Molecular Cancer Therapeutics

A Combined Preclinical Therapy of Cannabinoids and Temozolomide against Glioma

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Abstract

Glioblastoma multiforme (GBM) is highly resistant to current anticancer treatments, which makes it crucial to find new therapeutic strategies aimed at improving the poor prognosis of patients suffering from this disease. Δ^9-Tetrahydrocannabinol (THC), the major active ingredient of marijuana, and other cannabinoid receptor agonists inhibit tumor growth in animal models of cancer, including glioma, an effect that relies, at least in part, on the stimulation of autophagy-mediated apoptosis in tumor cells. Here, we show that the combined administration of THC and temozolomide (TMZ; the benchmark agent for the management of GBM) exerts a strong antitumoral action in glioma xenografts, an effect that is also observed in tumors that are resistant to TMZ treatment. Combined administration of THC and TMZ enhanced autophagy, whereas pharmacologic or genetic inhibition of this process prevented TMZ + THC-induced cell death, supporting that activation of autophagy plays a crucial role on the mechanism of action of this drug combination. Administration of submaximal doses of THC and cannabidiol (CBD; another plant-derived cannabinoid that also induces glioma cell death through a mechanism of action different from that of THC) remarkably reduces the growth of glioma xenografts. Moreover, treatment with TMZ and submaximal doses of THC and CBD produced a strong antitumoral action in both TMZ-sensitive and TMZ-resistant tumors. Altogether, our findings support that the combined administration of TMZ and cannabinoids could be therapeutically exploited for the management of GBM. *Mol Cancer Ther; 10(1); 90–103. ©2011 AACR.*

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. As a consequence, median survival after diagnosis is usually just 12 to 15 months (1–3). This dramatic behavior is mainly due to the high invasiveness and proliferation rate of GBM. In addition, GBM exhibits a high resistance to standard chemotherapy and radiotherapy. Current strategies for the treatment of GBM are only palliative and include surgical resection (which is frequently incomplete because of the proximity of the tumor to vital brain structures) and focal radiotherapy (1–3). A large number of chemotherapeutic agents [e.g., alkylating agents such as temozolomide (TMZ; Supplementary Fig. 1) and nitrosureas such as carmustine] have also been tested, but they display limited efficacy (1, 2). It is therefore essential to develop new therapeutic strategies for the management of GBM. Nowadays, it is believed that the development of new combinational therapies, together with an increase in the selectivity of the treatments based on a detailed molecular characterization of these tumors (4, 5), may contribute to enhance the survival of patients suffering from GBM.

Δ^9-Tetrahydrocannabinol (THC; Supplementary Fig. 1), the main active component of the hemp plant *Cannabis sativa* (6), exerts a wide variety of biological effects by mimicking endogenous substances—the endocannabinoids—that bind to and activate specific cannabinoid receptors (7). So far, 2 G protein–coupled, cannabinoid-specific receptors have been cloned and characterized from mammalian tissues: CB_1, abundantly expressed in the brain and at many peripheral sites, and CB_2, expressed in the immune system and also present in some neuron subpopulations and glioma cells (7, 8). One of the most active areas of research in the cannabinoid field is the study of the potential application of cannabinoids in the treatment of different pathologies (9, 10). Among these

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-10-0688

therapeutic applications, cannabinoids are being investigated as antitumoral agents (11, 12). Thus, cannabinoid administration curbs the growth of several types of tumor xenografts in rats and mice (11, 12) including gliomas (13–16). On the basis of this preclinical evidence, a pilot clinical trial has been recently conducted to investigate the antitumoral action of THC on recurrent gliomas (17). The mechanism of THC antitumoral action relies on the activation of an endoplasmic reticulum stress-related signaling route that leads to the upregulation of the transcriptional coactivator p8 and its target the pseudokinase tribbles homologue 3 (TRB3; refs. 13, 18). The stimulation of this pathway promotes autophagy and is indispensable for the proapoptotic and antitumoral action of THC (15, 19).

Aside from THC, *C. sativa* produces approximately 70 other cannabinoids, although, unlike THC, many of them exhibit little affinity for CB receptors (10, 20). Of interest, at least one of these components, namely, cannabinol (CBD; Supplementary Fig. 1), has been shown to reduce the growth of different types of tumor xenografts including gliomas (20–25). Although the mechanism of CBD antitumoral action has not been completely clarified yet, it has been proposed that CBD-induced apoptosis relies on an increased production of reactive oxygen species (ROS; ref. 22), a mechanism that seems to operate also in glioma cells (23, 25). Of note, the combined administration of THC and CBD is being therapeutically explored (10, 20, 26), although its effects on the proliferation and survival of cancer cells have only been analyzed *in vitro* (26).

The present work was therefore undertaken to study the potential synergic antitumoral action of TMZ (the benchmark agent for the management of GBM) and THC and CBD. Our results support that administration of TMZ and these cannabinoids could enhance the efficacy of standard TMZ-based antitumoral therapies for gliomas.

Materials and Methods

Reagents

TMZ was kindly provided by Schering-Plough. THC, CBD, THC-botanical drug substance (THC-BDS; THC content 67.6% w/w; CBD content 0.3% w/w; other individual plant cannabinoids <1.5% w/w), and CBD-botanical drug substance (CBD-BDS; CBD content 65.4% w/w; THC content 2.5% w/w; other individual plant cannabinoids <1.7% w/w) were kindly provided by GW Pharmaceuticals. THC, THC-BDS, and CBD-BDS were obtained as a resin, solvated in ethanol at a concentration of 100 mg/mL, and stored at −20ºC. Finally, the required amounts of each component were dried, weighted, and solvated in dimethyl sulfoxide. A Sativex-like (SAT-L) preparation was prepared by mixing THC-BDS and CBD-BDS in a 1:1 (w/w) proportion. The cannabinoid antagonists SR141716 (SR1) and SR144528 (SR2) were kindly donated by Sanofi-Aventis. Double-stranded RNA duplexes corresponding to human Atg1 (smartpool) and control siRNA were synthesized by Dharmacon. The human glioma cell lines U87MG (U87), A172, SW1783, U373MG (U373), T98G (T98), SW1088, and LN405 were from ATCC and DSMZ or were kindly provided by Dr. Javier Castresana (Universidad Pública de Navarra). Immunofluorescence of neural markers, gene expression profile with DNA arrays, and CGH analyses were done after resuscitation in all the cell lines used in this study to corroborate that they had the features of human glioma cells. Primary cultures of brain tumor cells (designated as HG19, HG2, and HG14) were obtained from biopsies donated by the Tumor Bank Network, coordinated by the Spanish Cancer Research Centre and from the Hospital Clínico Universitario.

Cell culture and viability

Human glioma cell lines were cultured in DMEM containing 10% FBS. The preparation of adherent primary cultures of brain tumor cells was as follows: tumor samples were homogenized, digested with type Ia collagenase (Sigma) for 1 hour, and incubated on ice for 10 minutes. The supernatant was collected and, after centrifugation to discard the remaining death-floating cells, was resuspended in DMEM containing 15% FBS. Finally, cells were seeded at a density of 400,000 cells/cm^2 and kept in culture for 2 weeks in DMEM containing 15% FBS and 1% glutamine. Cells were transferred to a serum-free medium (human glioma cell lines) or a 0.5% FBS medium (primary cultures of glioma cells) before doing the different treatments. When indicated, cells were preincubated with SR1, SR2, ISP-1, 3-MA, or QVDOPH for 1 hour before treatment with the antitumoral agents. Cell viability was determined after 72 hours of drug treatment by using the MTT test (Promega) according to manufacturer's instructions. Stock solutions of cannabinoid agonists and antagonists were prepared in dimethyl sulfoxide. Control incubations contained the same amount of dimethyl sulfoxide, and no significant effect was observed in any of the parameters determined throughout this study at the final concentration used (0.1%–0.2%, v/v).

Cell transfections

Seventy-five percent confluent U87 cells were transfected with control (siC) or human Atg1 (siAtg1) siRNAs, using the X-tremeGENE siRNA Transfection Reagent (Roche) according to manufacturer's instructions. Twenty-four hours after transfection, cells were trypsinized and seeded in complete medium at a density of 5,000 cells/cm^2. After 8 hours, cells were transferred to a serum-free medium for 18 hours and the different treatments were done. Transfection efficacy was monitored using a control fluorescent siRNA (Qiagen) and was greater than 80% in all the experiments.

