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CBD loaded microparticles as a potential formulation to improve paclitaxel and doxorubicin-based chemotherapy in breast cancer

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ABSTRACT

Cannabidiol (CBD) has emerged as a potential agent for breast cancer management. In this work, the potential use of cannabidiol in solution (CBD$_{\text{sol}}$) and encapsulated in polymeric microparticles when combined with paclitaxel (PTX) and doxorubicin (DOX) in breast cancer treatment has been evaluated for the first time using MCF-7 and MDA-MB-231 cells. CBD$_{\text{sol}}$, previously administered at suboptimal concentrations (cell death $<10\%$), enhanced the PTX and DOX effect in both breast cancer cells. The co-administration of CBD$_{\text{sol}}$ and PTX or DOX showed a synergistic effect. PLGA-502 was selected as the most suitable polymer to develop CBD-loaded microparticles. The developed formulation (CBD-Mps) was effective as monotherapy, showing extended antiproliferative activity for at least 10 days, and when combined with PTX or DOX. In fact, the use of CBD-Mps allows the combination of both, pre and co-administration strategies, with a single administration, also showing a significant increase in PTX and DOX antiproliferative activity. Finally, the anticancer effect of both CBD$_{\text{sol}}$ and CBD-Mps as monotherapy or in combination with PTX was also confirmed $\textit{in ovo}$, using MDA-MB-231-derived tumours. This data evidences the promising inclusion of CBD in conventional breast cancer chemotherapy and the use of CBD-Mps for the extended release of this cannabinoid, optimising the effect of the chemotherapeutic agents.

KEYWORDS: Cannabinoids, CAM model, Doxorubicin, drug delivery, Paclitaxel, synergism.
1. Introduction

Cancer is one of the major health problems of the 21st century, and in women breast cancer is the most frequent malignancy. It is estimated that more than 23 million cases will be diagnosed until 2030 (Bray et al., 2013; Bray et al., 2018). The implementation of early diagnostic technologies and the development of novel therapies are considerably increasing the rate of survival. Nevertheless, breast tumours continue being one of the leading causes of death in women (Ng et al., 2017).

Breast cancer is a heterogeneous pathology, grouped into several subtypes according to the level of aggressiveness (in situ and infiltrating carcinomas), the molecular profile (considering the expression of oestrogen and progesterone receptors and the overexpression of human epidermal growth factor receptors), the histopathology (tubular, ductal or medullary) and the stage of the disease (from stage 1, with a limited localisation in the breast, to stage 4, with spreading to distant organs like bone or brain) (Fedele et al., 2017; Hon et al., 2016; Sinn et al., 2013). Nowadays, several therapeutic approaches are available depending on cancer subtype: surgery, hormone therapy, radiotherapy, immunotherapy and chemotherapy (Iqbal et al., 2018).

In the case of breast cancer chemotherapy, it is usually recommended before (to shrink the tumour facilitating its elimination) or after surgery (to eliminate remnant cancer cells), and in advanced breast carcinomas (where it constitutes the main treatment strategy). It usually includes combinations of several anticancer agents: i) taxanes like paclitaxel or docetaxel, ii) anthracyclines like doxorubicin or epirubicin, iii) 5-fluorouracil, iv) platinum agents like carboplatin or cisplatin and v) gemcitabine, among others (Goetz et al., 2019; Harris et al., 2014). However, the high toxicity of conventional chemotherapy drugs limits their doses. In this way, the appearance of novel agents with a lower toxicity is really desirable.

In the last decades cannabinoids have attracted a great deal of interest in cancer. They have been demonstrated to be useful to improve appetite, pain and nausea and vomiting resulting from chemotherapy (Sledzinski et al., 2018; Wang et al., 2019b), and in fact, several formulations based on cannabinoids are already approved as palliative agents in cancer. However, cannabinoids are also of interest as antitumor drugs per se;
inhibiting proliferation, neovascularisation, invasion and chemoresistance of tumours (Fraguas-Sanchez et al., 2018; Hinz et al., 2018; Ramer et al., 2017).

Cannabidiol (CBD) is one of the most promising cannabinoids due to its lack of psychoactive properties and its high anticancer activity. CBD has been reported to exert a high antitumor activity in breast carcinomas, decreasing the proliferation of tumour cells (Ligresti et al., 2006; Shrivastava et al., 2011; Sultan et al., 2018) and reducing breast cancer metastasis, due to its ability to inhibit the expression of the Id-1 factor (Elbaz et al., 2015; Mcallister et al., 2007; Mcallister et al., 2011; Murase et al., 2014). In fact, a recent study undertaken in metastatic tumour patients has demonstrated that pharmaceutical grade synthetic CBD has a potential therapeutic interest in breast carcinomas, being safe and well-tolerated (Kenyon et al., 2018).

Some works have also reported the usefulness of CBD when combined with conventional chemotherapy and radiotherapy, improving their antitumor efficacy. That is the case of vincristine and vinblastine, anti-leukaemia drugs (Holland et al., 2006; Scott et al., 2017), and of temozolomide and radiotherapy in glioblastoma (Lopez-Valero et al., 2018; Scott et al., 2014). In fact, several clinical studies are being undertaken to evaluate the safety and efficacy of CBD in combination with chemo- and radiotherapy in gliomas (NCT03246113, NCT03529448), gastrointestinal malignancies and multiple myeloma (NCT03607643).

Despite the potential interest of CBD to be included in chemotherapy regimens, its low aqueous solubility and stability problems (it is sensitive to light, temperature and oxidation (Mechoulam, 1981; Munjal et al., 2006; Van Drooge et al., 2004), hinder the development of effective formulations. Microencapsulation could resolve these challenges, also allowing extended antitumor activity after a single administration, which would be desirable in cancer where long-term treatments are required.

In the present study, poly(lactic-co-glycolic acid) (PLGA) microparticles loaded with CBD were prepared. PLGA is an FDA-approved polymer widely used for the elaboration of parenteral controlled release systems due to its biocompatible and biodegradable properties.
The aim of this work was to evaluate, for the first time, the activity of CBD in solution and encapsulated in PLGA-microparticles, designed for parenteral administration, when combined with paclitaxel or doxorubicin in breast cancer cells in order to obtain a possible synergistic effect, being a first in vitro approach to the advantages of including CBD formulations in standardised chemotherapy regimens.

