present in the extract (Table 1), and combined it with THC, at the same

proportion as in the PCD as well. The terpene cocktail did not produce any effect on cell viability (Fig. 1C). In addition, its combination with THC did not improve THC antiproliferative action either (Fig. 1C). These observations suggest that other compounds (or compound combinations) present in the CDP are responsible for its superior potency over THC.

We next studied which were the primary targets of cannabinoid antiproliferative action in ER+/PR+ breast cancer cells. 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.

Next, we aimed at analyzing how cannabinoid-based therapies may impact the efficacy of current standard antitumor therapies for hormone-sensitive breast cancer. Specifically, we combined THC or the CDP 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, we tested whether all the observations made in cell cultures were also evident in a more physiological setting. Specifically, we generated ER+/PR+ tumors in immunedeficient mice 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). On the contrary, and administered at the same dose of THC, the CDP produced a remarkable decrease in tumor growth (Fig. 1F), confirming the higher potency of the botanical preparation *vs.* the pure cannabinoid as

observed *in vitro*. Tamoxifen, on the other hand, also impaired tumor growth, and in this case neither pure THC nor the CDP had any impact (negative or positive) on its antitumoral action (Fig. 1F).

3.2. 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 (1). Previous studies suggest that this breast cancer subtype may be responsive to cannabinoid treatments (12). However, as for most of the preclinical research conducted so far, those studies were carried out with pure cannabinoids. Here, we observed that both pure THC and the CDP decreased the viability of HCC1954 human HER2+ breast cancer cells (Fig. 2A). As for hormone-sensitive cells, the botanical preparation was more potent than the pure compound (IC_{50} THC = 2.7 μM; IC_{50} CDP = 2.0 μM). Of interest, 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 μM for pure THC and 2.7 μM for the CDP. Also in this case, neither the cocktail containing the 5 most abundant terpenes in the botanical extract produced any effect on cell viability, nor the combination of this cocktail with pure THC improved the effect of the cannabinoid on cell viability (Fig. 2C), which again points to different compounds or 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 CB_2R 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 CB_2R 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, we used lapatinib, a small molecule tyrosine kinase inhibitor that targets both HER2 and HER1 (EGFR) (4). 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).

We then tested the effect of the cannabinoid-based therapies alone or in combination with lapatinib in an animal model of HER2+ breast cancer. Specifically, we generated subcutaneous tumors 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).

3.3. 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 (7). To study the effect of cannabinoids on this breast cancer subtype, we used human MDA-MB-231 cells as a model. Both pure THC and the CDP decreased the viability of these cells in a concentration-dependent

manner (Fig. 3A). As in cells expressing ER or PR, or overexpressing HER2, the CDP was more potent than the pure cannabinoid (Fig. 3A), with IC_{50} values of 1.6 μM and 1.9 μM, 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 IC_{50} values were 2.8 μM for THC, and 2.1 μM for the CDP. Once again, the terpene cocktail did not have any significant effect on cell viability, and its combination with pure THC did not improve cannabinoid antiproliferative action (Fig. 3C). THC effect on the viability of MDA-MB-231 cells, when administered as a pure compound, was due, at least partially, to CB_2R activation and ROS generation. Thus, SR2 and TOC were able to prevent THC-induced decrease in cancer cell viability, while blockade of CB_1R 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.

The chemotherapy drugs that triple-negative patients receive include, among others, taxanes (aimed at interfering with the cytoskeletal rearrangements that occur on cell replication and migration), anthracyclines (antineoplastic antibiotics that interfere with DNA replication) and platinum analogs (which covalently bind to the DNA, also blocking its replication) (7). We first used a taxane (paclitaxel), and observed that the decrease in cell viability produced by either treatment alone (paclitaxel, THC or the CDP) was not altered upon combination (Fig. 3E). Similar results were obtained when an anthracycline (epirubicin) was studied (Fig. 3F). We then analyzed the combination between cannabinoids and the platinum-based drug cisplatin. In this case, combination with the CDP produced an increased antiproliferative response (Fig. 3G).