Reverse transcription and real-time quantitative PCR analysis

RNA was isolated using the RNeasy Protect kit (Qiagen), including a DNase digestion step using the

RNase-free DNase kit (Qiagen). cDNA was subsequently obtained using Transcriptor Reverse Transcriptase (Roche). Real-time quantitative PCR assays were performed using the FastStart Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library Set (Roche). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems). The following sense/antisense primers and probes were used for detecting human Atg1 (5′-TCATCTTCAGCCACGCTGT-3′ and 5′;-CACGGTGCTGGAACATCTC-3′, probe 37), and multispecies 18S RNA (GCTCTAGAATTACCACAGTTATCCAA and AAATCAGTTATGGTTCCTTTGGTC, probe 55). Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA levels as reference.

Tumor xenografts

Tumors were induced in nude mice by subcutaneous injection of 5×10^6 U87 cells or 10×10^6 T98 cells in PBS supplemented with 0.1% glucose. When tumors had reached an average size of 250 mm^3 (an average of 2 weeks for U87 tumors and 14 weeks for T98 tumors), animals were assigned randomly to various groups and injected peritumorally for 14 days with the different treatments or vehicle in 100 μL of PBS supplemented with 5 mg/mL defatted and dialyzed BSA [for combinations of TMZ + THC, THC + CBD, SAT-L (THC-BDS + CBD-BDS), TMZ + THC + CBD, or TMZ + SAT-L, the required amounts of each agent or BDS were placed in the same tube, solvated in PBS supplemented with 5 mg/mL defatted and dialyzed BSA, and administered in a single injection]. Tumors were measured with external caliper, and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$. At the end of the treatment, animals were sacrificed, tumors were excised, and their weights were determined.

Western blot analysis

Western blot analysis was done following standard methods. The antibodies used were anti–α-tubulin (1:4,000; Sigma) and anti–LC3 (1:1,000; polyclonal, MBL, ref PM036, Naka-Ku Nagoya).

Imunofluorescence of cell cultures

Cell cultures grown on 12-mm coverslips were washed in PBS, fixed with 4% paraformaldehyde (10 minutes at room temperature), permeabilized with 0.5% Triton X100 (5 minutes at room temperature), blocked to avoid nonspecific binding with 10% goat antiserum, and incubated (1 hour at room temperature) with rabbit polyclonal anti-cleaved caspase-3 Asp175 antibody (1:100; Cell Signaling Technology) and mouse monoclonal anti–LC3 antibody (1:100; Nanotools Antikörpertechnik GmbH & Co Antikörpertechnik GmbH & Co; clone 5F10) as primary antibodies. Incubation with Alexa-488–conjugated secondary antibody (Invitrogen) was done in the dark at room temperature for 1 hour. Cell nuclei were stained with Hoechst 33342 (Invitrogen). Finally, coverslips were mounted in ProLong Gold antifade reagent (Invitrogen) and visualized in a Leica TCS SP2 confocal microscope.

Immunofluorescence of tumor samples

Samples from tumors xenografts were dissected and frozen. Sections (8 μm) were permeabilized and blocked to avoid nonspecific binding with 10% goat antiserum and 0.25% Triton X-100 in PBS for 45 minutes, and subsequently incubated with mouse monoclonal anti-LC3 antibody (1:100; Nanotools Antikörpertechnik GmbH & Co; 4ºC, overnight), washed, and further incubated with the corresponding Alexa-488–conjugated secondary antibodies (Invitrogen; 90 minutes, room temperature). Nuclei were stained with Hoechst 33342 (Invitrogen; 10 minutes, room temperature) before montage with Mowiol (Merck) was done. Fluorescence images were acquired using an Axiovert 135 microscope (Carl Zeiss).

TUNEL

Tumor samples were fixed, blocked, permeabilized, and TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) was done as described (13).

Statistical analysis

Unless otherwise specified, results shown represent mean ± SD. Statistical analysis was done by ANOVA with a *post hoc* analysis by the Student–Neuman–Keuls test. Where indicated, data were analyzed using the Calcusyn software (BIOSOFT) to test for drug synergism. Combination index (CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively) was determined as described elsewhere (27, 28).

Results

Combined treatment with THC and TMZ strongly reduces the growth of glioma xenografts

To analyze the combined antitumoral action of TMZ and THC in gliomas, we first characterized the ability of the 2 individual agents to promote glioma cell death. In agreement with the results obtained by other groups (29, 30), we observed that TMZ treatment produced a dose-dependent reduction in cell viability that reached a value of 40% to 50% of viable cells even when high concentrations of this agent (up to 400 μmol/L) were used (Supplementary Fig. 2A). Likewise, THC reduced in a dose-dependent manner the viability of glioma cells (Supplementary Fig. 2B). We therefore selected submaximal doses of TMZ and THC to evaluate whether the combined administration of the 2 agents enhanced their ability to induce glioma cell death. In line with this possibility, combined treatment with low doses of THC and TMZ reduced in a synergic fashion the viability of several human glioma cell lines and of 2 primary cultures of glioma cells derived from human GBM biopsies (Fig. 1A and Supplementary Fig. 2B). Use of higher

