



Targeting Glioma Initiating Cells with A combined therapy of cannabinoids and temozolomide

Israel López-Valero^{a,b,c}, Cristina Saiz-Ladera^{a,b,c}, Sofía Torres^a, Sonia Hernández-Tiedra^{a,b}, Elena García-Taboada^{a,d}, Fátima Rodríguez-Fornés^a, Marina Barba^a, David Dávila^{a,b}, Nélida Salvador-Tormo^{a,b}, Manuel Guzmán^{a,c,d}, Juan M. Sepúlveda^e, Pilar Sánchez-Gómez^f, Mar Lorente^{a,b,c,1}, Guillermo Velasco^{a,b,c,*,1}

^a Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, Madrid, Spain

^b Instituto de Investigaciones Sanitarias San Carlos (IdISSC), 28040 Madrid, Spain

^c Instituto Universitario de Investigación Neuroquímica, Complutense University, 28040 Madrid, Spain

^d Centro de Investigación Biomédica en Red Sobre Enfermedades Neurodegenerativas, Spain

^e Neuro-oncology Unit, Hospital Universitario 12 de Octubre, Madrid, Spain

^f Neuro-oncology Unit, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

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ABSTRACT

Glioblastoma multiforme (GBM) is the most frequent and aggressive type of brain tumor due, at least in part, to its poor response to current anticancer treatments. These features could be explained, at least partially, by the presence within the tumor mass of a small population of cells termed Glioma Initiating Cells (GICs) that has been proposed to be responsible for the relapses occurring in this disease. Thus, the development of novel therapeutic approaches (and specifically those targeting the population of GICs) is urgently needed to improve the survival of the patients suffering this devastating disease. Previous observations by our group and others have shown that Δ^9 -Tetrahydrocannabinol (THC, the main active ingredient of marijuana) and other cannabinoids including cannabidiol (CBD) exert antitumoral actions in several animal models of cancer, including gliomas. We also found that the administration of THC (or of THC + CBD at a 1:1 ratio) in combination with temozolomide (TMZ), the benchmark agent for the treatment of GBM, synergistically reduces the growth of glioma xenografts. In this work we investigated the effect of the combination of TMZ and THC:CBD mixtures containing different ratios of the two cannabinoids in preclinical glioma models, including those derived from GICs. Our findings show that TMZ + THC:CBD combinations containing a higher proportion of CBD (but not TMZ + CBD alone) produce a similar antitumoral effect as the administration of TMZ together with THC and CBD at a 1:1 ratio in xenografts generated with glioma cell lines. In addition, we also found that the administration of TMZ + THC:CBD at a 1:1 ratio reduced the growth of orthotopic xenografts generated with GICs derived from GBM patients and enhanced the survival of the animals bearing these intracranial xenografts. Remarkably, the antitumoral effect observed in GICs-derived xenografts was stronger when TMZ was administered together with cannabinoid combinations containing a higher proportion of CBD. These findings support the notion that the administration of TMZ together with THC:CBD combinations – and specifically those containing a higher proportion of CBD – may be therapeutically explored to target the population of GICs in GBM.

1. Introduction

Glioblastoma multiforme (GBM), or grade IV astrocytoma, is the

most frequent class of malignant primary brain tumor and one of the most aggressive forms of cancer. Consequently, median survival upon diagnosis is just 12–15 months [1–3]. This dramatic behavior has been

Abbreviations: GBM, Glioblastoma multiforme; TMZ, temozolomide; THC, Δ^9 -Tetrahydrocannabinol; CBD, cannabidiol; GICs, Glioma-Initiating Cells; I.P., intraperitoneal

* Corresponding author at: Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University Calle José Antonio Nováis 12, 28040 Madrid, Spain.

E-mail address: gvelasco@ucm.es (G. Velasco).

¹ ML and GV are co-senior authors of this work.

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attributed to the high invasiveness and proliferation rate exhibited by these tumors. In addition, GBM is highly resistant to radiotherapy and standard chemotherapy. These features could be explained, at least partially, by the presence within the tumor mass of a small sub-population of cells called Glioma Stem-like Cells or Glioma Initiating Cells (GICs), due to their similarity with the normal stem cells and to their capacity to initiate and maintain tumor growth [4–6]. Current treatments against GBM include surgical removal of the tumor (which, in many occasions is partial depending on the proximity of the tumor mass to eloquent brain regions), focal radiotherapy [1,3] and treatment with different chemotherapeutic agents, being the most widely used the alkylating agent temozolomide (TMZ) [2,3,7]. All these treatments exhibit limited efficacy and thus it is crucial to develop new therapeutic strategies that help to fight more efficiently this disease. Today is considered that the development of new therapies based on the combination of various anticancer agents, including those targeting GICs, together with an increase in the selectivity of the treatments [8,9], may contribute to enhance the survival of patients with GBM.

Δ^9 -Tetrahydrocannabinol (THC), the main active ingredient derived from *Cannabis sativa* [10], exerts its biological effects by mimicking the actions of a family of endogenous bioactive lipid mediators named endocannabinoids. Thus, THC binds and activate two specific G protein-coupled cannabinoid receptors: CB₁ and CB₂ [11]. CB₁ is highly expressed in different brain regions although it is also present in many tissues outside of the central nervous system. CB₂ is abundant in the immune system although it is also present in other tissues and in many types of cancer cells including glioma cells [11–13]. Nowadays, cannabinoids are being investigated as potential therapeutic agents for the management of different pathologies [14–16], including cancer [17,18]. Thus, treatment with cannabinoid has been shown to inhibit tumor growth in different animal models of cancer [17–21]. These findings led to the development of a first pilot clinical study aimed at testing the anti-cancer activity of THC on recurrent GBM [22]. The mechanism underlying these anticancer actions of THC has been partially clarified and relies on the stimulation of an ER stress-related signaling pathway that triggers the up-regulation of Tribbles pseudokinase 3 and the subsequent stimulation of autophagy-mediated cancer cell death [19,23,17,18,20].