Finally, we tested the cannabinoid treatments, alone and in combination with cisplatin, in an *in vivo* setting. We generated triple-negative tumors in immunedeficient mice 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).

4. Discussion

A large body of preclinical evidence shows that cannabinoids produce antitumor responses in a variety of animal models of cancer, including breast, pancreas, lung or liver adenocarcinomas, glioblastomas or melanomas, among others (8, 9, 12). The solidness of these studies has set the bases for the first controlled clinical study of the combination of a cannabis-based medicine with a standard anticancer drug. Specifically, a phase 2 placebo-controlled clinical trial was performed in patients with recurrent glioblastoma multiforme, to address some safety and efficacy endpoints of the combination of Sativex [a cannabis extract containing equal amounts of THC and cannabidiol (CBD)] with temozolomide (an alkylating agent that constitutes the gold-standard treatment for this devastating type of brain tumors) (ClinicalTrials.gov identifiers NCT01812603 and NCT01812616). At the time this manuscript was submitted, the results of that study had not been publicated, but a press release of the sponsor company has partially unveiled positive results (https://www.gwpharm.com/). The vast majority of the preclinical research conducted in this field has used pure cannabinoids (mainly THC). However, the cannabis plant produces hundreds of other compounds with potential therapeutic properties, which makes whole-plant cannabis preparations potentially better therapeutic tools. For example, more than 100 different phytocannabinoids have been described so far (13), and we are just beginning to study and characterize them in terms of medical potential. In the context of cancer, and although research is not as exhaustive as that of THC and CBD yet, some plant-derived cannabinoids have been shown to produce antiproliferative actions, as well as invasion/migration inhibiting effects in cell cultures (14, 15), which makes them potentially interesting tools to include in cannabinoid-based therapies. The CDP used in this study had measurable amounts of CBG and THCA, for example. CBG has been shown to display antitumor responses via TRPM8 receptors in models of colon cancer (16). THCA on the other hand was recently shown to be a

PPAR-γ agonist with neuroprotective activity (17), and this receptor has been previously linked to apoptotic responses in cancer cells (18).

Aside from cannabinoids, *Cannabis sativa* produces other families of chemical compounds with potential interest in the oncology field. Among them, terpenes are receiving increasing attention. These volatile compounds are the responsible for the organoleptic properties of cannabis, and some are starting to show potentially interesting therapeutic properties. For example, β-myrcene is analgesic and antibacterial; α-pinene is anti-inflammatory, as well as β-caryophyllene, which is also analgesic and a gastric protectant (19). Although research in this field is in its infancy, the available information so far suggests that the presence of these compounds in cannabis-based therapies may be beneficial. It is also important to mention that the presence of certain terpenes [with sedative properties, like β-myrcene, for example (19)] and of CBD [with anxiolytic and antipsychotic activity (20)] in cannabis preparations may have additional advantages related with an improved tolerability of THC.

Although current medicine is mostly based on the use of pure compounds that have single targets, it is increasingly obvious that for diseases as complex as cancer, multi-target approaches could conceivably be more effective. In fact, the majority of oncologic patients receive several treatments simultaneously. The complex chemical composition of cannabis drug preparations makes them multi-drug preparations, which, in principle, could allow the concurrent tackling of different hallmarks of cancer, and of other symptomatology associated to this pathology (pain, anxiety, nausea, side effects of standard anticancer treatments, etc.). From a pharmacological point of view, this would the consequence of a battery of compounds activating cannabinoid receptor-dependent and independent signaling pathways in different target populations (cancer cells, neuronal circuits controlling pain perception and nausea reflex, etc.). In addition, the presence of different compounds in these preparations is a source of potential pharmacological interactions, either synergic or antagonistic. The synergistic interaction between endocannabinoid