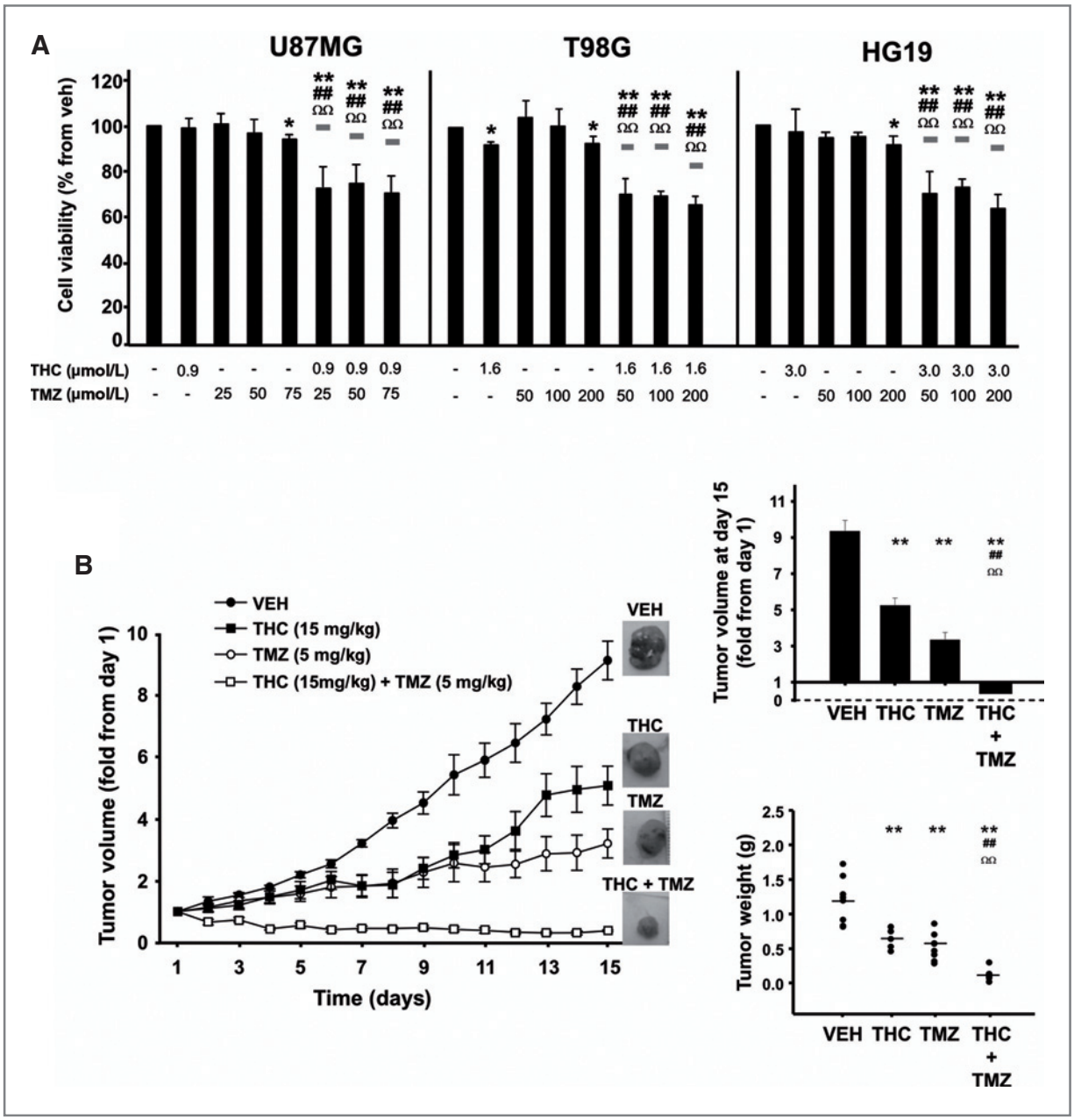


Figure 1. THC and TMZ strongly reduce the growth of glioma xenografts. A, effect of THC, TMZ, and THC + TMZ on the viability (72 hours) of U87MG, T98G (human glioma cell lines), and HG19 (a primary culture of human glioma cells) cells as determined by the MTT test (mean ± SD; $n = 6$ for U87MG and T98G and $n = 4$ for HG19 cells; **, $P < 0.01$ from vehicle-treated cells; ##, $P < 0.01$ from THC-treated cells; ΩΩ, $P < 0.01$ from TMZ-treated cells). Gray lines correspond to the reduction of cell viability predicted from the addition of the individual cell death–promoting actions of THC and TMZ at each concentration of these agents. B, left, effect of THC, TMZ, or THC + TMZ on the growth of U87MG cell–derived tumor xenografts [$n = 6$–8 for each condition; mean ± SEM; symbols of significance are omitted for clarity; THC + TMZ-treated tumors were significantly different from vehicle-, THC-, and TMZ-treated tumors from day 2 until the end of the treatment ($P < 0.01$); THC- and TMZ-treated tumors were significantly different from vehicle-treated tumors on days 5 and 6 ($P < 0.05$) and from day 7 until the end of the treatment ($P < 0.01$)]. Top right, data correspond to the mean fold-increase in tumor growth ± SEM at the last day of the treatment. Lower right, data represent tumor weight on the last day of the treatment ($n = 6$–8 for each condition; **, $P < 0.01$ from vehicle-treated tumors; ##, $P < 0.01$ from THC-treated tumors; and ΩΩ, $P < 0.01$ from TMZ-treated tumors).

doses of THC or TMZ also enhanced glioma cell death, although at these concentrations, the synergistic action of the 2 agents was not evident (Supplementary Fig. 2C and Supplementary Table 1),

To evaluate the *in vivo* relevance of these observations, we generated tumor xenografts by subcutaneous injection of U87MG cells in immunodeficient mice. Once the tumors had reached an average size of 250 mm^3, THC

(15 mg/kg/d), TMZ (5 mg/kg/d), or THC (15 mg/kg/d) + TMZ (5 mg/kg/d) were administered to the tumor-bearing mice. Strikingly, treatment with THC and TMZ decreased tumor growth to a much higher extent than the treatment with the individual agents (Fig. 1B).

Combined Treatment with THC and TMZ Enhances Autophagy-Mediated Cell Death

We have recently found that the mechanism of THC-induced cancer cell death relies on the stimulation of autophagy and the subsequent activation of apoptosis (15). In addition, TMZ has been shown to activate autophagy in glioma cells (29, 31). We therefore asked whether the combined treatment with these 2 agents might promote cancer cell death via enhanced stimulation of autophagy.

Upon autophagy induction, LC3 becomes conjugated to phosphatidylethanolamine, which targets this protein to the membrane of the autophagosome. The lipidated autophagosome-associated form of LC3 (LC3-II) can be monitored by immunofluorescence (autophagic cells exhibit a characteristic pattern of LC3 puncta) or Western blot (LC3-II has higher electrophoresis mobility than non-lipidated LC3; refs. 32, 33). We observed that THC but not TMZ enhanced autophagy of U87MG cells (Supplementary Fig. 3A). Of importance, when submaximal doses of THC and TMZ were coadministered to glioma cells, autophagy (Fig. 2A) and apoptosis (Fig. 2B) were strongly enhanced. Furthermore, pharmacologic inhibition of autophagy [by using the class III phosphatidylinositol 3 kinase inhibitor 3-methyl adenine (3-MA)] and apoptosis (by using the pan-caspase inhibitor QVDOPH) or genetic inhibition of autophagy (by silencing the expression of the essential autophagy gene *Atg1*; ref. 33), prevented THC + TMZ-induced cell death (Fig. 2C and Supplementary Fig. 3B and C). Moreover, immunofluorescence analysis of samples obtained from tumor xenografts that had been treated with THC, TMZ, or the combination of these 2 agents revealed that administration of THC + TMZ strongly enhanced autophagy (as determined by LC3 immunostaining) and apoptosis (as determined by TUNEL) in these tumors (Fig. 2D). Taken together, these observations support that the combined antitumoral action of THC and TMZ relies on an enhanced stimulation of autophagy and apoptosis.

Combined treatment with THC and CBD greatly enhances glioma cell death

Several studies have shown that CBD can reduce the growth of glioma xenografts (23, 34). We therefore investigated whether the combined utilization of THC and CBD could be effective in reducing the viability of glioma cells.

Treatment with submaximal doses of THC and CBD greatly reduced the viability of several human glioma cell lines as well as of primary cultures of human glioma cells (Fig. 3A and Supplementary Fig. 4). Moreover, combined administration of submaximal doses of THC (7.5 mg/kg/d) and CBD (7.5 mg/kg/d) reduced the growth of U87MG cell–derived subcutaneous xenografts at a higher extent than the treatment with the individual agents and at the same extent than an effective dose of THC (15 mg/kg/d; Fig. 3B and Supplementary Fig. 5), supporting that the combined use of submaximal doses of THC and CBD could be equally effective compared with THC in reducing the growth of glioma xenografts.

The cannabinoid-based medicine Sativex is BDS presented as oromucosal spray that results from the combination of THC-BDS and CBD-BDS at a 1:1 (w/w) ratio and to which excipients are subsequently added. It therefore contains an approximate proportion (1:1:1 w/w/w) of THC, CBD, and a (well characterized) fraction of additional plant constituents (10). To investigate the potential antitumoral activity of Sativex, we prepared a SAT-L medicine by mixing THC-BDS and CBD-BDS in a 1.1 (w/w) ratio, which is therefore only devoid of the excipients present in Sativex. Of note, the IC_{50} of SAT-L preparation on the viability of U87MG or T98G cells was that expected for the amounts of THC and CBD present in this extract (Supplementary Fig. 6), indicating that the minor constituents present in SAT-L mixture do not significantly modify the cell death–promoting activity of THC and CBD in glioma cells. Furthermore, treatment with SAT-L reduced the growth of U87MG tumor xenografts to the same extent as an identical dose of THC (Supplementary Fig. 7A).

THC but not CBD has been shown to engage cannabinoid receptors to promote tumor cell death (16, 23, 34). Thus, THC triggers the accumulation of *de novo*–synthesized ceramide, which leads in turn to stimulation of autophagy and apoptosis (15). The mechanism of CBD antitumoral action seems to rely on an increased production of reactive oxygen species (ROS), which leads to apoptosis (25). In line with these observations, blockade of CB_1 or CB_2 receptors [using the CB_1 and CB_2 receptor antagonists SR141716 (SR1) and SR144528 (SR2), respectively; Supplementary Fig. 8A)], inhibition of ceramide biosynthesis (by using ISP-1, an inhibitor of serine palmitoyltransferase, the enzyme that catalyzes the first step of sphingolipid biosynthesis; Supplementary Fig. 8B) or pharmacologic or genetic inhibition of autophagy (Supplementary Fig. 8B and C) abrogated THC- but not CBD-induced glioma cell death. In addition, incubation with the antioxidants *N*-acetyl cysteine and α-tocopherol abrogated CBD killing action but only partially prevented THC-induced cell death (Supplementary Fig. 8D). Furthermore, pharmacologic blockade of apoptosis prevented both THC- and CBD-induced cell deaths (Supplementary Fig. 8B). These observations further support that THC and CBD activate different intracellular mechanisms to promote the apoptotic death of glioma cells.