2. Materials and methods

2.1. Materials

Cannabidiol (CBD) was obtained from THC-Pharma (Frankfurt, Germany). Poly-(lactide-co-glycolic acid-resomer (PLGA) RG® 502 (MW: 7000-17000, i.v. 0.2dL/g) and RG® 504 (MW: 38000-64000, i.v. 0.5 dL/g) was obtained from Evonik® Industries (Essen, Germany). Doxorubicin hydrochloride and Paclitaxel (Acros Organics) were obtained from Fisher Scientific (Madrid, Spain). Polyvinyl alcohol (PVA, Mw= 30,000–70,000), Sigmacote® and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from SigmaAldrich (Missouri, USA). Acetonitrile, dichloromethane (DCM), dimethyl-sulfoxide (DMSO) and methanol (all HPLC grade) were obtained from Fisher Scientific (Madrid, Spain). Potassium di-hydrogen phosphate (KH₂PO₄), disodium hydrogen phosphate dihydrate (Na₂HPO₄•2 H₂O) and Tween®-80 were supplied by Panreac (Barcelona, Spain). RPMI-1640, DMEM, Gentamicin (10 mg/ml) and Geltrex® were obtained from Gibco (Life technologies, California, USA). Foetal bovine serum (FBS) was supplied by Biowest (Nuaillé, France). 24-well Inserts (3µm pore size) were purchased from Falcon (Corning, Nueva York, USA). Demineralized Milli-Q® water (Millipore, Spain) was used. All chemicals and reagents were used as received.

All glass material was pre-treated with Sigmacote® to avoid cannabidiol binding.

2.2. In vitro antitumor activity of CBD in solution

2.2.1. Cell culture

MCF-7 (oestrogen receptor positive) and MDA-MB-231 (oestrogen, progesterone and HER-2 receptors negative also called triple negative) breast cancer cells were obtained from an American Type Culture Collection. MCF-7 and MDA-MB-
231 cells were grown in a RPMI-1640 and DMEM medium, respectively, supplemented with 10% (v/v) of FBS and 1% (v/v) of gentamicin. Cells were maintained at 37 ±0.5°C in a humid atmosphere containing 5% of CO₂.

For cytotoxic studies, cells in an exponentially growing phase were used.

2.2.2. Cell viability studies with CBD as monotherapy

The antiproliferative activity of CBD in solution (CBD<sub>sol</sub>) was tested in both MCF-7 and MDA-MB-231 cell lines. Briefly, cells were seeded in a 96-well-plate at a density of 1.5·10⁴ cells/cm² and treated, 24 hours after seeding, with CBD<sub>sol</sub> (1.25-50 μM). Cell viability was determined by MTT 24 and 48 hours after treatments. Briefly, the medium was removed and 100μL of an MTT solution (0.5 mg/ml) were added. Then the cells were incubated for 3.5 hours. After that, the medium was gently discarded and 100 μL of DMSO were added to dissolve formazan crystals. Plates were stirred for 10 minutes and read at 570 nm (Varioskan Flash, Thermo Scientific). Cell culture medium and Triton X at 1% were used as negative and positive controls of cell death.

The effect of low CBD concentrations (100-1000 nM) was also investigated during 24-96 hours of incubation using the aforementioned protocol.

2.2.3. Combination studies: modulatory effect

The ability of sub-optimal concentrations of CBD<sub>sol</sub> (with a cell death lower than 10%) to increase the antiproliferative activity of paclitaxel (PTX) and doxorubicin (DOX) was evaluated in both MCF-7 and MDA-MB-231 cells. CBD<sub>sol</sub> concentrations of 2.5, 5 and 10μM were used in MCF-7 cells, and concentrations of 1.25; 2.5 and 5μM in MDA-MB-231 cells. DOX and PTX stocks were prepared in a sterile physiological solution and cell grade DMSO, respectively. The final well concentration of DMSO was lower than 0.01% (v/v). Nevertheless, the maximum amount of DMSO was tested to evaluate its effect on cell viability. For all these experiments, cells were seeded in a 96-well-plate at a density of 1.5·10⁴ cells/cm² and treated 24 hours after seeding. Briefly, in a first step, cells were treated with CBD<sub>sol</sub> for 24 hours. Afterwards, the medium was removed and solutions of DOX (0.1-20μM) or PTX (10-500nM) were added and
incubated for 48 hours. DOX stock solution was prepared in saline solution at a concentration of 1.8mM and then diluted in cell culture medium. PTX stock solution was prepared in DMSO at a concentration of 10 µM and then diluted in cell culture medium. Cell viability was determined by MTT as previously described.

IC$_{50}$ of PTX and DOX of non-pre-treated, and CBD pre-treated cells were determined for comparison purposes. A reduction in IC$_{50}$ values would allow the same antiproliferative activity to be achieved with a lower concentration of these antineoplastic agents. For this reason, the results of combination studies were considered as follows:

- Highly positive effect: statistically significant differences in IC$_{50}$ at p value <0.01
- Positive effect: statistically significant differences in IC$_{50}$ at 0.01<p<br>&lt;0.05
- No effect: no statistically significant differences in IC$_{50}$ at p value &gt;0.05.

2.2.4. Combination studies: synergistic effect

The co-administration of CBD$_{sol}$ with PTX or DOX was also evaluated. For these studies, MCF-7 cells were treated with CBD at 10; 15 and 20 µM. In MDA-MB-231 cells CBD concentrations of 5; 7.5 and 10µM were used due to the highest sensitivity of these cells to CBD. Briefly, MCF-7 and MDA-MB-231 cells were seeded at a density of 1.5·10$^4$ cells/cm$^2$ in 96-well plates and treated, 24 hours after seeding, with different drug pairs (CBD$_{sol}$+PTX or CBD$_{sol}$+DOX). 48 hours later cell viability was determined by MTT. From data of dose vs cell death(%) (Kommineni et al., 2018), combination index (CI) values were obtained using CompuSyn Software (ComboSyn, Inc. NJ, USA). The CI values were used to define the effect of drug combinations: synergism (CI < 1), additive effect (CI ≈1) and antagonism (CI>1)(Chou, 2010).

2.3. Elaboration and characterisation of CBD-loaded microparticles.

PLGA microparticles with two different PLGA resomers (PLGA-502 and PLGA-504) and loaded with CBD at 10% (CBD-Mps) (w/w) were prepared using the oil-in-water (O/W) emulsion–solvent evaporation technique; maintaining constant the
viscosity of the internal phase of the emulsion (i.v. 0.2 dl/g). Briefly, PLGA and CBD were dissolved in 5 mL of DCM and dropped onto 250 mL of a 0.5% (w/v) PVA aqueous solution while stirring at 4000 rpm for 6 min. Afterwards, the resulting O/W emulsion was continuously stirred at 200 rpm using a turbine homogenizer for 3-4 hours to allow the evaporation of the organic solvent and the hardening of the microparticles. Finally, microparticle suspension was filtered using a 5 μm PTFE membrane. Collected microparticles were washed with demineralised water to remove residual PVA and lyophilised for 18h at −50 °C and 0.2 mbar.