compounds has been previously reported. Thus, Mechoulam and coworkers described that the biological activity of the endocannabinoid 2-arachydonoylglicerol was augmented by other 2-acylglycerols that, when used alone, did not produce biological responses (21). This was called "entourage effect", a term that is currently used to refer to the potential synergies between chemical compounds present in the cannabis plant (19). In summary, and although the pharmacology of cannabis drug preparations extracts is obviously more complex to study, this therapeutic approach has the potential to produce better therapeutic responses than pure cannabinoids. Results presented herein support that idea. We have observed that both in cell cultures and in animal models of breast cancer, a THC-rich CDP is more potent than pure THC in producing antitumor responses. We were unable to identify the compounds or compound combinations responsible for this increased potency, but we ruled out the possibility that it resided in the presence of the 5 most abundant terpenes. In addition, our results suggest that additional or different molecular targets and mechanisms of action are activated when the CDP is used as compared to pure THC, supporting the idea that its increased potency may be due to a multi-target response.

Of interest, our results show that all breast cancer subtypes respond to cannabinoids, including the highly aggressive triple-negative. This observation suggests that the susceptibility of breast cancer cells to cannabinoid treatment is not related to the expression (or lack of expression) or specific oncogenic signaling triggered by hormone receptors or HER2. In line with this idea, many different types of cancer cells, including pancreas, skin, liver or lung adenocarcinomas, glioblastomas, hematological tumors, sarcomas, etc., have shown antiproliferative or death-inducing effects in response to cannabinoids (8, 9, 12). In fact, to the best of our knowledge, no overtly cannabis-resistant tumors have been described so far. Considering how different cancer subtypes are, and the fact that the viability of non-transformed cells is not affected by cannabinoids at the concentrations they kill tumor cells (8, 9, 12), it is

tempting to speculate that these compounds tackle essential, as yet unidentified, cellular functions that all cancer cells share, and that are absent in their non-cancerous counterparts.

Here, we also analyzed whether there was any kind of interaction, either positive or negative, between cannabinoids and some of the most common treatments for breast cancer patients. Targeted therapies for ER+/PR+ and HER2+ tumors showed an improved antiproliferative activity in cell cultures when combined with THC or the CDP. This effect was not that evident when cannabinoids were combined with two of the chemotherapeutic agents used in this study (paclitaxel and epirubicin). Previous work, however, showed a clear additive response when combining paclitaxel with the endocannabinoid anandamide in gastric cancer cell lines (22). Additional studies should be carried out to clarify whether this discrepancy is related to these precise cancer subtypes, the specific cannabinoid used, or other additional factors. Synergistic responses between cannabinoids and other chemotherapeutic agents have been previously reported. For example, a positive interaction between the antimetabolite 5-fluorouracil and the synthetic cannabinoid HU-210 was found in colorectal cancer cell cultures (23), and between the alkylating agent temozolomide and THC in glioblastoma, both *in vitro* and *in vivo* (24). Intriguingly, the additive effects we observed between tamoxifen or lapatinib and cannabinoids in cell cultures was not evident *in vivo*. As shown in the corresponding figures, the doses we chose for tamoxifen, lapatinib, cisplatin and the CDP produced very prominent antitumor responses. It would be interesting to determine whether combination of lower doses of all of them (producing submaximal responses by themselves) would trigger the additive effects we observed *in vitro*. In addition, it is important to highlight that combination with the CDP did not, in any case, diminished the antitumor efficacy of any of the standard treatments, which suggest that cannabis-based therapies would not interfere with the usual therapies these patients receive. On the contrary, cannabinoids have been shown to protect tissues from damage produced by certain chemotherapy drugs. Thus, these compounds prevent cisplatin- and doxorubicin-

induced neuropathic pain (25), a very distressing and common side effect that frequently leads to treatment discontinuation. Of interest, this protective effect is also produced by β-caryophyllene (26), one of the most common terpenes in cannabis, and one of the constituents of the cannabis drug preparation used in this study. Cannabinoids also prevent doxorubicin-induced cardiomyopathy (27-29). In summary, and even if no synergistic interactions with other chemotherapy drugs (in terms of antitumor efficacy) were to occur in patients, the combination of current standard treatments and cannabis-based therapies, containing not only THC but other plant-derived accompanying compounds, would have a positive impact in preventing the highly toxic effects of their treatments and consequently on their quality of life.