Aside from THC, *C. sativa* produces more than 150 other cannabinoids [24] although, unlike THC, many of them exhibit little affinity for CB receptors [15,25]. Of importance, one of these phytocannabinoids, namely cannabidiol (CBD), has been shown to exhibit antineoplastic activity in animal models of cancer including gliomas [25–29]. The mechanism by which CBD exerts its anti-cancer activity is not completely understood, although it has been proposed to rely on the ability to promote the accumulation of reactive oxygen species (ROS) [30], a mechanism that it is also activated in glioma cells [26,28]. The potential therapeutic interest of the combined administration of THC and CBD is being investigated for the treatment of different diseases [15,25,31]. Specifically, THC and CBD have been shown to inhibit the proliferation and survival of cancer cells *in vitro* [31] and in animal models of cancer [32,33]. Moreover, we found that the co-administration of TMZ with THC and with THC + CBD (at a 1:1 ratio) exerts a strong anti-tumoral action in glioma xenografts [33,34]. These findings led to the development of a clinical study where the effect of TMZ administered in combination with the cannabinoid-based medicine Sativex (containing THC and CBD in a 1:1 ratio) has been investigated in patients with recurrent GBM (NCT01812603 and NCT01812616).

One issue that remained to be analyzed is whether CBD alone or combinations of THC and CBD other than those containing the same amount of the two cannabinoids may also have anticancer activity when administered together with other anticancer agents. Results presented here support the idea that administration of TMZ in combination with cannabinoid preparations containing a higher proportion of CBD than of THC (but not CBD alone) target more efficiently the GICs population.

2. Materials and methods

2.1. Reagents

Pure THC and CBD were obtained from THC Pharm Company (Frankfurt, Germany). THC botanical drug substance (THC-BDS containing 67.6% THC w/w; 0.3% CBD w/w; other individual plant cannabinoids < 1.7% w/w) and CBD-botanical drug substance (CBD-BDS containing 65.4% CBD w/w; 3.2% THC w/w; other individual plant cannabinoids < 1.7% w/w) were obtained from GW Pharmaceuticals (Cambridge, UK). THC-BDS and CBD-BDS were obtained as a resin, dissolved in ethanol at a concentration of 100 mg/mL, and stored at –20 °C. The required amounts of each component were dried, weighted, and diluted in dimethyl sulfoxide (DMSO). Treatments containing different THC:CBD ratios were prepared from pure THC and CBD or from the corresponding Botanical drug substances (BDS) extracts enriched in each of the two cannabinoids. In this latter case the total amount of THC and CBD present in the BDS extracts was used to calculate the amounts to be administered to obtain the appropriate proportions of each cannabinoid. TMZ was purchased from Merck (Darmstadt, Germany). Drugs were prepared in DMSO for *in vitro* experiments. Control incubations contained the same amount of DMSO and no significant effect was observed in any of the parameters determined throughout this study at the final concentration used (< 0.5%, v/v).

2.2. Cell cultures

The human brain U87MG (ATCC® HTB-14™) cell line was purchased from ATCC (Manassas, Virginia, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. Glioblastoma patient-derived cells with stem-like properties (Glioma Initiating Cells, GICs) were obtained from human GBM tumor samples from the Spanish National Cancer Center (CNIO, Madrid, Spain) biobank (GH2-GICs cells) [35] and from Hospital 12 de Octubre (Madrid, Spain) (12O12-GICs cells) [36]. All procedures involving samples of human origin were performed with the approval of the corresponding ethical committees from each institution as well as of the ethical committee of Complutense University. Briefly, GICs cultures were obtained by using the following procedure: tumors samples were mechanically and enzymatically dissociated with Collagenase type Ia from *Clostridium histolyticum* (Sigma #C9722, Saint Louis, MS, USA) for 2 h at 37 °C and filtered using a 100 µm nylon filter (Millipore, ref 352360, Burlington, MA, USA). Cells obtained after this procedure were then plated and maintained as non-adherent cultures of neuro-spheres for at least 3 consecutive passages in a DMEM:Ham's F-12 media supplemented with 1% penicillin–streptomycin and HEPES buffer (Lonza, Basel, Switzerland), 0.5% ultraglutamine 200 nM (Lonza), 20 ng/ml EGF and FGFb (Gibco, Carlsbad, USA), 2 µg/ml heparin sodium salt (Sigma), 1% B27 (Invitrogen, Carlsbad, USA) and 1% leukemia inhibitory factor LIF (Millipore). Enrichment in GICs was analyzed by testing the expression of stem cell markers in these cultures. All cell cultures were incubated at 37 °C, 5% CO₂. Experiments were performed using U87MG cultures of < 28 passages and GICs cultures between passages 3 and 15.

2.3. Generation of tumor xenografts

For heterothopic/subcutaneous xenografts, 5×10^6 U87MG cells resuspended in 100 µl of PBS supplemented with 0.1% glucose were subcutaneously injected in the right flank of 5 week-old (male or female) nude mice (Harlan Laboratories, Indiana, USA) weighting approximately 25 g. Tumors were daily measured with an external caliper, and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$. When tumors had reached an average size of 200 mm³, animals were randomly assigned to different groups and treatments with the corresponding drugs commenced. Cannabinoids were diluted in sesame oil

and orally administered by using an oral gavage. TMZ was diluted in PBS supplemented with 5 mg/ml BSA. Once the treatments were completed, animals were sacrificed and tumors excised for further analyses.

For the generation of intracranial/orthotopic xenografts, 3×10^5 U87 MG cells resuspended in 4 μ l of PBS or 7.5×10^4 12O12 GICs resuspended in 4 μ l of supplemented GIC-medium were stereotactically injected into the right cerebral hemisphere of nude mice at a depth of 3 mm. Animals were previously anesthetized with isoflurane and subsequently treated with a mixture of buprenorphine (0.1 mg/kg) and meloxicam (1 mg/kg). Tumor growth was followed by magnetic resonance imaging (MRI) analysis. In the experiments with U87MG-cell derived orthotopic xenografts, once the tumors were detected, mice were randomly assigned to different experimental groups and treatments started at the indicated time points. In the experiment with GICs, treatments started the following day after the injection of the cells. The monitoring of tumor growth by magnetic resonance imaging was performed at the Nuclear Magnetic Resonance Centre of Complutense University (Madrid, Spain) using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany). Tumor volume was calculated using the Image J software from T1-weighted images. All procedures involving animals were performed with the approval of the corresponding ethical committees from Complutense University and Madrid region according to European regulations.

2.4. Analysis of self-renewal and proliferation capacity of GICs

Nonadherent cultures of GICs were plated at a density of 10^4 cells/ml (passage 0, P0) and incubated with the different treatments for 5 days. The spheres formed in each well were then dissociated, counted (passage 1, P1) and equal number of cells re-plated and incubated again with the corresponding treatments for 5 additional days. This procedure was repeated for two consecutive passages (passage 2, P2).