Next, we asked about the cellular processes responsible for THC + CBD action in glioma cells. Coadministration of submaximal doses of THC and CBD stimulated autophagy (Fig. 4A) and apoptosis (Fig. 4B) in these cells. Moreover, blockade of CB receptors (Supplementary

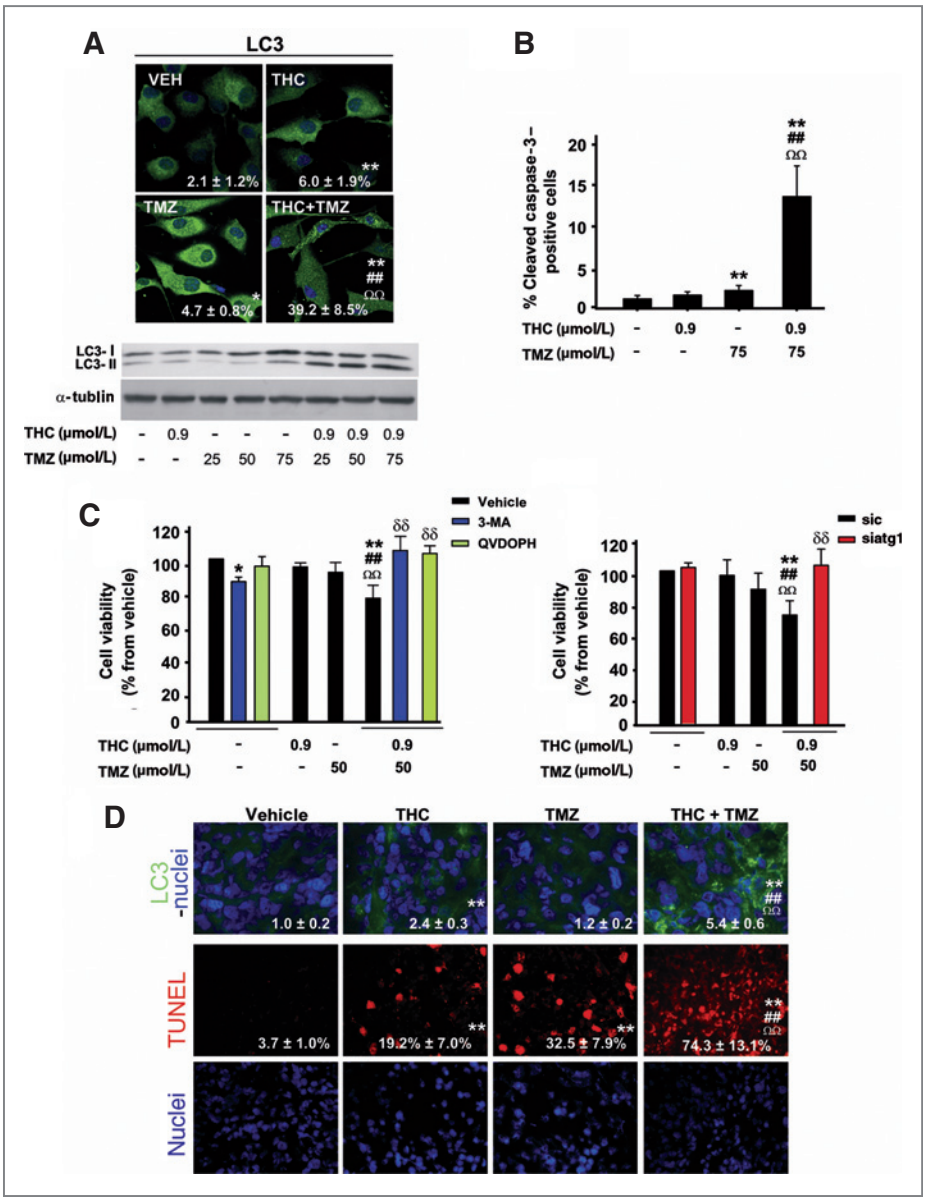


Figure 2. The combined administration of THC and TMZ enhances autophagy and apoptosis in human glioma cells. A, top, effect of THC (0.9 μmol/L), TMZ (75 μmol/L), and THC + TMZ (0.9 μmol/L + 75 μmol/L; 24 hours) on LC3 immunostaining of U87MG cells. Values in the bottom right corner of each photomicrograph correspond to the percentage of cells with LC3 dots relative to the total number of cells (mean ± SD; $n = 3$; representative photomicrographs of each condition are shown; **, $P < 0.01$ or * P, < 0.05 from vehicle-treated cells; ##, $P < 0.01$ from THC-treated cells; and $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated cells). Bottom, effect of THC, TMZ, and THC + TMZ on LC3 lipidation (24 hours). A representative experiment of 6 is shown. B, effect of THC, TMZ, and THC + TMZ on apoptosis (24 hours; as determined by active caspase-3 immunostaining) of U87MG cells. Data correspond to the percentage of active caspase-3–positive cells relative to the total number of cells (mean ± SD; $n = 3$; **, $P < 0.01$ from vehicle-treated cells; ##, $P < 0.01$ from THC-treated cells; and $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated cells). C, left, effect of 3-MA (5 mmol/L) and QVDPOH (15 μmol/L) on the viability (72 hours) of U87MG cells treated with THC, TMZ, or THC + TMZ ($n = 6$; mean ± SD, **, $P < 0.01$ from vehicle-treated cells; ##, $P < 0.01$ from THC-treated cells; $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated cells; and $^{\delta\delta}$, $P < 0.01$ from THC + TMZ-treated cells). Additional controls are omitted for clarity [no significant differences in viability relative to THC (0.9 μmol/L) were found in cells treated with THC + 3-MA or THC + QVDOPH or relative to TMZ (50 μmol/L) in cells treated with TMZ + 3-MA and TMZ + QVDOPH]. Right, effect of THC, TMZ, or THC + TMZ on the viability (72 hours) of U87MG cells transfected with control (siC) or Atg1-selective (siAtg1) siRNA ($n = 6$; mean ± SD, **, $P < 0.01$ from siC-transfected, vehicle-treated cells; ##, $P < 0.01$ from siC-transfected, THC-treated cells; $^{\Omega\Omega}$, $P < 0.01$ from siC-transfected, TMZ-treated cells; and $^{\delta\delta}$, $P < 0.01$ from siC-transfected, THC + TMZ-treated cells). Atg1 mRNA levels (as determined by real-time quantitative PCR) were reduced in siAtg1-transfected cells relative to their corresponding siC-transfected cells by 61% ($n = 5$). Additional controls are omitted for clarity [no significant differences in viability relative to siC-transfected, THC-treated cells were found in cells transfected with siAtg1 and treated with THC or relative to siC-transfected, TMZ-treated cells in cells transfected with siAtg1 and treated with TMZ]. D, effect of THC, TMZ, or THC + TMZ on LC3 immunostaining and TUNEL of U87MG cell–derived tumor xenografts. Values correspond to the LC3-stained area normalized to the total number of nuclei in each section (mean fold change ± SD) or to the percentage of TUNEL-positive cells relative to the total number of nuclei in each section ± SD (10 sections of 3 different tumors from each condition were analyzed; **, $P <$ 0.01 from vehicle-treated tumors; ##, $P < 0.01$ from THC-treated tumors; and $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated tumors).