Unloaded microparticles (PLGA-Mps) were prepared by the same protocol.

Microparticles were characterized in terms of size, polydispersity, morphology, drug loading and in vitro drug release. Particle size was determined by laser diffraction using a Microtrac® S3500 Series Particle Size Analyser (Pennsylvania, USA). The mean particle size was calculated as volume diameter. The polydispersity index was evaluated by calculating SPAN values as follows:

\[ \text{SPAN} = \frac{D_{90} - D_{10}}{D_{50}} \]

Where \( D_{10}, D_{50} \) and \( D_{90} \) indicate the percentage of particles with 10%, 50% and 90% of the diameter lower than or equal to the given value, respectively.

Microparticle morphology was evaluated by scanning electron microscopy (SEM, Jeol, JSM-6400, Tokyo Japan). Briefly, freeze-dried microparticles were placed on aluminium stubs. Then, samples were coated with gold and examined by SEM.

For the quantification of encapsulated CBD in PLGA microparticles and CBD released from microparticles, High Performance Liquid Chromatography (HPLC, Agilent 1200 series, California, USA) method was used. The analytical conditions were as follows: i) a mobile phase containing a mixture of methanol: acetonitrile: water at pH-4.5 at a ratio of 52:30:18, ii) a flow rate of 1.8ml/min; iii) a reversed-phase Mediterranea® C18 column (15x0.46 cm, 5 μm) (Teknokroma® , Barcelona, Spain) , iv) 20μL of injection volume and v) detection at 228nm.

The amount of CBD encapsulated in PLGA microparticles was determined as follows: 10 mg of microparticles were dissolved in 1 ml of DCM and the CBD was
extracted with the addition of 9 ml of mobile phase, which also promoted polymer precipitation. The samples were filtered using PTFE 0.45μm syringe filters and analysed by HPLC.

The encapsulation efficiency (EE) was calculated using the following equation:

\[
EE(\%) = \frac{[(CBD: PLGA ratio_{\text{experimental}}) - (CBD: PLGA ratio_{\text{initial}})]}{(CBD: PLGA ratio_{\text{initial}})} \times 100
\]

The CBD released from microparticles was determined indirectly by calculating the remaining CBD into microparticles. The experiment was designed according to standard conditions for parenteral drug delivery systems. Briefly, vials with 10 mg of microparticles suspended in 5 ml of PBS (pH 7.4) containing Tween® 80 at 0.5% (w/v) to maintain sink conditions were disposed in a thermostatic shaking water bath at 37 ±0.5°C and an agitation rate of 100rpm. At specific time points (2h, 8h, 1, 2, 4, 7, 10, 14, 21, 28, 31 and 42 days) the supernatant was removed, CBD was extracted from microparticles, as mentioned above, and samples were analysed by HPLC.

Polymeric matrix degradation during release studies was also evaluated by SEM. CBD microparticles were incubated using release study conditions (10 mg of microparticles suspended in 5 ml of PBS with Tween® 80 at 0.5% (w/v) at 37±0.5°C with an agitation rate of 100 rpm). At pre-determined points, particles were collected, washed three times with demineralised water, lyophilised and examined by SEM using the aforementioned protocol.

2.4. In vitro antitumor activity of CBD microparticles

2.4.1 Cell viability studies with CBD microparticles as monotherapy

MCF-7 and MDA-MB-231 cells were seeded in a 24-well plate at a density of 1.5·10^4 cells/cm^2. 24 hours after seeding, the medium was removed, and the treatments were added. For these experiments, release studies of CBD from Mps were undertaken in parallel. At pre-determined time points (2, 4, 6 and 8 days) release medium was removed after centrifugation, and microparticles were collected, suspended in cell culture medium and positioned in the top of 3μm pore size inserts, which were placed in
each well (24 well-plates were used). The amount of microparticles was calculated according to *in vitro* CBD release. The results were compared with those obtained when an equivalent concentration of CBD$_{sol}$ was administered. Unloaded microparticles were also tested.

Cell viability measurement was evaluated by MTT as previously described in section 2.2.1 with some modifications.

### 2.4.2 Combination studies with CBD microparticles

The antiproliferative efficacy of CBD-Mps when combined with PTX or DOX was also tested. MCF-7 and MDA-MB-231 cells were seeded as previously described in a 24 well-plate. Briefly, 24 hours after seeding, an amount of CBD-Mps were added in the top of 3μm pore size inserts and placed in each well to achieve a daily administration of CBD of 5 and 10 μM in MDA-MB-231 and MCF-7, respectively. 24 hours later, PTX (10-500 nM) or DOX (0.1-20 μM) was added to cells. After 48h of incubation, cell viability was determined by MTT as mentioned above. The activity of microparticles was compared to the daily administration of the equivalent amount of CBD$_{sol}$.

### 2.5. Chicken embryo chorioallantoic membrane (CAM) assay

#### 2.5.1. CBD as monotherapy

A CAM assay has been used as an *in vivo* model to evaluate the antitumor effect of CBD and CBD microparticles, using the method described by Zuo and co-workers (Zuo et al., 2017). Briefly, fertilized chicken eggs were placed in an egg incubator at 37°C and 47% humidity under rotation. On embryo development day (EDD) 4, a small window (≈3-mm) is drilled in the eggshell and sealed to avoid desiccation. Eggs were placed once again in the incubator without rotation. At EDD 8, the eggshell window was enlarged, and CAM membrane was gently scratched. Then MDA-MB-231 cells (suspended in Geltrex® matrix) were inoculated, at a density of 2·10$^6$ cells/egg. At EED 11, the tumour was formed, photographed and surrounded with a silicone O-ring. The tumour area was measured using Image J software. Then, the tumour was treated topically with: i) DMEM medium that served as control, ii) CBD$_{sol}$ at 100μM
administered daily until EDD 14, iii) CBD-Mps single administration (a daily CBD release of 100µM was reached) and iv) unloaded microparticles. At EDD 14, tumours were photographed, and their area was measured. Tumour growth was evaluated using the following equation:

Tumour growth (%) = \frac{\text{Tumour area }_{\text{FINAL}} \times 100}{\text{Tumour area }_{\text{INITIAL}}}

where [Tumour area ]_{\text{INITIAL}} is the area measured at EDD11 (before treatments) and [Tumour area ]_{\text{FINAL}} is the area measured at EDD 14 (after treatments).