2.5. Limiting dilution assays (LDA)

Nonadherent cultures of GICs were plated at density of 10^4 cells/ml and incubated with the different treatments for 5 days. Spheres formed were dissociated and plated in 96-well plates at different densities (200, 100, 50, 20 and 10 cells per well, respectively). One week later, each well was scored for tumorsphere formation and wells in which there was at least one neurosphere were considered positive. Results correspond to the number of wells for each experimental condition in which neurospheres were found. Results were analyzed using ELDA software application [37].

2.6. Western blot

Western blot analysis was performed following standard methods. Briefly, cells were lysed in a buffer containing 50 mM Tris-HCl, 0.1% Triton X-100, 50 mM NaF, 10 mM sodium glycerophosphate, 5 mM sodium pyrophosphate and 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 mM EDTA, EGTA, 200 μ M β -mercaptoethanol and 200 μ M microcystin and centrifuged at 12,000 rpm for 15 min. Protein concentration was determined by Bradford assay. Proteins were electrophoretically separated in SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA), transferred onto PVDF membranes (Bio-Rad), blocked in a 5% skim milk solution or 5% BSA (Sigma) and incubated at 4 °C overnight with a primary antibody: cleaved-PARP Asp 214 [(1:1000); #9541 Cell Signaling (Danver, MS, USA)] and α -tubulin (1:4000, Sigma). Immunoreactivity was detected using the enhanced chemiluminescence (ECL) system (Bio-Rad). Densitometric quantification was performed by ImageJ software.

2.7. Statistical analysis

Unless otherwise indicated, data are expressed as mean \pm standard

error of the mean (SEM). Analysis of variance (ANOVA) test was performed for multi-component comparisons (one-way or two-way ANOVA) using the GraphPad Prism 6.0 software. χ^2 -test was used for LDA experiments. The survival of nude mice was analyzed by Kaplan-Meier curves and differences were compared by log-rank test analysis. P-value of < 0.05 was considered significant.

3. Results

3.1. Effect of THC and CBD mixtures containing a higher proportion of CBD in combination with TMZ on the growth of U87MG cell-derived xenografts

Previous research by our group has shown that the administration of THC and of THC and CBD at a 1:1 ratio in combination with temozolomide (TMZ) exerts a strong anticancer activity in glioma xenografts [33] and (López Valero et al submitted manuscript). Since CBD has also been found to have anticancer activity in different cancer models including gliomas [38–40] we first asked whether the combination of CBD and TMZ may also produce a synergistic reduction on the growth of glioma xenografts. In disagreement with this hypothesis, administration of CBD and TMZ produced a lower decrease in tumor growth than the administration of TMZ alone (Fig. 1A), indicating that CBD does not enhance TMZ anticancer activity and supporting the notion that the presence of at least a certain amount of THC is required to observe an enhanced anticancer activity of TMZ.

Therefore, next we investigated the anticancer activity of combinations of THC and CBD containing a higher proportion of CBD on the growth of glioma xenografts. As shown in Fig. 1B, the administration of BDS extracts containing THC and CBD at a 1:4 ratio produced a similar reduction on the growth of U87MG cell-derived subcutaneous xenografts (and enhanced the effect of TMZ at a similar extent) than the administration of THC and CBD at a 1:1 ratio. To further characterize the effect of THC-CBD combinations on this model, we next investigated whether this enhanced effect of the combination of THC and CBD with TMZ was preserved using lower doses of THC. In line with this hypothesis, administration of BDS extracts containing THC and CBD at a 1:4 and 1:6 ratio produced a similar reduction on the growth of tumor xenografts (and enhanced the effect of TMZ at a similar extent) than the administration of THC:CBD at a 1:1 ratio (Fig. 1C).

To investigate the potential relevance of these findings in a model that resembles more closely the treatments that take place in patients with GBM, next, we analyzed the effect of BDS extracts containing THC and CBD at a 1:4 ratio in combination with TMZ in orthotopic xenografts generated by intracranial injection of U87MG cells (Fig. 2A). The combined administration of THC and CBD at a 1:4 ratio together with TMZ significantly enhanced the effect of this alkylating agent on the growth of the tumors (as determined by MRI) (Fig. 2B) as well as in the survival of the animals (Fig. 2C). Taken together, these observations support the notion that combinations of THC and CBD containing a higher proportion of CBD produce a similar anticancer effect on glioma xenografts (and enhance the anticancer activity of TMZ at a similar extent) than the administration of THC and CBD at a 1:1 ratio.

3.2. Effect of THC and CBD mixtures containing a higher proportion of CBD in combination with TMZ on the population of Glioma Initiating Cells

The population of cancer stem-like cells derived from glioma or Glioma Initiating Cells (GICs) is a small cell subpopulation present in GBM tumors that has features of stem cells and that has been proposed to be responsible for the relapses occurring in most patients with this disease [41]. Therefore, finding new therapeutic approaches capable of targeting this cell subpopulation have great interest to enhance the efficacy of current therapies against GBM [41]. One of the characteristics of GICs is that they are highly resistant to most anticancer therapies [41]. In addition, GICs exhibit a relatively slow proliferation rate and can self-renew undergoing asymmetric divisions [41]. We