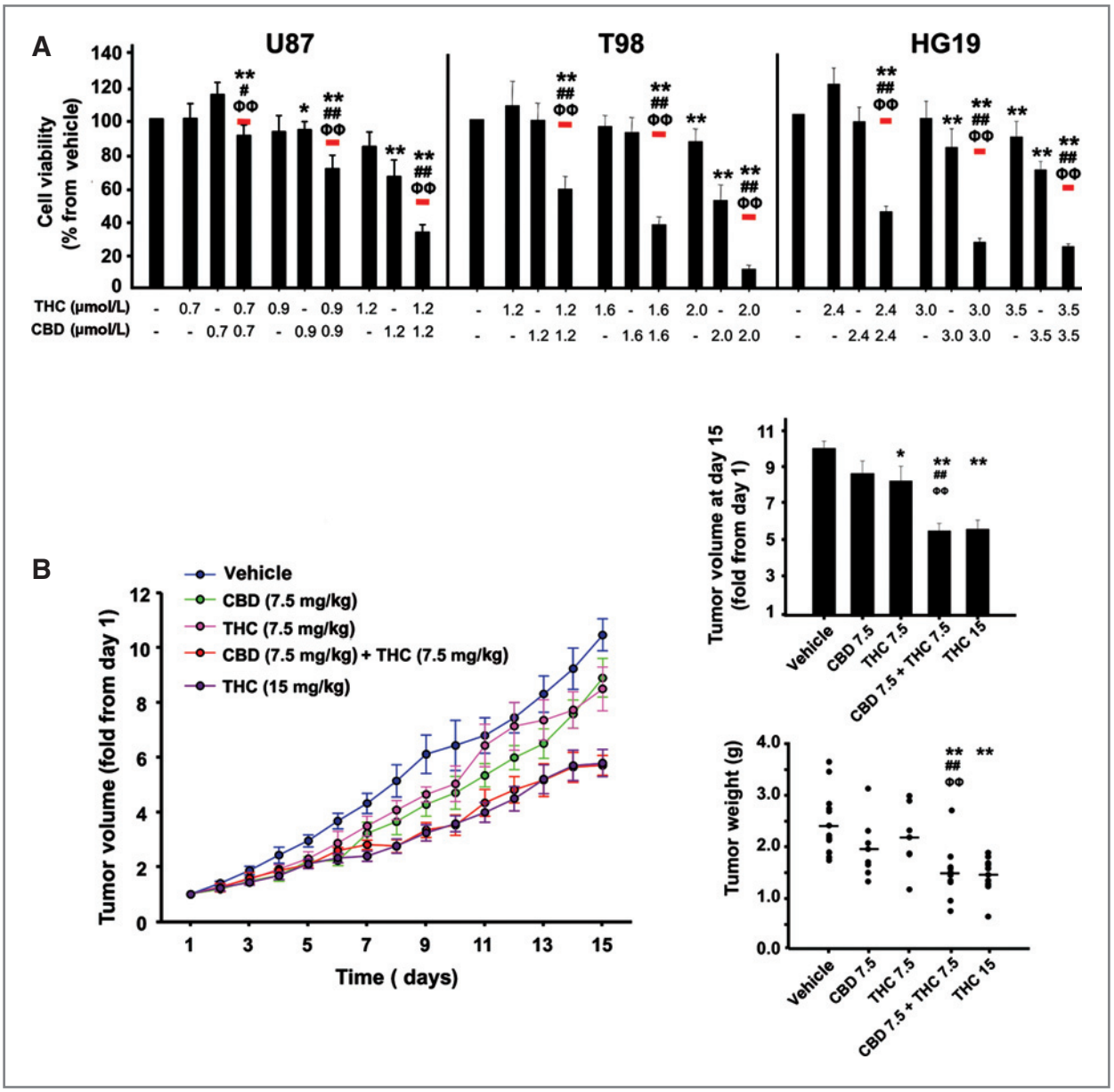
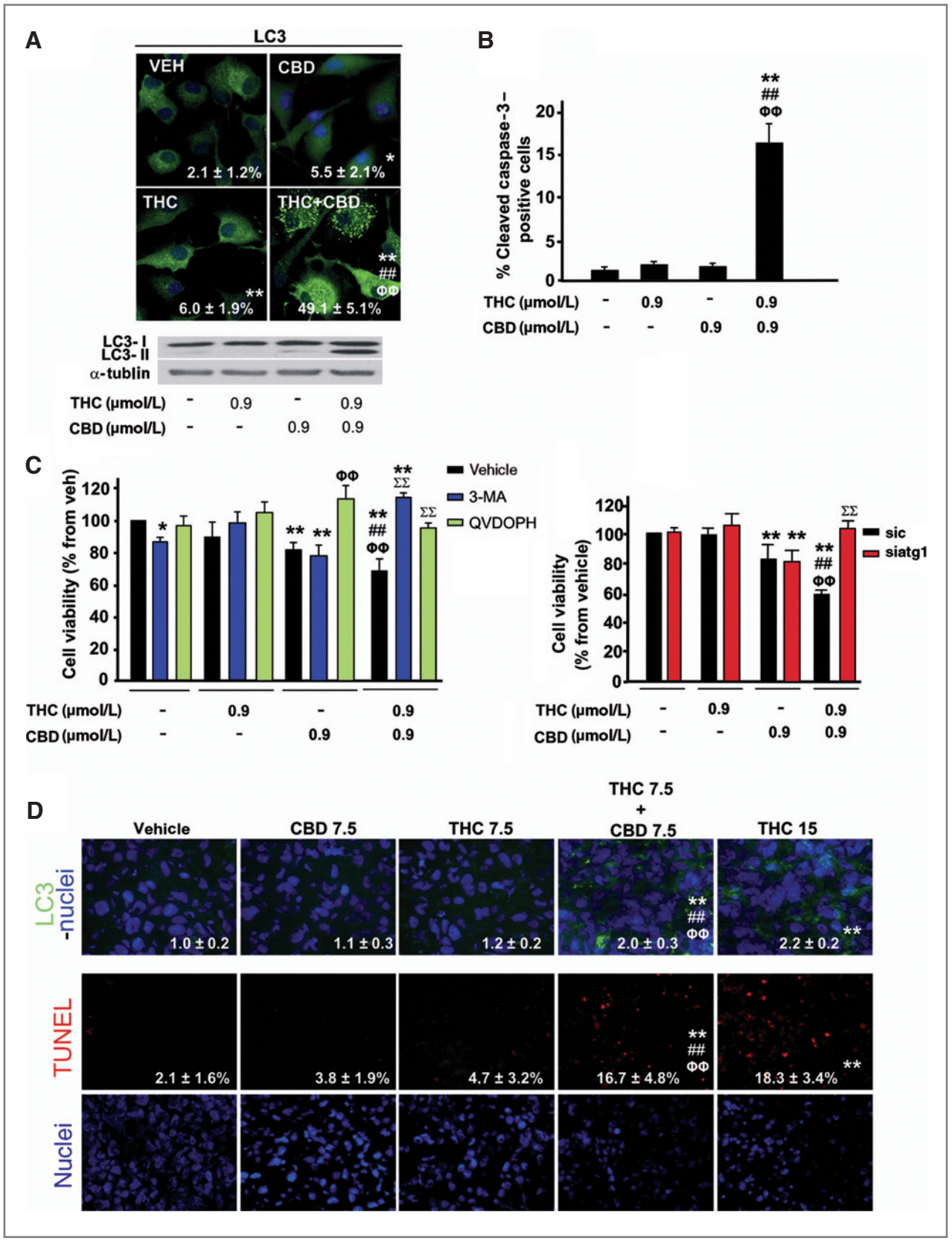


Figure 3. Combined administration of submaximal doses of THC and CBD reduces the growth of U87MG cell–derived tumor xenografts. A, effect of THC, CBD, and THC + CBD on the viability (72 hours) of U87MG, T98G (human glioma cell lines), and HG19 (a primary culture of human glioma cells) cells as determined by the MTT test (mean ± SD; $n = 12$ for U87MG and T98G and $n = 4$ for HG19 cells; **, $P < 0.01$ from vehicle-treated cells; $^{\#\#}$, $P < 0.01$ from THC-treated cells; $^{\Phi\Phi}$, $P < 0.01$ from CBD-treated cells). Red lines correspond to the reduction of cell viability obtained from the addition of the individual cell death–promoting actions of THC and CBD at each concentration of these agents. B, left, effect of THC, CBD, or THC+CBD on the growth of U87MG cell–derived tumor xenografts [$n = 6$–8 for each condition; mean ± SEM; symbols of significance are omitted for clarity; THC (7.5 mg/kg) + CBD (7.5 mg/kg)-treated tumors were significantly different from vehicle-treated tumors on days 6 and 7 ($P < 0.05$), and from day 8 until the end of the treatment ($P < 0.01$); and from THC (7.5 mg/kg)- and CBD (7.5 mg/kg)-treated tumors from day 8 until the end of the treatment ($P < 0.01$). THC (15 mg/kg)-treated tumors were significantly different from vehicle-treated tumors on day 6 ($P < 0.05$) and from day 7 until the end of the treatment ($P < 0.01$). THC (7.5 mg/kg)-treated tumors were significantly different from vehicle-treated tumors on days 14 and 15 ($P < 0.05$)]. Top right, data correspond to the mean fold-increase in tumor growth ± SEM on the last day of the treatment. Bottom right, data represent tumor weight on the last day of the treatment ($n = 6$–8 for each condition; **, $P < 0.01$ from vehicle-treated tumors; $^{\#\#}$, $P < 0.01$ from THC -treated tumors; and $^{\Phi\Phi}$, $P < 0.01$ from CBD-treated tumors).