2.5.2. CBD in combination with paclitaxel

MDA-MB-231 cells were grafted onto CAM membrane as mentioned above. Then, at EDD 11, tumours were photographed and treated topically with CBD sol or CBD-Mps for 24 hours. Next, at EDD12, PTX (at a concentration of 100 µM) were administered. CBD sol (100 µM) was administered daily since EDD11 until EDD 14. However, CBD-Mps were administered singly at EDD 11, providing a daily CBD release of 100µM. At EDD 14, tumours were photographed again.

Tumour growth was evaluated as mentioned above, measuring the area at EDD 11(initial) and 14(final).

2.6. Statistical analysis

The data was reported as a mean ± S.D (standard deviation) of at least three experiments (n=3). Multiple groups were compared using a one-way variance analysis (ANOVA), while a t-Student test was used to determine the differences between two groups. Significant differences were reported as follows: *0.01<p < 0.05 and **p < 0.01. All the graphs were compiled and statistical analyses performed using Origin 2017 software (Origin lab, Massachusetts, USA).

3. Results and discussion

3.1. Antitumor activity of CBD in solution

Breast cancer is one of the tumour types where CBD has shown a high anticancer activity. In fact, numerous authors have reported that CBD inhibits the
proliferation, migration and invasion of both oestrogen-receptor-positive and triple negative breast tumour cells (Elbaz et al., 2015; Mcallister et al., 2011; Shrivastava et al., 2011). The antiproliferative effect of CBD is due to its ability to induce apoptosis, with the direct or indirect activation of the specific cannabinoid receptor type 2 and the vanilloid transient receptor (Ligresti et al., 2006, 86), accompanied by the inhibition of mTOR and cyclin D1 and the up-regulation of PPARγ protein expression (Sultan et al., 2018). The anti-invasive properties of this cannabinoid were related to the inhibition of Id-1 protein expression (Mcallister et al., 2007). In our study, CBD\textsubscript{sol} inhibited the proliferation of both MDA-MB-231 and MCF-7 breast cancer cells, with IC\textsubscript{50} values after 48 hours of incubation of 11.37 ±1.82 µM and 20.04 ± 2.97 µM, respectively (cell viability curves of CBD\textsubscript{sol} are depicted in Fig.S1 of supplementary material).

However, some authors have reported a biphasic effect of cannabinoids on tumour cells, so that at low concentrations (in the nanomolar range), some cannabinoids may stimulate cancer cell proliferation (Fraguas-Sanchez et al., 2016). This could challenge the use of microparticles as carriers for cannabinoid administration due to the slow drug release phases from these systems. In the case of breast cancer, the pro-cancer effect of cannabinoids has been reported for $\Delta^9$-tetrahydrocannabinol (THC) in triple negative breast tumours (4-T1 cell line was used) (Mckallip et al., 2005). However, to the best of our knowledge, data on the effect of CBD at nanomolar concentrations has not been published. In our work, we have demonstrated that CBD did not increase the proliferation of MCF-7 or MDA-MB-231 cells in a range of concentrations of 100-1000nM (Fig.S2 of supplementary material).

In this work, the combination of CBD and conventional chemotherapy has been evaluated using two models of drug interactions: i) modulatory studies to evaluate whether the previous administration of sub-effective concentrations of CBD alters the sensitisation of breast cancer cells to PTX and DOX and ii) synergistic studies to evaluate whether the simultaneous administration of CBD potentiates the effect of these antineoplastic agents.

Firstly, modulatory studies have demonstrated that CBD\textsubscript{sol} sensitises MCF-7 and MDA-MB-231 breast cancer cells against PTX. In MCF-7 cells, the previous administration of sub-effective concentrations of CBD\textsubscript{sol} enhanced the activity of PTX, showing a positive effect at CBD concentrations of 2.5 and 5 µM and a highly positive
effect when it was administered at 10µM (Fig. 1 A). In MDA-MB-231 cells, that were more sensitive to CBD action, pre-treatment with CBD$_{sol}$ was also effective (Fig. 1B), showing a highly positive effect at all CBD-tested concentrations (1.25, 2.5 and 5 µM) (Table 2). However, in these cells the reduction of PTX IC$_{50}$ values was smaller compared to MCF-7 cells. While the pre-administration of CBD$_{sol}$ at the highest concentrations (5µM in MDA-MB-231 and 10µM in MCF-7 cells) conducted to a ≈7-fold reduction in PTX concentration to obtain a cell death of 50% in MCF-7 cells (Table 1), this reduction was markedly lower (≈3-fold) in MDA-MB-231 cells (Table 2).

The co-administration of CBD$_{sol}$ plus PTX was also effective in both tested breast cancer cell lines (Fig. 1C and D). In MCF-7 cells, a highly positive effect was seen with all CBD concentrations (Table 1). In fact, as it could be observed in Fig. 1E, combination indexes below 1, in the range of 0.59-0.83, were obtained for all tested combinations, indicating a moderate synergistic effect between CBD and PTX (Table S1 supplementary material). In MDA-MB-231 cells, CBD$_{sol}$ co-administered with PTX also showed a highly positive effect in all tested concentrations (Table 2). As illustrated in Fig. 1F, an additive or synergistic effect (CI≤1) was detected in most of combinations tested, especially at PTX concentrations of 500 nM, with CI values of 0.54-0.63 (in table S3 of supplementary material IC values obtained from each combination are described). The synergistic effect of PTX and CBD could be related to the increase in apoptosis. Miyato et.al reported in gastric cancer models like anandamide, an endocannabinoid, enhanced the antiproliferative activity of PTX due to an increase in the apoptosis (Miyato et al., 2009). Due to the fact that the induction of apoptosis by CBD in breast cancer cells has been widely demonstrated, the synergistic effect of CBD with PTX in breast cancer could also be related to the increase in apoptosis induction.

Regarding the combination with DOX, the pre-administration of sub-effective concentrations of CBD$_{sol}$ was more effective in MDA-MB-231 cells than in MCF-7 cells (Fig. 2A and B). In MCF-7 a positive effect was only detected when CBD was administered at the highest concentration (10 µM), while in the cells pre-treated with CBD at 2.5 or 5 µM, the reduction in IC$_{50}$ were not significant (Table 1). However, in MDA-MB-231 cells, a positive effect was detected at all CBD tested concentrations (1.25, 2.5 and 10 µM) (Table 2).
The co-administration of CBD<sub>sol</sub> plus doxorubicin was effective in both breast cancer cells. In MCF-7 cells, a positive effect was detected (Table 1), except when CBD<sub>sol</sub> was used at 10µM (the lowest CBD concentration), where a non-significant reduction in DOX IC<sub>50</sub> was detected. Combination indexes below 1, except for the combination of DOX 1 µM with CBD 10 µM (Table S2 supplementary material), were obtained, indicating a slightly synergistic effect between CBD and DOX in these oestrogen receptor positive cells (Fig.2E). In MDA-MB-231 cells, the co-administration of CBD and DOX was also effective, and a positive effect was detected when DOX was combined with CBD 5 µM and a highly positive effect in the other combinations (CBD 7.5 and 10 µM) (Table 2). Moreover, CI in the range of 0.76-1.1 were found (Fig. 2 F), indicating that an additive effect or a moderate synergism was produced (in table S4 of supplementary material the CI values are described for each combination).