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Figure legends

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 24h. 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 CB_1R-selective antagonist SR141716 (SR1, 1μM), the CB_2R-selective antagonist SR144528 (SR2, 1μM) or the antioxidant agent α-tocopherol (TOC, 10μM) (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 (45mg/Kg), CDP (containing 45mg/Kg THC), TAM (2.5mg/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).

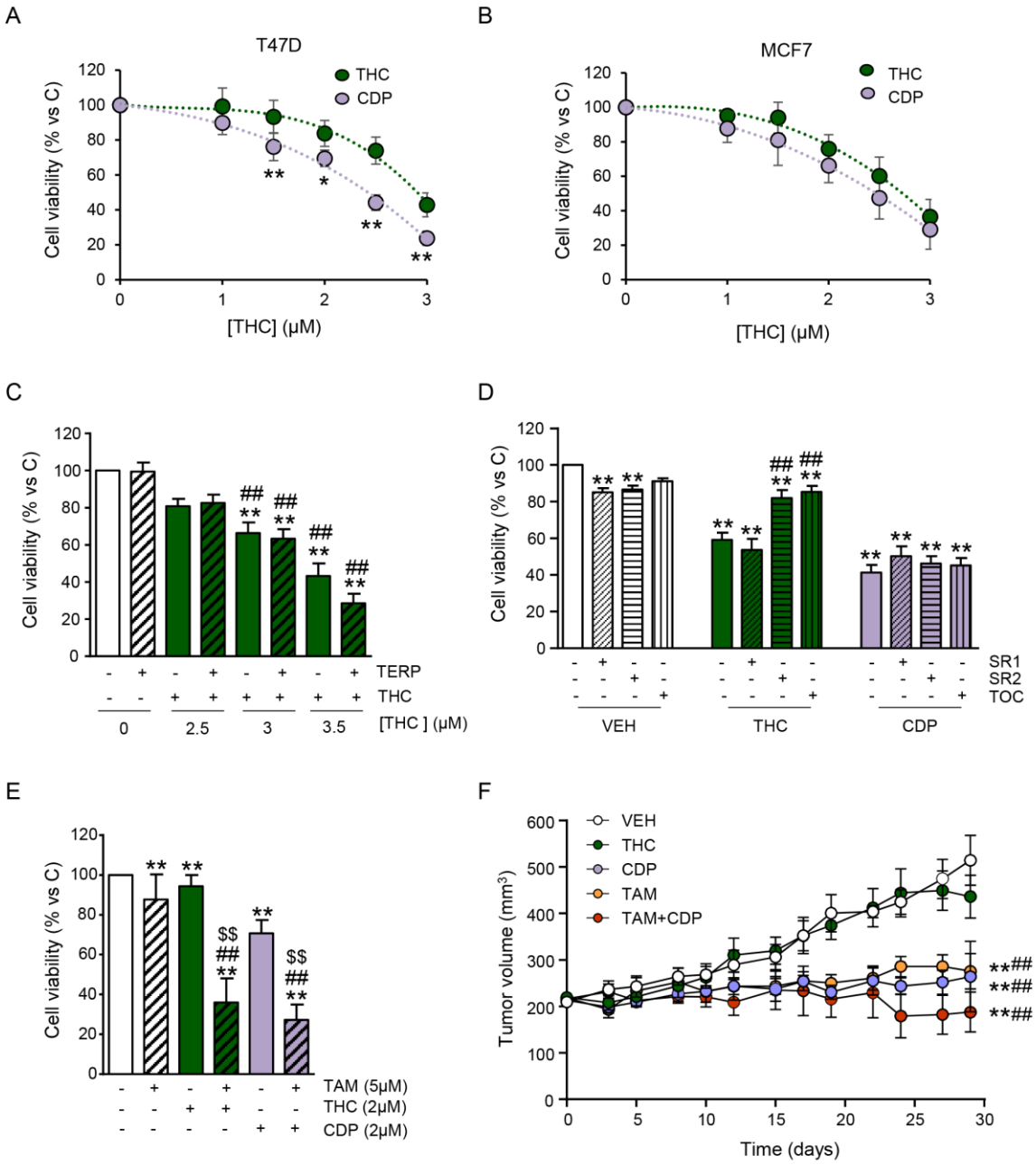
Fig. 2. Effects on preclinical models of HER2+ breast cancer. (A-E) Cell viability, as determined by crystal violet staining, in response to the indicated treatments for 24h. Results are expressed as % *versus* vehicle-treated cells, set at 100%. (A) Viability of HCC1954 (A) and BT474 (B) human breast 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 CB_1R-selective antagonist SR141716 (SR1, 1μM), the CB_2R-selective antagonist SR144528 (SR2, 1μM) or the antioxidant agent α-tocopherol (TOC, 10μM) (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 (45mg/Kg), CDP (containing 45mg/Kg THC), LAPA (100mg/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).