Fig. 9A), inhibition of ceramide biosynthesis (Supplementary Fig. 9B), pharmacologic or genetic inhibition of autophagy (Fig. 4C and Supplementary Fig. 9B and C), or inhibition of apoptosis (Fig. 4D and Supplementary Fig. 9B) prevented THC + CBD-induced cell death. Furthermore, *in vivo* administration of THC + CBD (Fig. 4D) or of the SAT-L preparation (Supplementary Fig. 7B) enhanced autophagy and apoptosis of U87MG



tumor xenografts, supporting that the combined treatment of THC + CBD activates a similar mechanism as that of THC to promote glioma cell death.

Combined treatment with THC + CBD and TMZ strongly reduces the growth of glioma xenografts

In view of the aforementioned results, we investigated the ability of the combined administration of TMZ and THC + CBD to stimulate glioma cell death. Treatment with submaximal doses of THC, CBD, and TMZ strongly reduced the viability of U87MG and T98G glioma cells (Supplementary Fig. 10A). Moreover, treatment with TMZ and THC + CBD enhanced both autophagy and apoptosis of glioma cells (Supplementary Fig. 10B and C), and pharmacologic inhibition of autophagy and apoptosis prevented TMZ + THC + CBD-induced cell death (Supplementary Fig. 10D).

To validate the potential therapeutic relevance of these observations, we treated U87MG cell–derived tumor xenografts with TMZ in combination with THC, THC + CBD, or the SAT-L mixture. Of importance, the combined administration of TMZ with THC (15 mg/kg/d), a submaximal dose of THC + CBD, or the SAT-L preparation was equally efficient in reducing tumor growth (Fig. 5A). Furthermore, autophagy and apoptosis were strongly enhanced in the tumors that had been treated with TMZ in combination with a submaximal dose of THC + CBD or with the SAT-L mixture (Fig. 5B). These observations support that the combination of TMZ with THC + CBD (even at concentrations at which the latter drug combination does not reduce tumor growth by itself) exhibits a strong antitumoral action.

Combined treatment with TMZ and cannabinoids overcomes the resistance of glioma xenografts to TMZ antitumoral action

Resistance to TMZ antitumoral action frequently contributes to the poor life prognosis of patients with GBM. Increased expression of the enzyme O^6-methylguanine methyltransferase (MGMT) has been widely associated with the resistance to TMZ (35). In line with previous studies (36), we observed that T98G cells exhibited much higher MGMT mRNA levels than U87MG cells, which correlated with a higher resistance of T98G cells to TMZ-induced cell death (Fig. 1 and Supplementary Fig. 11A and B). To evaluate the efficacy of the combined administration of TMZ and cannabinoids in tumors that were potentially resistant to the antineoplasic action of these agents, we generated xenografts with T98G cells. In agreement with the aforementioned *in vitro* data, T98G tumors had higher MGMT mRNA levels than tumors derived from U87MG cells (Supplementary Fig. 11C). Moreover, unlike their U87MG counterparts, T98G tumors were resistant to TMZ treatment (Fig. 6A). In addition, and in line with our recent results (Lorente et al., unpublished observations), T98G tumors were also resistant to THC action (Fig. 6A). Of importance, treatment with TMZ + THC or TMZ + SAT-L preparation strongly reduced the growth of T98G tumors (Fig. 6A), supporting that the combined administration of TMZ and cannabinoids overcomes the resistance of gliomas to the antitumoral action of these agents.

It is worth noting that levels of MGMT were not modified upon administration of THC, the SAT-L mixture, or the combination of these agents with TMZ (Supplementary Fig. 11B and C), indicating that cannabinoids do not influence the expression of MGMT, and that additional mechanisms are responsible for the combined action exerted by these agents and TMZ in T98G tumors. Immunofluorescence analyses revealed that similar to the results obtained in U87MG cell xenografts, autophagy and apoptosis were strongly increased in THC + TMZ and SAT-L + TMZ-treated tumors (Fig. 6B). Taken together, these observations support that the treatment with cannabinoids and TMZ activates an autophagy-mediated cell death mechanism that contributes to the

Figure 4. Combined administration of THC and CBD enhances autophagy and apoptosis in human glioma cells. A, top, effect of THC (0.9 μmol/L), CBD (0.9 μmol/L), and THC + CBD (0.9 + 0.9 μmol/L) on LC3 immunostaining (24 hours) of U87MG cells. Values in the bottom right corner of each photomicrograph correspond to the percentage of cells with LC3 dots relative to the total number of cells (mean ± SD; $n = 3$; representative photomicrographs of each condition are shown; **, $P < 0.01$ or *, $P < 0.05$ from vehicle-treated cells; ##, $P < 0.01$ from THC-treated cells; and $^{\Phi\Phi}$, $P < 0.01$ from CBD-treated cells). Bottom, effect of THC, CBD, and THC + CBD on LC3 lipidation (24 hours). A representative experiment of 6 is shown. B, effect of THC and CBD (24 hours) on apoptosis (as determined by active caspase-3 immunostaining) of U87MG cells. Data correspond to the percentage of active caspase-3–positive cells relative to the total number of cells (mean ± SD; $n = 3$; **, $P < 0.01$ from vehicle-treated cells; ##, $P < 0.01$ from THC-treated cells; and $^{\Phi\Phi}$, $P < 0.01$ from CBD-treated cells). C, left, effect of 3-MA (5 mmol/L) and QVDPOH (15 μmol/L) on the viability (72 hours) of U87MG cells treated with THC, CBD, or THC + CBD ($n = 4$; mean ± SD, **, $P < 0.01$ from vehicle-treated cells; ##, $P < 0.01$ from THC-treated cells; $^{\Phi\Phi}$, $P < 0.01$ from CBD-treated cells; and $^{\Sigma\Sigma}$, $P < 0.01$ from THC + CBD-treated cells). Right, effect of THC, CBD, or THC+CBD on the viability (72 hours) of U87MG cells transfected with control (siC) or Atg1-selective (siAtg1) siRNA ($n = 6$; mean ± SD, **, $P < 0.01$ from siC-transfected, vehicle-treated cells; ##, $P < 0.01$ from siC-transfected, THC-treated cells; $^{\Phi\Phi}$, $P < 0.01$ from siC-transfected, CBD-treated cells; and $^{\Sigma\Sigma}$, $P < 0.01$ from siC-transfected THC + CBD-treated cells). Atg1 mRNA levels (as determined by real-time quantitative PCR) were reduced in siAtg1-transfected cells relative to their corresponding siC-transfected cells by 72% ($n = 5$). E, effect of THC (7.5 mg/kg), CBD (7.5 mg/kg), THC (15 mg/kg), or THC (7.5 mg/kg) + CBD (7.5 mg/kg) on LC3 immunostaining and TUNEL of U87MG cell–derived tumor xenografts. Values correspond to the LC3-stained area normalized to the total number of nuclei in each section (mean fold change ± SD; arrows point cells with LC3 dots) or to the percentage of TUNEL-positive cells relative to the total number of nuclei in each section ± SD [10 sections of 3 different tumors from each condition were analyzed; **, $P < 0.01$ from vehicle-treated tumors; ##, $P < 0.01$ from THC (7.5 mg/kg)-treated tumors; and $^{\Phi\Phi}$, $P < 0.01$ from CBD (7.5 mg/kg)-treated tumors].