In both breast cancer cells, MCF-7 and MDA-MB-231; the treatment with CBD, at 10 and 5µM, respectively, prior to PTX or DOX administration, was more efficient than the co-administration of both CBD+PTX or CBD +DOX, since lower IC<sub>50</sub> values of these chemotherapy drugs were obtained (Table 1 and 2). However, statistically significant differences (p value<0.05) were only achieved with PTX in MDA-MB-231 cells.

<table>
<thead>
<tr>
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<th>PTX (nM)</th>
<th>DOX (µM)</th>
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<tbody>
<tr>
<td>Single drug</td>
<td>228.28 ± 55.92</td>
<td>3.09 ± 1.32</td>
</tr>
<tr>
<td>+ Pre-treatment CBD&lt;sub&gt;sol&lt;/sub&gt; 2.5 µM</td>
<td>111.60 ± 22.50</td>
<td>2.3± 0.89</td>
</tr>
<tr>
<td>+ Pre-treatment CBD&lt;sub&gt;sol&lt;/sub&gt; 5 µM</td>
<td>72.53 ± 8.76</td>
<td>1.22 ± 0.1</td>
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<tr>
<td>+ Pre-treatment CBD&lt;sub&gt;sol&lt;/sub&gt; 10 µM</td>
<td>33.46 ± 11.74</td>
<td>0.88± 0.13</td>
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<tr>
<td>+ CBD 10 µM</td>
<td>55.19 ± 11.11</td>
<td>1.11 ± 0.98</td>
</tr>
<tr>
<td>+ CBD 15 µM</td>
<td>24.88 ± 1.43</td>
<td>0.30 ± 0.17</td>
</tr>
<tr>
<td>+CBD 20 µM</td>
<td>5.52 ± 4.83</td>
<td>0.03 ± 0.025</td>
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Table 1: IC<sub>50</sub> values of paclitaxel and doxorubicin in MCF-7 cells when combined with CBD<sub>sol</sub>. * (0.01<p <0.05) and ** (p <0.01) describe significant differences between non-CBD-treated and CBD-treated cells.
Table 2: IC\textsubscript{50} values of paclitaxel and doxorubicin in MDA-MB-231 cells when combined with CBD in solution. * (0.01<p <0.05) and ** (p<0.01) describe significant differences between non CBD treated and CBD treated cells.

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<tr>
<th></th>
<th>PTX (nM)</th>
<th>DOX (µM)</th>
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<tbody>
<tr>
<td>Single drug</td>
<td>142.77 ±18.24</td>
<td>10.73 ± 2.49</td>
</tr>
<tr>
<td>+ Pre-treatment CBD\textsubscript{sol} 1.25 µM</td>
<td>102.09 ± 7.66**</td>
<td>6.11± 1.01***</td>
</tr>
<tr>
<td>+ Pre-treatment CBD\textsubscript{sol} 2.5 μM</td>
<td>67.08 ± 7.01**</td>
<td>6.29± 0.68***</td>
</tr>
<tr>
<td>+ Pre-treatment CBD\textsubscript{sol} 5 µM</td>
<td>49.46 ± 2.47**</td>
<td>4.84± 0.68***</td>
</tr>
<tr>
<td>+ CBD\textsubscript{sol} 5 µM</td>
<td>79.22 ± 10.35**</td>
<td>5.49± 1.04***</td>
</tr>
<tr>
<td>+ CBD\textsubscript{sol} 7.5 µM</td>
<td>44.82 ± 4.60**</td>
<td>3.84± 1.05***</td>
</tr>
<tr>
<td>+CBD\textsubscript{sol} 10 µM</td>
<td>22.86 ± 4.8**</td>
<td>1.41± 0.51***</td>
</tr>
</tbody>
</table>

All these studies carried out on MCF-7 and MDA-MB-231 cell lines indicate, firstly, that the combination of CBD and both antineoplastic drugs could be really interesting, especially in oestrogen positive breast tumours, where a more pronounced significant reduction in IC\textsubscript{50} and a synergistic effect was seen in both CBD\textsubscript{sol}+ PTX and CBD\textsubscript{sol}+DOX combinations. In triple negative breast tumour cells, the decrease observed in the IC\textsubscript{50} values was statistically significant, although no synergism was detected in all combinations tested. In this case, an additive effect could also be interesting, especially if the invasiveness, the poor prognosis and the difficulty of the treatment of this tumour type are considered. In fact, the main advantage of the combination is due to the possibility of decreasing the dose of PTX and DOX with the reduction of toxicity related to chemotherapy. Moreover, it has been demonstrated in murine models that CBD palliates the peripheral neuropathy related to PTX (King et al., 2017; Ward et al., 2011; Ward et al., 2014) and the cardiotoxicity associated with doxorubicin (which is the major problem of doxorubicin-based chemotherapy) (Fouad et al., 2013; Hao et al., 2015), making the combination CBD plus PTX or CBD plus DOX more promising.
The combinations of other cannabinoids and PTX or anthracyclines in breast tumour models have been previously described, with different results. On the one hand, botanical cannabis extracts containing THC and other minor cannabinoids but not CBD did not enhance the anticancer activity of PTX or epirubicin in oestrogen receptor positive or in triple negative breast cancer model antineoplastic agents (Blasco-Benito et al., 2018). However, the combination of Dox and WIN55, 212-2, a synthetic cannabinoid, potentiated the activity of this antineoplastic agent in triple negative breast cancer models (Greish et al., 2018); which is in accordance with our results. This data suggests that the potential use of cannabinoids in combination with taxanes or anthracyclines in breast cancer management depends on the cannabinoid type. Whereas WIN55, 212-2 shows psychoactive properties that limit its therapeutic uses (Wiley et al., 2014), CBD, that lacks of psychotropic effects, could be a better alternative to improve conventional chemotherapy in triple negative breast cancer.