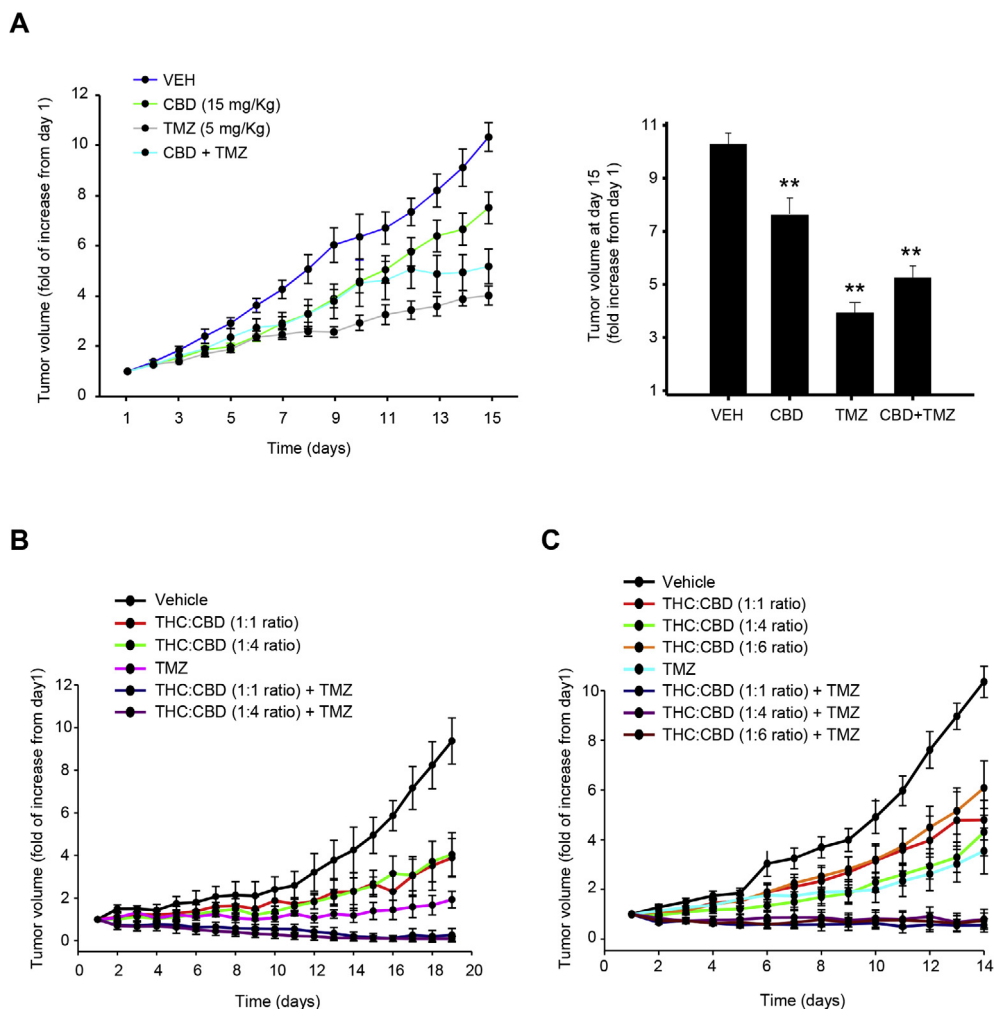


Fig. 1. THC and CBD combinations containing a higher proportion of CBD inhibit the growth of subcutaneous U87 MG cell-derived-glioma xenografts and enhance the anticancer activity of TMZ to a similar extent than THC and CBD at a 1:1 ratio. (A) Left panel: Effect of daily oral administration of CBD (15 mg/kg) and TMZ (5 mg/kg, I.P. administration) on the growth of U87MG cell-derived subcutaneous xenografts (mean ± SEM, n = 6–8 animals for each condition). Symbols of significance are omitted for clarity. CBD-treated tumors were significantly different from vehicle-treated tumors from day 5 to day 13 ($P < 0.05$), and from day 14 until the end of the treatment ($P < 0.01$); TMZ-treated tumors were significantly different from vehicle-treated tumors at day 4 ($P < 0.05$) and from day 5 until the end of the treatment ($P < 0.01$); CBD + TMZ-treated tumors were significantly different from vehicle-treated tumors from day 5 to day 12 ($P < 0.05$), and from day 13 until the end of the treatment ($P < 0.01$). Right panel: Data correspond to the change in tumor volume in the last day of the treatment and are expressed as the mean fold-change in tumor volume ± SEM relative to the first day of the treatment. ** $P < 0.01$ from vehicle-treated tumors. (B) Effect of daily oral administration of THC:CBD (1:1 ratio) [THC (5 mg/kg) + CBD (5 mg/kg)], THC:CBD (1:4 ratio) [THC (6.5 mg/kg) + CBD (24.5 mg/kg)] and TMZ (5 mg/kg, I.P. administration) on the growth of U87MG cell-derived subcutaneous xenografts (mean ± SEM, n = 6–7 animals for each condition). The total mg of THC and CBD administered in each case were obtained by

mixing the corresponding amounts of THC:BDS and CBD:BDS extracts. Symbols of significance are omitted for clarity. THC:CBD (1:1 ratio)-treated tumors were significantly different from vehicle-treated tumors from day 12 until the end of the treatment ($P < 0.001$). THC:CBD (1:4 ratio)-treated tumors were significantly different from vehicle-treated tumors at days 10,11 ($P < 0.05$) and from day 12 until the end of the treatment ($P < 0.001$); TMZ-treated tumors were significantly different from vehicle-treated tumors from day 8 to day 11 ($P < 0.01$) and from day 12 until the end of the treatment ($P < 0.001$); THC:CBD (1:1 ratio) + TMZ-treated tumors were significantly different from vehicle-treated tumors from day 5 until the end of the treatment ($P < 0.001$); significantly different from THC:CBD (1:1)-treated tumors from day 7 to day 9 ($P < 0.01$) and from day 10 until the end of the treatment ($P < 0.001$); and significantly different from TMZ-treated tumors at days 14 ($P < 0.05$), 15 ($P < 0.01$) and from day 16 until the end of the treatment ($P < 0.001$). THC:CBD (1:4 ratio) + TMZ-treated tumors were significantly different from vehicle-treated tumors from day 5 until the end of the treatment ($P < 0.001$); significantly different from THC:CBD (1:4)-treated tumors from day 7 to day 10 ($P < 0.01$) and from day 11 until the end of the treatment ($P < 0.001$); and significantly different from TMZ-treated tumors from day 13 to day 15 ($P < 0.01$); and from day 16 until the end of the treatment ($P < 0.001$). (C) Effect of daily oral administration of THC:CBD (1:1 ratio) [THC (3.5 mg/kg) + CBD (3.5 mg/kg)], THC:CBD (1:4 ratio) [THC (4.5 mg/kg) + CBD (16.5 mg/kg)], THC:CBD (1:6 ratio) [THC (5.2 mg/kg) + CBD (29.5 mg/kg)] and TMZ (5 mg/kg, I.P. administration) on the growth of U87MG cell-derived subcutaneous xenografts (mean ± SEM, n = 6–7 animals for each condition). The total mg of THC and CBD administered in each case were obtained by mixing the corresponding amounts of THC:BDS and CBD:BDS extracts. Symbols of significance are omitted for clarity. At day 14 (the last day of treatments), all the treatments were significantly different from vehicle ($P < 0.001$). THC:CBD (1:1 ratio) + TMZ-treated tumors were significantly different from individually-treated tumors ($P < 0.001$); THC:CBD (1:4 ratio) + TMZ and THC:CBD (1:6 ratio) + TMZ treated tumors were significantly different from individually-treated tumors ($P < 0.01$). THC:CBD (1:4 ratio)-treated tumors were significantly different from THC:CBD (1:6 ratio)-treated tumor ($P < 0.05$).