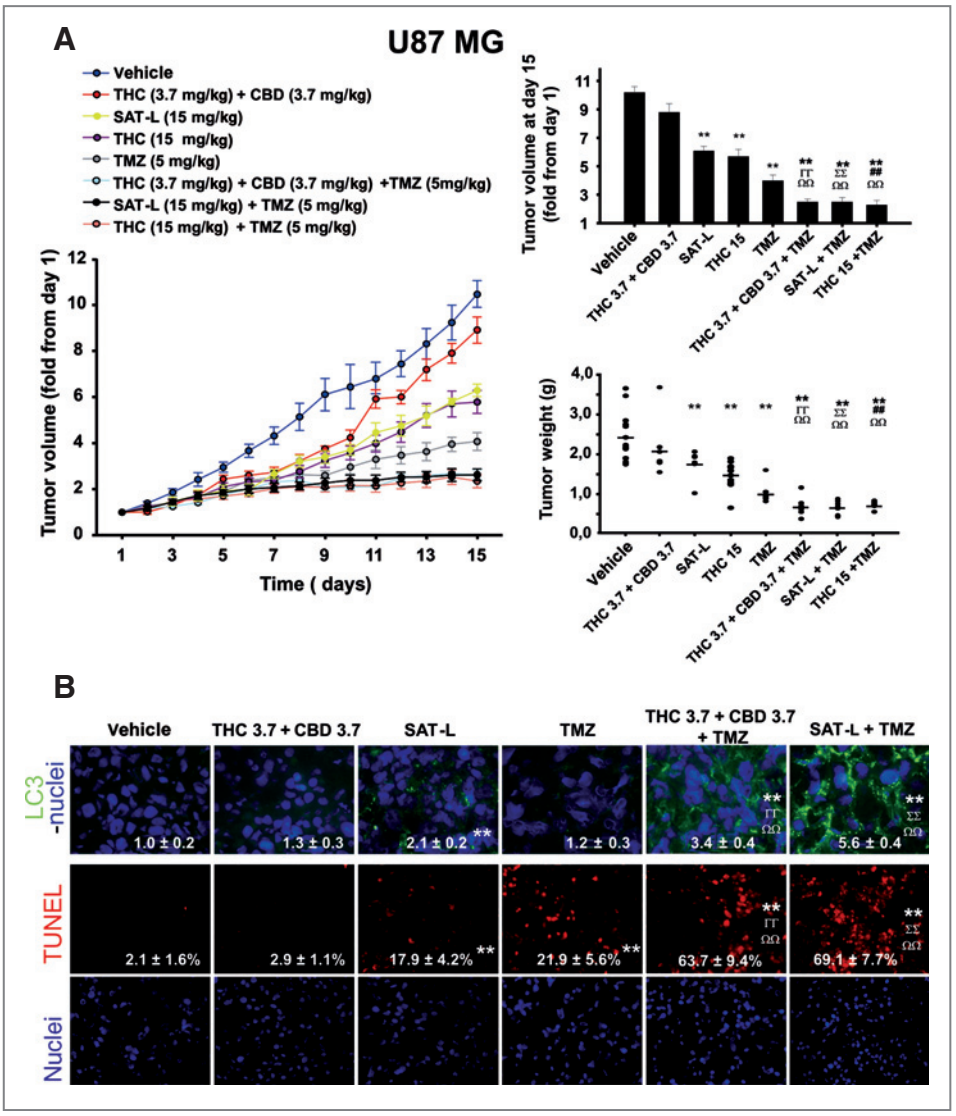


Figure 5. Combined administration of THC, CBD, and TMZ strongly reduces the growth of U87MG cell–derived tumor xenografts. A, effect of THC (15 mg/kg), TMZ (5 mg/kg), THC (3.7 mg/kg) + CBD (3.7 mg/kg), SAT-L [THC-BDS (7.5 mg/kg) + CBD-BDS (7.5 mg/kg)], THC (15 mg/kg) + TMZ (5 mg/kg), THC (3.7 mg/kg) + CBD (3.7 mg/kg) + TMZ (5 mg/kg), or SAT-L + TMZ (5 mg/kg) on the growth of U87MG cell–derived tumor xenografts ($n = 6$–8 for each condition; mean ± S.E.M; symbols of significance are omitted for clarity; see the Supplementary Information section for a description of the statistical differences for each treatment). Top right, data correspond to the mean fold-increase in tumor growth ± SEM on the last day of the treatment. Bottom right, data represent tumor weight at the last day of the treatment **, [$P < 0.01$ from vehicle-treated tumors; $^{\#\#}$, $P < 0.01$ from THC (15 mg/kg)-treated tumors; $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated tumors; $^{\Gamma\Gamma}$, $P < 0.01$ from THC (3.7 mg/kg) + CBD (3.7 mg/kg)-treated tumors; $^{\Sigma\Sigma}$, $P < 0.01$ from SAT-L–treated tumors]. B, effect of the different treatments on LC3 immunostaining and TUNEL of U87MG cell–derived tumor xenografts. Values correspond to the LC3-stained area normalized to the total number of nuclei in each section (mean fold change ± SD; arrows point cells with LC3 dots) or to the percentage of TUNEL-positive cells relative to the total number of nuclei in each section ± SD [10 sections of 3 different tumors from each condition were analyzed; **, $P < 0.01$ from vehicle-treated tumors; $^{\#\#}$, $P < 0.01$ from THC (15 mg/kg)-treated tumors; $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated tumors; $^{\Gamma\Gamma}$, $P < 0.01$ from THC + CBD-treated tumors; and $^{\Sigma\Sigma}$, $P < 0.01$ from SAT-L–treated tumors].

strong antitumoral action exerted by the combination of TMZ and cannabinoids.

Discussion

The standard therapy for the management GBM includes surgical resection, focal radiotherapy, and treatment with the alkylating agent temozolomide (1, 2). Unfortunately this therapeutic approach increases only modestly the survival of GBM patients, whose life expectancy after diagnosis remains approximately to 12 to 15 months. It is therefore urgent to search for novel therapeutic approaches aimed at improving the poor prognosis of GBM patients. Results obtained by our group and others during the last decade have shown that THC, the main active component of marijuana, reduces the growth