3.2. Microparticle preparation and characterisation

Despite the potential clinical interest of CBD, the low aqueous solubility of this compound hampers its parenteral administration as a solution. The use of microparticles as CBD carriers would resolve this challenge. In this work, PLGA-RG-502® and PLGA-RG-504® resomers were used. Microparticles with both polymers were elaborated by maintaining constant the viscosity of the internal phase of the emulsion (i.v.: 0.2 dl/g) in order to evaluate the effect of the polymer molecular weight on CBD microparticles.

When characterising the elaborated microparticles, a similar mean particle size (expressed as volume diameter) was obtained in both unloaded and CBD loaded formulations, with values around 24 µm in all cases, which is suitable for their parenteral administration. Regarding particle size dispersion, although unimodal distribution curves were observed in all formulations, SPAN values were around or above 2, indicating that size distribution was not monodisperse. A slightly higher polydispersity was seen in microparticles prepared with PLGA-504 (Table 3). However, statistically significant differences between formulations elaborated with both PLGA resomers were not detected (p value>0.05). Regarding particle morphology, as illustrated in figure 3A, spherical particles with a smooth surface were observed in the formulations prepared with both PLGA polymers, PLGA-502 and PLGA-504. No CBD
crystals on particle surfaces were observed in loaded formulations, indicating that the drug was encapsulated in the polymeric matrix.

As regards CBD content, considerable differences were detected between formulations prepared with both PLGA resomers. While CBD-502-Mps exhibited a higher loading capacity (CBD content was 8.601±0.42mg /100 mg Mps), with a high encapsulation efficiency of over 90%; in CBD-504-Mps, drug loading (CBD content was 5.212±0.58mg /100 mg Mps) was significantly lower (p value<0.01), with an encapsulation efficiency of around 57% (Table 3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (µm)</th>
<th>SPAN</th>
<th>Process yield (%)</th>
<th>Loading (mg CBD/100 mg Mps)</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA-502-Mps</td>
<td>24.03±2.01</td>
<td>1.98±0.05</td>
<td>89.25±1.01</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>CBD-502-Mps</td>
<td>24.17±2.32</td>
<td>2.02±0.08</td>
<td>87.36±2.77</td>
<td>8.601±0.42mg</td>
<td>94.62±4.62</td>
</tr>
<tr>
<td>PLGA-504-Mps</td>
<td>23.02±2.25</td>
<td>2.12±0.25</td>
<td>79.27±5.21</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>CBD-504-Mps</td>
<td>24.12±1.25</td>
<td>2.28±0.17</td>
<td>77.95±3.24</td>
<td>5.212±0.58</td>
<td>57.63±6.34</td>
</tr>
</tbody>
</table>

Table 3: Characteristics of developed microparticles

In general, the use of polymers with high molecular weights leads to an increase in the viscosity of the internal phase of the emulsion, which hampers the diffusion of the drug to the external phase, facilitating its encapsulation (Fu et al., 2005; Krishnamachari et al., 2007). However, in this work the internal phase viscosity was maintained constant in order to obtain a better comparison of the polymer molecular weight. The lower loading capacity of CBD-504-Mps could be attributed to a lower interaction between the drug and the polymer. The –OH groups of the CBD could chemically interact with – COOH and ester groups of the polymer, which are more numerous in PLGA-502. Other authors have also found similar results, reporting that PLGA copolymers with a higher molecular weight showed a lower loading capacity of doxycycline and atorvastatin (Mylonaki et al., 2018; Wang et al., 2019a), attributed to the interaction between the drug and the polymer (Wang et al., 2019a).
The CBD release profiles from CBD-502-Mps and CBD-504-Mps are depicted in Fig 3B, where both formulations show an extended CBD release for more than 40 days. CBD-502-Mps exhibited a faster release than CBD-504-Mps, with around 95 and 80% of CBD released at day 42, respectively. A slight burst effect (~4%) was seen in both microparticles over the first 2 hours of the study. This “faster” drug release over the first hours of the experiment could be related to the most superficial CBD, which has direct and rapid access to the particle surface (Gasmi et al., 2016).

In CBD-502-Mps, a tri-phasic release profile could be seen, with a 1st faster phase until day 2, followed by a 2nd slower phase from day 2 to 14 and a 3rd final phase from day 14 to 42. However, in CBD-504-Mps, a biphasic release profile could be seen, with a 1st faster phase until day 7 with around 35% of the CBD released and a 2nd slower phase from day 7 to day 42 with around 80% of the CBD released.

The slower drug release from CBD-504-Mps compared to CBD-502-Mps could be attributed to the polymer molecular weight. Polymers with a higher molecular weight exhibit slower polymer matrix degradation and, as a consequence, a slower drug release. In this work, SEM images during release studies confirmed slower polymer degradation in CBD microparticles elaborated with PLGA-504. In CBD-502-Mps, polymer degradation seemed to be evident at day 14, when signs of corrugations on microparticle surfaces started to be obvious. However, at this point, signs of corrugations were not observed in CBD-504-Mps but started to be detected at day 21. Finally, at day 28, while CBD-502-Mps completely lost their spherical shape and intense polymer degradation was appreciated, CBD-504-Mps did not lose their spherical shape. These polymer degradation results coincide with those obtained in other formulations (Wang et al., 2019a).

Due to the higher CBD content and better release profile, CBD-502-Mps were selected for efficacy studies.

3.3. In vitro antitumor efficacy of CBD-Mps

The use of microparticles as CBD carriers would lead to extended antitumor activity after a single administration. In fact, polycaprolactone microparticles loaded
with CBD have shown a promising extended antitumor efficacy in triple negative breast tumour cells (Hernan Perez De La Ossa et al., 2012).

When unloaded microparticles were administered, no cytotoxic effect was seen in MCF-7 or MDA-MB-231 cells. Nevertheless, CBD-Mps inhibited cell proliferation throughout the time interval evaluated (10 days) in both breast cancer cells (Fig.4A and B). In MCF-7 cells, a concentration of 2.55 mg/ml of CBD-Mps was administered. However, in MDA-MB-231 cells, due to their higher CBD sensitivity, 1.95 mg/ml of CBD-Mps were administered. These values were calculated using the equation obtained for zero-order kinetics fitting since day 2 of CBD to in vitro release studies, as the aim of this study was to evaluate whether the microencapsulation of CBD allows extended antiproliferative activity, and closely correspond with IC$_{70}$ values after 48 hours of incubation previously calculated (an overall concentration of CBD $\approx$25 and 18µM in MCF-7 and MDA-MB-231 cells, respectively).