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 24h 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 CB_1R-selective antagonist SR141716 (SR1, 1μM), the CB_2R-selective antagonist SR144528 (SR2, 1μM) or the antioxidant agent α-tocopherol (TOC, 10μM) (D); or in response to paclitaxel (PCT) (E), epirubicin (EPI) for 48h (F), or cisplatin (CIS) (G) for 48h, 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 (45mg/Kg), CDP (containing 45mg/Kg THC), CIS (3mg/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.

Fig. 1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THC
CDP
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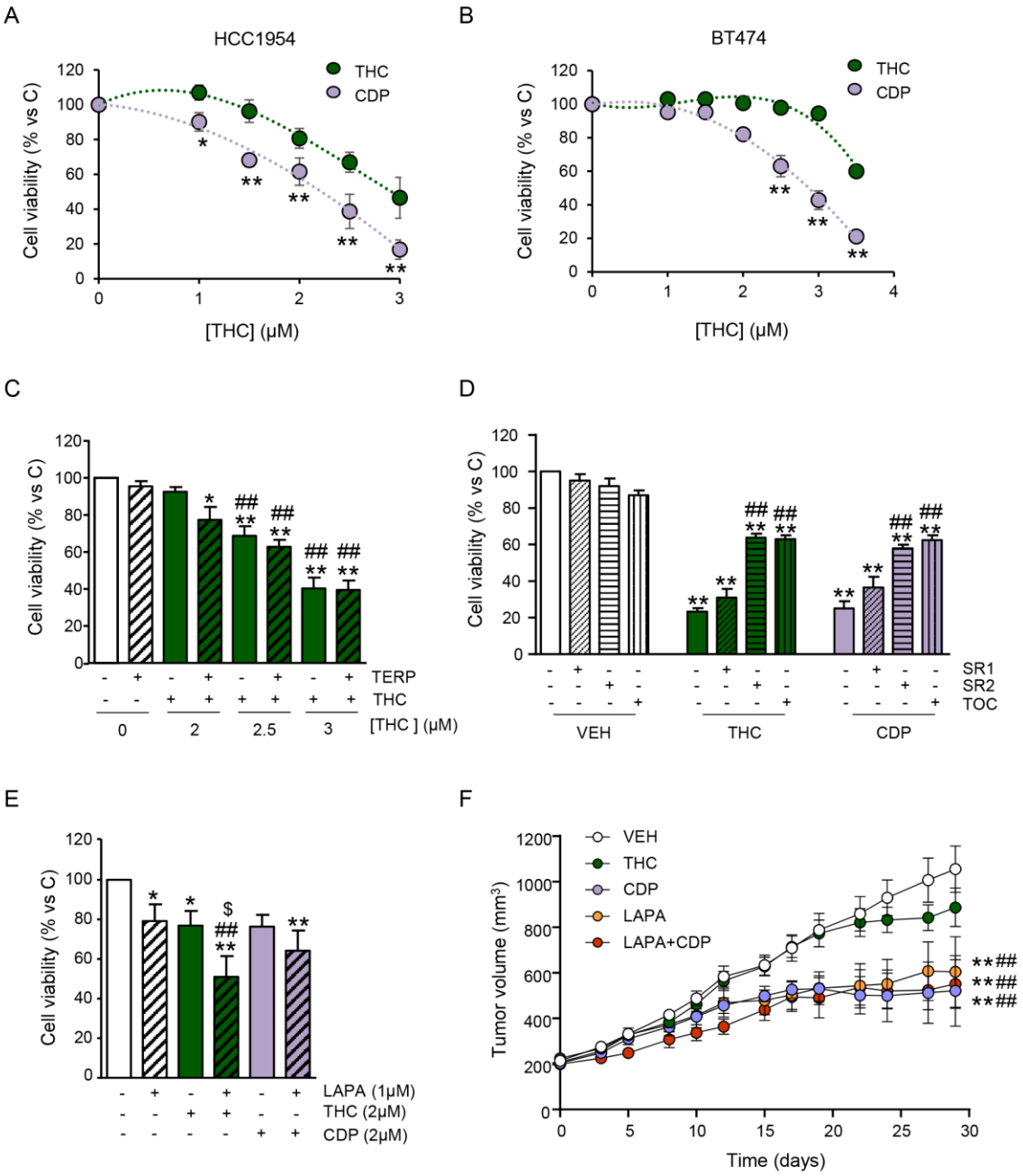
B
MCF7
THC
CDP
Cell viability (% vs C)
[THC] (μM)
C
Cell viability (% vs C)
TERP
THC
[THC] (μM)
0
2.5
3
3.5
D
Cell viability (% vs C)
SR1
SR2
TOC
VEH
THC
CDP
E
Cell viability (% vs C)
TAM (5μM)
THC (2μM)
CDP (2μM)
F
VEH
THC
CDP
TAM
TAM+CDP
Tumor volume (mm3)
Time (days)