therefore asked whether different ratios of THC and CBD in combination with TMZ may target the population of GICs. As a first approach to test this hypothesis we analyzed the effect of the different treatments on the proliferation during two consecutive passages of primary cultures of GICs derived from human GBM samples grown as non-adherent spheroid cultures. As shown in Fig. 3A, treatment with THC and CBD at a 1:5 ratio produced a stronger reduction (and enhanced at a further extent the effect of TMZ) on the proliferation of GH2-GICs than THC and CBD at a 1:1 ratio or than TMZ alone. Similar results were obtained with 12O12 cells where the strongest effect was consistently observed upon exposure to THC:CBD 1:5 + TMZ (Fig. 3B). In any case, differences between THC:CBD 1:5 + TMZ and the rest of the treatments were

non-significant. Next, we analyzed the effect of these drug combinations on the ability to generate “neurospheres” of primary cultures of GICs (an estimation of the self-renewal capacity of these cells) by performing the limiting dilution assay (LDA). As shown in Fig. 3C and D treatment with THC and CBD at a 1:5 ratio inhibited the formation of neurospheres at a higher extent than TMZ or than the combination of THC and CBD at a 1:1 ratio. Likewise, the combination of TMZ with THC and CBD at a 1:5 ratio produced a stronger inhibition on neurosphere formation than the treatment with THC and CBD at a 1:1 ratio in combination with TMZ, and almost completely inhibited the formation of neurospheres. Moreover, treatment with THC:CBD at a 1:5 ratio induced apoptosis of GICs (as determined by the cleavage of the caspase 3

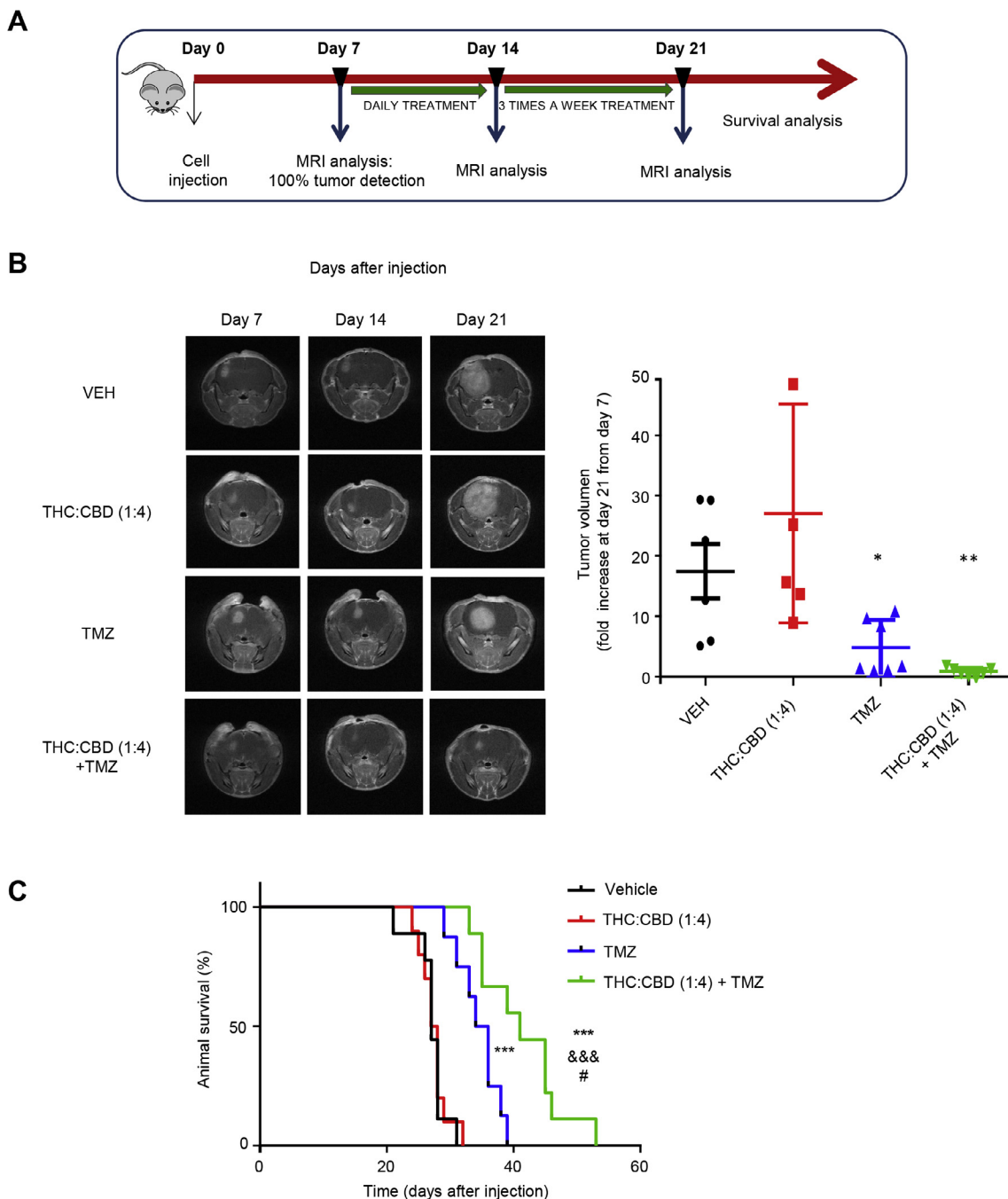


Fig. 2. The administration of THC:CBD (1:4 ratio) and TMZ strongly reduce tumor growth and enhances the survival of mice bearing U87MG cell-derived intracranial xenografts. (A) Scheme of the experimental design. (B) Effect of the oral administration of THC:CBD (1:4 ratio) [THC (6.5 mg/kg) + CBD (24.5 mg/kg)] and TMZ (5 mg/kg, I.P. administration) on the size of glioma xenografts (as determined by MRI) generated by intracranial injection of 3×10^5 U87MG cells ($n = 6-7$). The total mg of THC and CBD administered in each case were obtained by mixing the corresponding amounts of THC:BDS and CBD:BDS extracts. Left panel: Representative MRI images at different time points for each experimental condition are shown. Right panel: Data correspond to the increase in tumor volume at day 21 and are expressed as the mean fold change in tumor volume relative to day 7 \pm SEM; ($n = 5-7$) * $P < 0.05$ or ** $P < 0.01$ from vehicle-treated tumors. (C) Effect of the different treatments on the survival of mice bearing orthotopic tumor xenografts generated by intracranial injection of 3×10^5 U87MG cells. Data correspond to the percentage of alive animals along the experiment for each treatment and are depicted in Kaplan-Meier plot ($n = 8-10$) *** $P < 0.001$ from vehicle-treated tumors. && $P < 0.001$ from THC:CBD (1:4 ratio)-treated tumors; # $P < 0.05$ from TMZ-treated tumors.