The higher antiproliferative activity during the first two days of the experiment is related to CBD release profile. This period of time corresponds to the 1$^{st}$ faster phase, in which a high free CBD concentration, above 100µM, is reached in both MDA-MB-231 and MCF-7 cells. This explains the high cell viability inhibition, above 85%, detected in both cell lines. According to the release study, after day 2, the 2$^{nd}$ slow release phase of CBD from Mps starts. As a consequence, during the rest of the antitumor activity assay (2-10 days) an overall free CBD concentration around 18 and 25 µM was achieved in MDA-MB-231 and MCF-7 cells respectively, leading to a cell proliferation inhibition of 60% that was similar to that obtained with CBD$_{sol}$ administered daily. Therefore, these results indicated that the CBD encapsulated into polymeric microparticles maintained its antiproliferative activity against both mammary breast tumour cells. This antiproliferative activity remained constant during at least 10 days, indicating that this formulation could be a good strategy to obtain an extended CBD antiproliferative activity after a single administration.

3.4. Combination activity: CBD-loaded microparticles

As previously mentioned, the results obtained in the combination studies of CBD in solution with PTX and DOX showed that the administration of CBD before or during PTX or DOX treatments could be interesting strategies to enhance the antiproliferative activity of these drugs in breast carcinomas. Now, both strategies (CBD
pre and co-administration) were combined looking for an optimal effect of CBD on the antiproliferative activity of PTX or DOX. For this, CBD was administered in two different formulations: as a solution and in microparticles. CBD-Mps were added in a single administration 24h before the administration of PTX or DOX. However, CBD$_\text{sol}$ was added daily: in a 1st step of pre-treatment, after 24 hours in co-administration with PTX or DOX and 24 hours later. It should be noted that the administration of CBD in Mps led to its continuous release before and after PTX or DOX administration. Additionally, in order to protect CBD from possible degradation due to external agents, it has been reported that it is unstable in aqueous medium at 37°C. In fact, to the best of our knowledge, the use of microparticles to combine both treatment strategies in cancer has not been previously described.

In these experiments, the amount of administered microparticles was calculated using in vitro release data during the first 72 hours, the total duration of this study. In MCF-7 cells, a concentration of 0.432 mg/ml of CBD-Mps were administered, achieving a daily free CBD concentration of ≈10 µM. In MDA-MB-231 cells, 0.216 mg/ml of CBD-Mps were used, corresponding to a daily free CBD concentration of ≈5 µM. CBD$_\text{sol}$ was administered daily at 5 and 10 µM in MDA-MB-231 and MCF-7 cells, respectively.

Regarding PTX, the combination with CBD using this protocol (pre+co administration) was really effective. In MCF-7 cells, the combination of PTX with both CBD$_\text{sol}$ and CBD-Mps showed a significantly higher cell death than PTX alone (Figure 5A). The single administration of CBD-Mps reported a similar efficacy to the daily administration of CBD in solution, with no statistically significant differences (p value >0.05). It should be noted that by using this protocol, the administration of the lowest PTX concentration (10 nM) exhibited an inhibition of cell proliferation of higher than 50%. This PTX concentration (10nM) is much lower than the IC$_{50}$ values obtained in pre-administration (33.46 ± 11.74 nM) or co-administration (55.19 ± 11.11 nM) protocols, indicating that the previous administration of CBD followed by its co-administration with PTX could be a good strategy to improve its anticancer efficacy in oestrogen- receptor-positive breast tumours.

In MDA-MB-231 cells, a higher and statistically significant (p value<0.01) antiproliferative activity was seen in both CBD$_\text{sol}$ and CBD-Mps treated cells, compared
to single PTX, except for the highest PTX concentration, probably due to the high cell
death detected when it was administered at 500 nM. Interestingly, in these cells, the
administration of CBD-Mps was more effective than CBD\textsubscript{sol}, especially when combined
with PTX 10 nM, where statistically significant differences (p value<0.01) between
both CBD treatments were detected (Figure 5B). As observed in MCF-7 cells, the
combination of CBD-Mps and PTX 10 nM showed a cell death of higher than 50%.
This PTX concentration is also much lower than the IC\textsubscript{50} values of this antineoplastic
obtained in pre (49.46 ± 2.47 nM) and co-administration (79.22 ± 10.35 nM) strategies
with CBD at 5 µM. However, when CBD was administered in solution using a pre+co-
administration schedule, with PTX 10 nM, the cell viability inhibition detected was
lower than 50% (=35%), although still higher than in pre or co-administration strategies
at this PTX concentration (=15 and 20%, respectively). This data suggests that the
combination of CBD and PTX in triple negative breast tumours could be really
interesting, especially when the formulation of CBD-Mps is used.

As regards DOX, the combination with CBD was also really effective. In MCF-7,
the combination of DOX with CBD\textsubscript{sol} or CBD-Mps (using a pre+co administration
protocol) also showed a statistically significant higher cell death (p value <0.05) than
DOX alone (Figure 5C). In this combination, the single administration of CBD-Mps
showed a similar or even better efficacy to the daily administration of CBD in solution.
Nevertheless, no statistically significant differences were seen between both treatments,
except in the combination of CBD-Mps plus doxorubicin 1µM. The administration of
both CBD\textsubscript{sol} and CBD-Mps (pre+co-administration protocol with CBD 10µM
administered daily) with the lowest DOX concentration (0.1µM) produced a cell death
considerably greater than 50%. This DOX concentration is lower than the IC\textsubscript{50} of DOX
calculated in these cells when combined with CBD 10µM using pre-(IC\textsubscript{50}=0.88±0.13µM) and co-administration (IC\textsubscript{50}=1.11±0.98) strategies, suggesting the
potential combination of DOX and CBD, administered in solution or encapsulated in
microparticles, using a pre+co administration protocol in oestrogen-receptor positive
breast cancer.

Finally, in MDA-MB-231 a significantly higher antiproliferative activity (p
value < 0.01) was also detected in CBD treated cells, compared to cells only treated
with DOX (Figure 5 D). Interestingly, CBD-Mps reported a higher activity than CBD\textsubscript{sol},
with statistically significant differences (p value < 0.01) in all tested combinations. In fact, in this case the pre+co administration protocol was really effective when CBD was administered in CBD-Mps. When this formulation was combined with DOX 0.1µM (lowest concentration), the cell death achieved was much higher than 50%. This DOX concentration was considerably lower than the IC₅₀ obtained in pre- (4.84 ± 0.68) and co-administration (5.49 ± 1.04) strategies. However, in a pre+co-administration protocol when CBD was administered in solution and combined with DOX 0.1µM, the cell death detected was much lower than 50% (≈30%), similar to that observed in pre- and co-administration strategies (≈20 and 25%, respectively). This suggests that the combination of CBD and DOX using a pre+co-administration strategy is useful in triple negative breast tumours only when CBD is administered as CBD-Mps.