Fig. 2


A
HCC1954
THC
CDP
Cell viability (% vs C)
[THC] (μM)
B
BT474
THC
CDP
Cell viability (% vs C)
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C
Cell viability (% vs C)
TERP
THC
[THC] (μM)
0
2
2.5
3
D
Cell viability (% vs C)
SR1
SR2
TOC
VEH
THC
CDP
E
Cell viability (% vs C)
LAPA (1μM)
THC (2μM)
CDP (2μM)
F
VEH
THC
CDP
LAPA
LAPA+CDP
Tumor volume (mm3)
Time (days)

Fig. 3

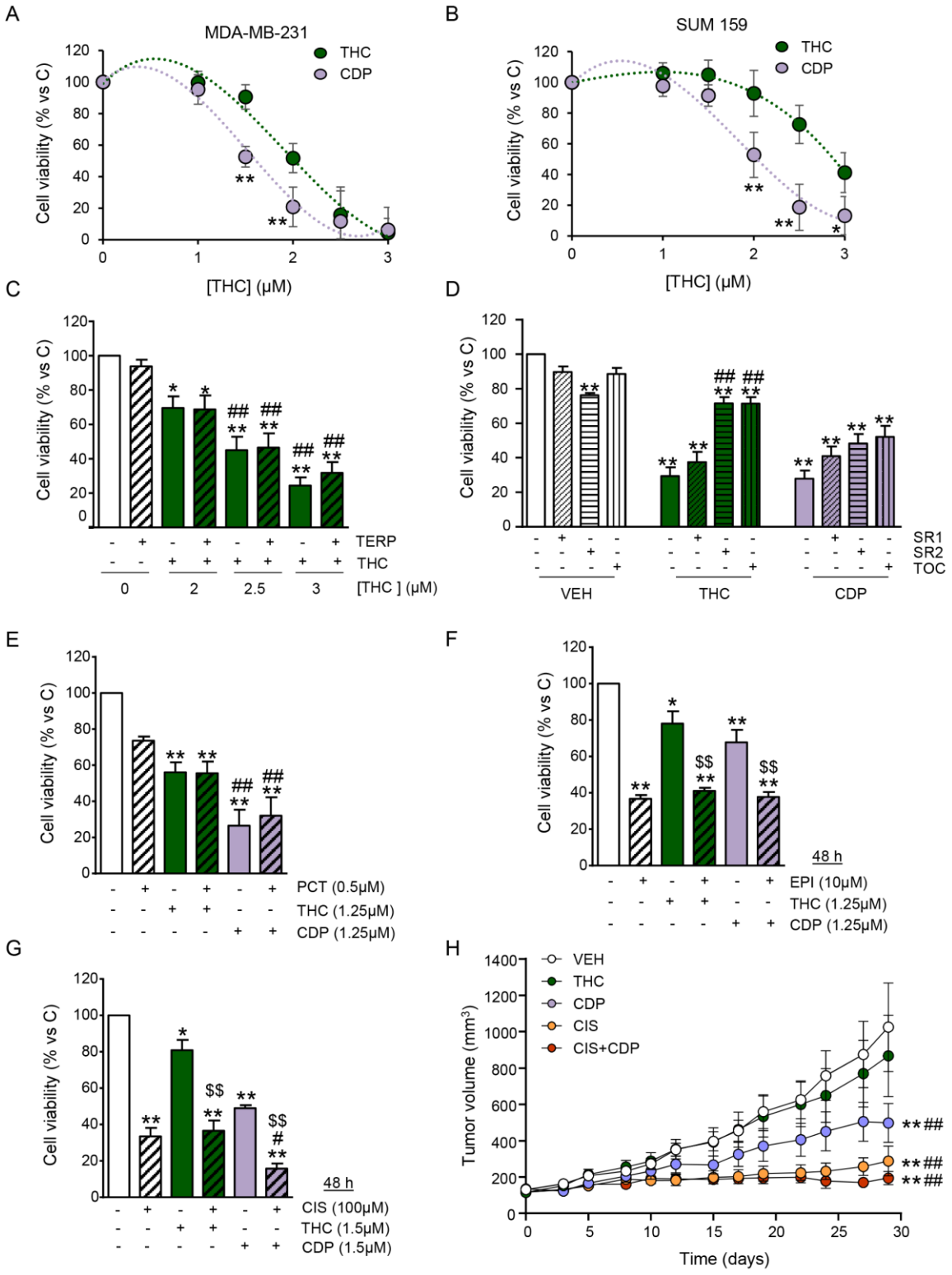
A
MDA-MB-231
THC
CDP
Cell viability (% vs C)
[THC] (μM)
B
SUM 159
THC
CDP
Cell viability (% vs C)
[THC] (μM)
C
Cell viability (% vs C)
TERP
THC
0
2
2.5
3
[THC] (μM)
D
Cell viability (% vs C)
SR1
SR2
TOC
VEH
THC
CDP
E
Cell viability (% vs C)
PCT (0.5μM)
THC (1.25μM)
CDP (1.25μM)
F
Cell viability (% vs C)
48 h
EPI (10μM)
THC (1.25μM)
CDP (1.25μM)
G
Cell viability (% vs C)
48 h
CIS (100μM)
THC (1.5μM)
CDP (1.5μM)
H
VEH
THC
CDP
CIS
CIS+CDP
Tumor volume (mm³)
Time (days)

Table 1. Cannabinoid and terpene composition of the cannabis drug preparation

CANNABINOID	Concentration (mg/g)
THCA	3.449
THC	551.308
THCV	ND
CBD	ND
CBDA	ND
CBG	3.667
CBN	ND
CBC	ND

TERPENE	Concentration (mg/g)
α-Bisabolol	0.177
Camphene	BDL
3-Carene	BDL
β-Caryophyllene	1.948
Caryophyllene Oxide	0.032
p-Cymene	0.178
Geraniol	ND
Guaiol	ND
α-Humulene	0.557
Isopulegol	0.023
D-Limonene	ND
Linalool	0.620
β-Myrcene	0.025
Nerolidol 1	0.357
Nerolidol 2	0.081
Ocimene	0.049
α-Pinene	0.015
β-Pinene	0.317
α-Terpinene	0.013
γ-Terpinene	0.013
Terpinolene	0.017

ND = Not detected
BDL = Below detectable limit