substrate PARP) at higher extent than treatment with TMZ or with THC:CBD at a 1:1 ratio alone (Fig. 3E and F). Similarly, the combined administration of TMZ and THC:CBD at a 1:5 ratio activated apoptosis at a higher extent than TMZ + THC:CBD at a 1:1 ratio (Fig. 3E and F). To test the in vivo relevance of these observations we analyzed the effect of the different treatments on orthotopic xenografts generated by intracranial injection of 12O12 GICs in immunodeficient mice (Fig. 4A).

As shown in Fig. 4B, oral administration of THC and CBD at a 1:1 or 1:5 ratio alone did not significantly affect tumor size (as determined by MRI). However treatment with THC:CBD at a 1:5 ratio alone but not with THC:CBD at a 1:1 ratio increased the survival of the animals (Fig. 4C). More importantly, the combined administration of TMZ and THC:CBD at a 1:1 and 1:5 ratios reduced tumor growth and had a more potent effect on the survival of the animals than treatment with TMZ

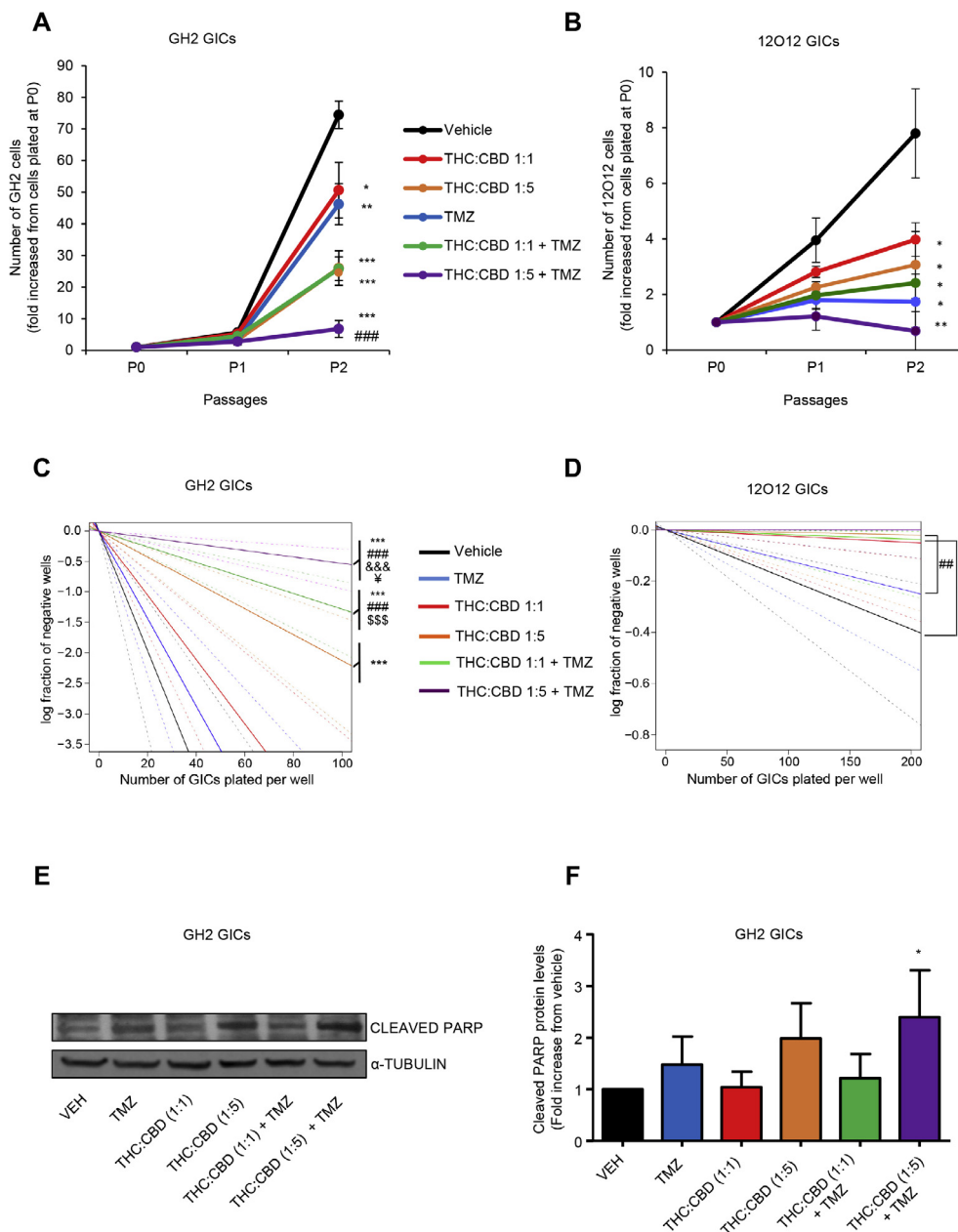


Fig. 3. THC:CBD (1:5 ratio) inhibits GICs proliferation and self-renewal of GICs to a higher extent than THC-CBD (1:1 ratio). (A–B) Effect of the treatment with THC:CBD (1:1 ratio) [2.5 μM THC + 2.5 μM CBD]; THC:CBD (1:5 ratio) [0.83 μM THC + 4.17 μM CBD] and TMZ (100 μM, panel A or 20 μM, panel B) during 2 consecutive passages on the proliferation and self-renewal capacity of GH2-GICs (panel A) and 12O12-GICs (panel B) grown as spheroids. Data correspond to the total number of cells counted upon disaggregation of spheroid cultures in each passage and are expressed as the mean fold-change from the number of cells plated at P0 ± SEM. n = 3. *P < 0.05, **P < 0.01 or ***P < 0.001 from vehicle-treated cells; ###P < 0.001 from TMZ-treated cells. Treatment of GH2-GICs with THC:CBD (1:1 ratio) + TMZ or THC:CBD (1:5 ratio) + TMZ were significantly different (P < 0.05) from treatment with THC:CBD (1:1 ratio) and THC:CBD (1:5 ratio). Treatment with THC:CBD (1:5) + TMZ was significantly different (P < 0.05) from THC:CBD (1:1) + TMZ. (C–D) Effect of THC:CBD (1:1 ratio) [2.5 μM THC + 2.5 μM CBD]; THC:CBD (1:5 ratio) [0.83 μM THC + 4.17 μM CBD] and TMZ (100 μM, panel C or 20 μM, panel D) on the self-renewal ability (as determined by limiting dilution assays) of GH2-GICs (panel C) and 12O12-GICs (panel D). A representative experiment of 2 (Panels C and D) is shown. Data corresponds to the fraction of cells with ability to generate new spheres cultures. Graphs were obtained using the ELDA software application that fits the data obtained for each experimental condition to the limiting dilution model. The slope of the depicted solid lines corresponds to the fraction of cells with ability to generate new spheres cultures. Decreased slopes indicate a lower fraction of cells with capacity to generate new spheres. The dotted lines indicate the 95% confidence interval. **P < 0.01 or ***P < 0.001 from vehicle-treated cells; ##P < 0.01 or ###P < 0.001 from TMZ-treated cells; \$\$\$P < 0.001 from