To sum up, this data suggests that the use of polymeric microparticles could be a good strategy for CBD administration, so that the activity of both PTX and DOX in oestrogen-receptor positive breast cancer cells can increase after a single administration of CBD-Mps. CBD instability could explain, in part, the higher efficacy of CBD-Mps compared to CBD_sol. As previously mentioned, CBD is unstable in aqueous medium. While CBD encapsulated in microparticles is protected, CBD in solution is more susceptible to degradation, decreasing its final concentration and, as a consequence, its activity. It should also be noted that MDA-MB-231 cells (where considerable differences between CBD_sol and CMD-Mps were detected) are more sensitive to CBD than MCF-7 cells, and a constant CBD release from microparticles could be more effective, explaining the higher efficacy of CBD-Mps compared to the daily administration of CBD in solution.

3.5. In ovo studies

The CAM model has emerged as a potential tool in cancer research due to the feasibility to induce tumour formation onto the CAM, allowing the evaluation of new anticancer formulations in a more representative in vivo tumour model (Vargas et al., 2007). In this work, the in ovo approach of the CAM model has been used to test the efficacy of CBD and CBD-Mps as monotherapy or in combination (pre+co-administration) with PTX. MDA-MB-231 cells (triple negative breast cancer) were selected due to their high invasiveness.
As illustrated in figure 6B, the use of CBD$_{\text{sol}}$ administered daily as monotherapy, at a concentration of 100µM, significantly decreased the growth of an MDA-MB-231 derived tumour (p value <0.01) compared to control (eggs treated with cell culture medium). Interestingly, the administration of a single dose of CBD-Mps (3.65 mg/ml of microparticles with a daily CBD release of 100 µM) also showed a significant reduction in tumour growth (p value <0.01). In fact, microparticles were even more effective, and while CBD$_{\text{sol}}$ treatment showed around a 1.6 fold reduction, CBD-Mps treatment exhibited a ≈1.8 reduction (Fig. 6C). However, this difference was not statistically significant (p value > 0.05).

The combination studies undertaken in this model also demonstrated the synergism of both CBD and PTX previously detected in cell cultures. The combination of this taxane with either CBD$_{\text{sol}}$ or CBD-Mps showed a statistically significant reduction in tumour growth compared to PTX alone (p value <0.05). In this case, a slightly better antitumor effect was also detected with CBD-Mps(≈2.3 and 2.5 fold reductions were detected with PTX + CBD$_{\text{sol}}$ and PTX+ CBD-Mps, respectively) (Fig. 6B and C). However, non-statistically significant differences between the two CBD formulations (solution and microparticles) were detected.

Although further in vivo studies are necessary, the results obtained in this in vivo model of a triple negative breast tumour reinforce the potential use of CBD in combination with PTX for the treatment of this highly invasive neoplasm. In this way, the use of CBD-Mps could be especially interesting due to the optimisation of the treatments, allowing a reduction in the number of administrations.

4. CONCLUSION

The combination of cannabidiol with paclitaxel or doxorubicin leads to a reduction of the effective concentration of these antineoplastic agents in MDA-MB-231 and MCF-7 cells.

Polymer microparticles are shown as an interesting tool to administer CBD and optimise its anticancer activity as monotherapy, showing extended antiproliferative activity for at least ten days; or in combination with PTX or DOX using the pre+co-administration protocol in both breast cancer cells. While in MCF-7 cells CBD-Mps produced similar or slightly higher cell viability inhibition than CBD$_{\text{sol}}$, in MDA-MB-
the cell death was significantly higher when CBD was administered in microparticles.

The antitumor efficacy of CBD-loaded microparticles as monotherapy or in combination with PTX in triple negative breast tumours was also confirmed in the chick chorioallantoic membrane model, exhibiting, after a single administration, a slightly better activity than the equivalent dose of CBD in a solution administered daily.

To sum up, this work provides, for the first time, *in vitro* and *in ovo* evidences of the potential use of CBD-loaded microparticles in combination with paclitaxel or doxorubicin in the treatment of both oestrogen receptor positive and triple negative breast cancer.

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REFERENCES


FIGURE CAPTIONS

**Figure 1:** Combination studies of CBD_{sol} with PTX. Viability of MCF-7 (A) and MDA-MB-231 (B) cells pre-treated with CBD_{sol} for 24 hours followed by the administration of PTX for 48 hours. Viability of MCF-7 (C) and MDA-MB-231 (D) cells treated with CBD_{sol}+PTX for 48 hours. Combination indexes obtained for CBD_{sol}+PTX in MCF-7 (E) and MDA-MB-231 (F) cells.

**Figure 2:** Combination studies of CBD_{sol} with DOX. Viability of MCF-7 (A) and MDA-MB-231 (B) cells pre-treated with CBD_{sol} for 24 hours followed by the administration of DOX for 48 hours. Viability of MCF-7 (C) and MDA-MB-231 (D) cells treated with CBD_{sol}+DOX for 48 hours. Combination indexes obtained for CBD_{sol}+DOX in MCF-7 (E) and MDA-MB-231 (F) cells.

**Figure 3:** SEM images (A), release profile (B) and polymer matrix degradation during release studies (C) of CBD laoded microparticles prepared with both PLGA- RG*-502 (CBD-502-Mps) and PLGA-RG*-504 (CBD-504-Mps).

**Figure 4:** Antiproliferative activity of CBD-loaded microparticles in MCF-7 (A) and MDA-MB-231 cells (B). Images obtained by optical microscopy of MCF-7 (C) and MDA-MB-231 cells (D) after CBD_{sol} and microparticle formulation treatments. Scale bar: 100µm.

**Figure 5:** Viability of breast cancer cells pre-treated with CBD in solution or encapsulated into polymeric microparticles for 24 hours followed by the administration of these treatments plus PTX (A and C) or doxorubicin (B and D) for 48 hours.

**Figure 6:** Images of MDA-MB-231 derived tumour formed on CAM membrane before and after treatment with CBD-Mps (A). Scale bar: 1mm. The *in ovo* antitumor efficacy of CBD_{sol} (daily administered) and CBD-Mps (single administration) as monotherapy or in combination with paclitaxel in MDA-MB-231 derived tumour (B). Fold-reduction of tumour growth of each treatments in MDA-MB-231 derived tumours (C) ** significant differences (p value<0.01) with control (cell culture medium). † Significant differences (p value<0.05) with PTX alone.
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
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Graphical abstract