THC:CBD (1:1 ratio); &&&P < 0.001 from THC:CBD (1:5 ratio); and ¥P < 0.05 from THC:CBD (1:1) + TMZ. (E) Effect of THC:CBD (1:1 ratio) [3.5 μM THC + 3.5 μM CBD]; THC:CBD (1:5 ratio) [1.16 μM THC + 5.83 μM CBD] and TMZ (100 μM) on apoptosis (as determined by the cleavage of the CASPASE 3 substrate PARP) of GH2-GICs (48 h). Left panel: A representative western blot experiment is shown (n = 3). Right panel: Densitometric analysis of cleaved PARP levels. Data are expressed as mean fold change in cleaved PARP levels relative to tubulin levels in each experimental condition ± SEM. *P < 0,05 from vehicle-treated cells.

alone. Furthermore, the combined administration of THC and CBD at a 1:5 ratio and TMZ reduced tumor growth and increased animal survival at a higher extent than treatment with THC:CBD at a 1:1 ratio in combination with TMZ. Altogether, these observations support the idea that the combined administration of TMZ with THC:CBD preparations has anticancer activity in GICs *in vitro* and *in vivo*. In addition, our findings also support that cannabinoid combinations containing a higher proportion of CBD target the population of GICs more efficiently than THC:CBD at a 1:1 ratio.

4. Discussion

Previous findings by our group and others have shown that THC as well as CBD have anticancer activity in animal models of glioma [17,18,40,42,43]. Moreover, it was also found that the combination of

THC and CBD at a 1:1 ratio produces a similar effect as THC and that the combination of TMZ [the benchmark agent for the treatment of GBM] and THC [or THC and CBD (at a 1:1 ratio)], strongly enhances the tumor inhibitory activity of the administration of THC, TMZ or THC + CBD at a 1:1 ratio alone [33].

Of note, cannabinoid preparations containing CBD only or different amounts of THC and CBD are currently being explored for different therapeutic applications [25] and therefore it could be relevant from the clinical point of view to know whether CBD alone or combinations of THC and CBD other than those containing the same amount of the two cannabinoids may also have anticancer activity when administered together with other antineoplastic agents. Results presented here indicate that, the combined administration of TMZ and CBD does not enhance the tumor-inhibitory activity of TMZ in glioma xenografts and that therefore cannabinoid-based combinational antitumoral therapies

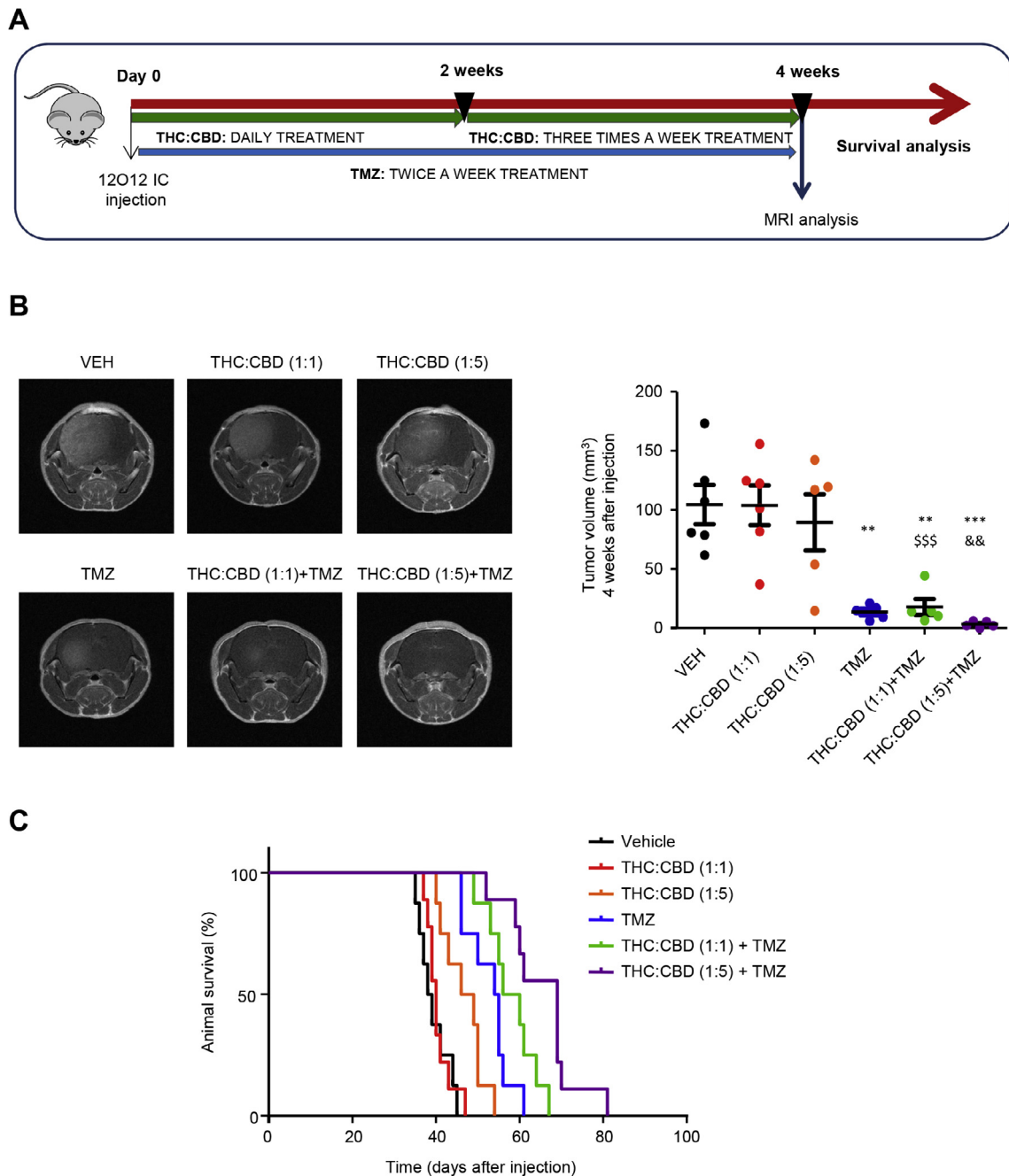


Fig. 4. The combination of THC:CBD (1:5 ratio) + TMZ reduces tumor growth and increases animal survival of mice bearing 12O12 GICs-derived intracranial xenografts to a higher extent than combination of THC:CBD (1:1 ratio) + TMZ. (A) Scheme of the in vivo experiment. (B) Effect of oral administration of THC:CBD (1:1 ratio) [THC (5 mg/kg) + CBD (5 mg/kg)], THC:CBD (1:5 ratio) [THC (5 mg/kg) + CBD (25 mg/kg)] and TMZ (5 mg/kg, I.P. administration) on the size of glioma xenografts (as determined by MRI) generated by intracranial injection of 7.5×10^4 12O12 GICs (n = 6–7). Left panel: Representative MRI images of each experimental condition after 4 weeks of treatment are shown. Right panel: Data correspond to the volume of the tumors at day 28 after the injection of the cells and the commencement of the treatments and are expressed as mean \pm SEM; (n = 5–7) $**P < 0.01$ or $***P < 0.001$ from vehicle-treated tumors; $$$$P < 0.001$ from THC:CBD (1:1 ratio)-treated tumors and $\&\&P < 0.01$ from THC:CBD (1:5)-treated tumors. (C) Effect of the different treatments on the survival of mice bearing 12O12 GICs derived intracranial xenografts. Data correspond to the percentage of alive animals along the experiment for each treatment and are depicted in Kaplan-Meier plot (n = 8–10). Symbols of significance are omitted for clarity. Survival of THC:CBD (1:5 ratio)-treated animals was significantly different ($P < 0.01$) from vehicle-treated animals; Survival of TMZ, THC:CBD (1:1) + TMZ- and THC:CBD (1:5 ratio) + TMZ-treated animals was significantly different ($P < 0.001$) from vehicle-treated animals. Survival of THC:CBD (1:1 ratio) + TMZ-treated animals was significantly different ($P < 0.001$) from THC:CBD (1:1 ratio)-treated animals. Survival of THC:CBD (1:5 ratio) + TMZ-treated animals was significantly different ($P < 0.01$) from TMZ-treated animals and from THC:CBD (1:5 ratio)-treated animals ($P < 0.001$). Survival of THC:CBD (1:5 ratio) + TMZ-treated animals was significantly different ($P < 0.05$) from THC:CBD (1:1 ratio) + TMZ-treated animals.

containing CBD may require the presence of THC (even if at sub-maximal concentrations) to produce an enhanced anticancer effect when administered in combination with TMZ. In support of this idea, the administration of extracts containing a higher proportion of CBD

than of THC enhanced TMZ anticancer activity at the same extent than THC:CBD at a 1:1 ratio. These observations suggest that therapies based on the use of cannabinoid-based medicines containing a higher proportion of CBD than of THC (and that therefore are expected to have a

lower psychoactive profile than those containing a higher proportion of THC [44,45]) in combination with chemotherapeutic agents (and specifically with TMZ), may also be of potential interest for the design of novel therapeutic strategies to treat gliomas.

The aggressiveness of GBM is, at least in part, due to the presence within the tumor mass of a small cell population of GICs that exhibits a high resistance to therapy and that has been proposed to be responsible for the relapses that take place in most if not all the patients suffering this devastating disease [46]. Since targeting this cell population might be particularly relevant in the context of novel anti-GBM therapies, in this work we also investigated the effect of THC:CBD combinations administered together with TMZ on GICs derived from human GBM tumors. Of note, previous work showed that synthetic agonists of cannabinoid receptors can promote the differentiation of GICs [35]. Likewise, CBD has been shown to inhibit the self-renewal of GICs and increase the survival of animals bearing intracranial xenografts derived from these cells [38,40]. Findings presented here now show that treatment with the combination of THC, CBD and TMZ leads to the activation of apoptosis, and in turn to a very significant reduction (or even to a complete elimination) of this cell population *in vitro*. In addition, and in contrast with the results obtained with differentiated glioma cells (Figs. 1 and 2), our *in vitro* and *in vivo* observations support the idea that the treatments with THC:CBD combinations containing a higher proportion of CBD targets more efficiently the population of GICs than THC:CBD at a 1:1 ratio.

Preclinical work performed during the past couple of decades has contributed to set the bases for the development of the first clinical studies to analyze the potential anticancer activity of cannabinoid-based medicines. Specifically, a clinical trial to investigate the effect of the combination of the cannabinoid-based medicine Sativex® and TMZ on recurrent GBM has been recently completed (NCT01812616) and it is expected that additional studies will follow in the near future. Results presented in this manuscript now support the notion that cannabinoid combinations containing a higher amount of CBD than of THC in combination with TMZ target more efficiently the GICs population (maintaining the same anticancer efficacy on differentiated glioma cells) than THC:CBD 1:1 combinations. These findings suggest that the utilization of cannabinoid-based medicines containing a higher amount of CBD than of THC in combination with TMZ may have an advantage over preparations containing the same amount of THC and CBD in the treatment of GBM. Whether this therapeutic approach could be beneficial at least for a fraction of GBM patients is an interesting possibility that might deserve to be therapeutically explored in future clinical studies.

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Conflict of interest

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