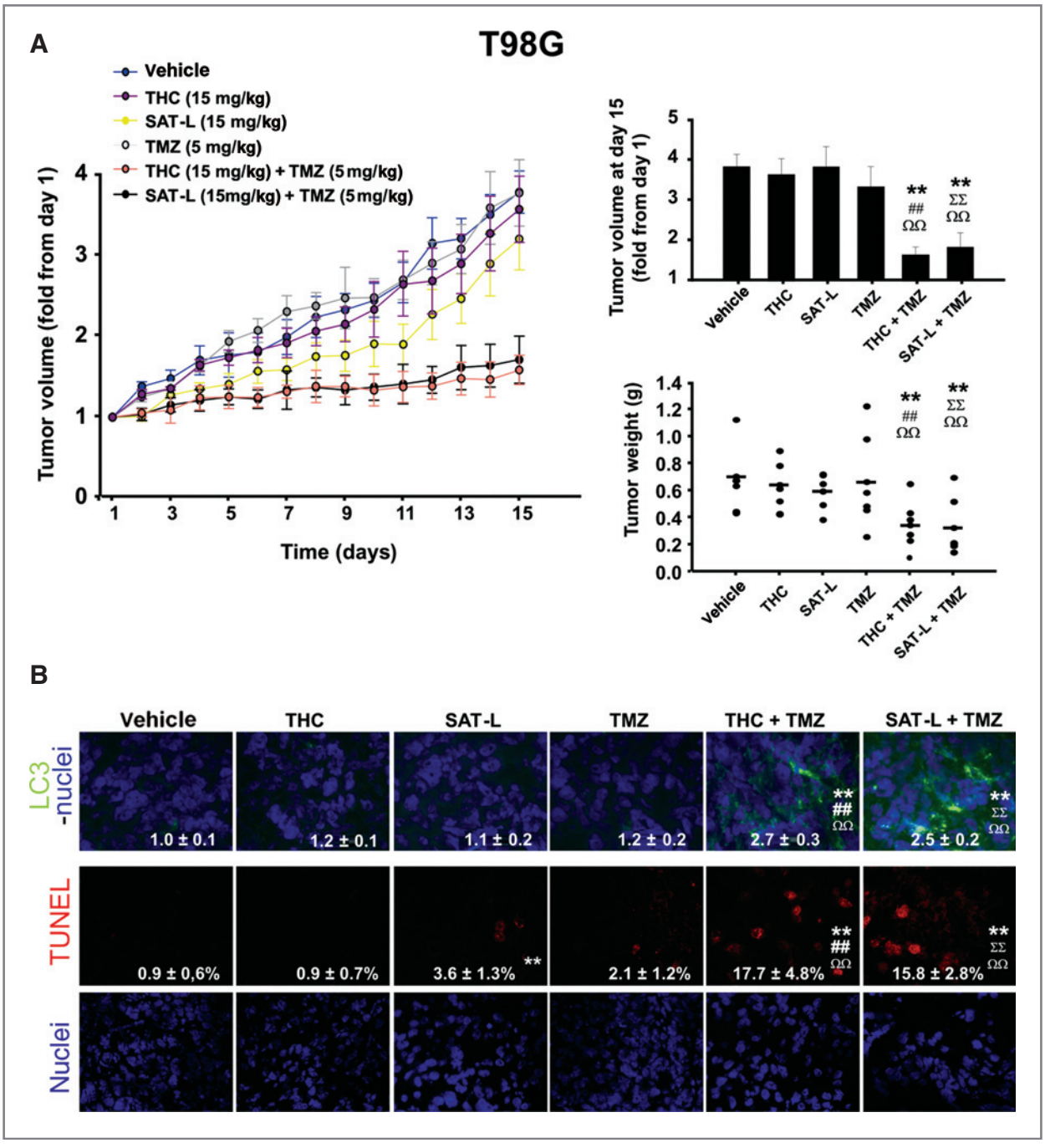


Figure 6. Combined administration of THC, CBD, and TMZ strongly reduces the growth of T98G cell–derived tumor xenografts. A, effect of THC, TMZ, SAT-L [THC-BDS (7.5 mg/kg) + CBD-BDS (7.5 mg/kg)], THC + TMZ, or SAT-L + TMZ on the growth of T98G cell–derived subcutaneous tumor xenografts. Once the tumors (generated by subcutaneous injection of 10×10^6 T98G cells) had reached 250 mm^3, treatments were daily administered for 14 days with a single peritumoral injection ($n = 6$–8 for each condition; mean ± SEM). Symbols of significance are omitted for clarity [THC (15 mg/kg) + TMZ- or SAT-L + TMZ-treated tumors were significantly different from vehicle-, TMZ-, and THC-treated tumors on days 12 and 13 ($P < 0.05$) and from day 14 until the end of the treatment ($P < 0.01$)]. Top right, data correspond to the mean fold-increase in tumor growth ± SEM on the last day of the treatment. [$n = 6$–8 for each condition; **, $P < 0.01$ from vehicle-treated tumors; ##, $P < 0.01$ from THC (15 mg/kg)-treated tumors; $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated tumors; and $^{\Sigma\Sigma}$, $P < 0.05$ from SAT-L–treated tumors]. Lower right, data represent tumor weight on the last day of the treatment [**, $P < 0.01$ from vehicle-treated tumors; ##, $P < 0.01$ from THC (15 mg/kg)-treated tumors; $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated tumors; and $^{\Sigma\Sigma}$, $P < 0.01$ from SAT-L–treated tumors]. B, analysis of LC3 immunostaining and TUNEL. Values correspond to the LC3-stained area normalized to the total number of nuclei in each section (mean fold change ± SD) or to the percentage of TUNEL-positive cells relative to the total number of nuclei in each section ± SD [10 sections of 3 different tumors from each condition were analyzed; **, $P < 0.01$ from vehicle-treated tumors; ##, $P < 0.01$ from THC (15 mg/kg)-treated tumors; $^{\Omega\Omega}$, $P < 0.01$ from TMZ-treated tumors; and $^{\Sigma\Sigma}$, $P < 0.01$ from SAT-L–treated tumors].

of different types of tumor xenografts including gliomas (11, 13–16, 18, 37–40). In this report, we find that the combined administration of TMZ and THC exerts a potent antitumoral action in glioma xenografts. Thus, treatment with these 2 agents inhibited tumor growth to much higher extent than the treatment with TMZ or THC alone. Furthermore, coadministration of TMZ and THC strongly reduced the growth of tumors that are resistant to the individual treatment with either of these 2 agents.

Our data indicate that the mechanism of TMZ + THC antitumoral action is based on the stimulation of autophagy, which is a cellular process by which cytoplasmic components—including organelles—are targeted for degradation to the lysosome (32, 33). The final outcome of the activation of the autophagy program seems to be highly dependent on the cellular context and the strength and duration of the stress-inducing signals. Thus, besides its role in cellular homeostasis, autophagy can be a form of programmed cell death or play a cytoprotective role, for example, in situations of nutrient starvation (41). Accordingly, autophagy plays a dual role in cancer. On the one hand, this cellular process may help to overcome the stress evoked at the initial steps of tumorigenesis, and on the other, autophagy has been proposed to work as a tumor-suppressing mechanism (42–44). Moreover, different anticancer treatments activate autophagy in tumor cells, which either enhance cancer cell death or act as a mechanism of resistance to chemotherapy (29, 42, 45). We had previously shown that the mechanism of THC antitumoral action relies on autophagy stimulation (15, 19). In this work, we find that coadministration of THC and TMZ strongly enhances this cellular process both *in vitro* and in tumor xenografts. Moreover, inhibition of autophagy prevents THC + TMZ-induced cell death, supporting that autophagy plays a crucial role in the antitumoral action of this drug combination.

Resistance to TMZ and other alkylating agents has been associated with increased expression of the enzyme MGMT, which catalyzes the removal of methyl groups added to the O^6-position of DNA guanines, thereby neutralizing the cytotoxic effect promoted by this chemical modification (35, 36). Interestingly, coadministration of TMZ and cannabinoids overcomes the resistance of T98G glioma xenografts (which express high levels of MGMT) to TMZ. We observed that the treatment with TMZ alone hardly activates autophagy *in vitro* or *in vivo* (even in tumors that respond to TMZ treatment), whereas the combination of TMZ + THC strongly enhances this cellular process. Of note, the treatment with TMZ and THC does not modify MGMT expression in these tumors. These observations further support that the combination of TMZ and cannabinoids promotes cell death via stimulation of autophagy and supports that TMZ together with cannabinoids could efficiently reduce the growth of TMZ-resistant tumors.

Recent results by McAllister's group indicate that CBD, which had been previously shown to reduce the growth of different types of tumor xenografts by itself (21–23, 25), enhance the inhibitory effects of THC on the viability of glioma cells (26). In line with this idea, in this report we find that the combination of submaximal doses of THC and CBD reduce tumor growth with a similar potency compared with an effective dose of THC. Similar results were obtained with the SAT-L mixture. This ability of a nonpyscoactive compound such as CBD to enhance THC antitumoral action could allow reducing the amount of THC and therefore the psychoactive effects of a potential cannabinoid-based therapy. Of importance, the effects of the combination of THC and CBD can be reproduced by using a SAT-L extract. Our data support that the cell death–promoting activity of the SAT-L mixture relies on the presence of THC and CBD rather than on the other plant constituents. Thus, Sativex could be used with similar efficacy compared with the mix of THC and CBD or a higher dose of THC to reduce tumor growth. Of note, although THC and CBD promote glioma cell death through the stimulation of different signaling routes (refs. 23, 25; and this report), our results indicate that THC + CBD antitumoral action is based on the activation of a similar mechanism as the one triggered by THC. Thus, although the CBD-induced decrease in cell viability is prevented only by apoptosis inhibitors, this agent still enhances THC-related autophagy to a great extent, suggesting that the signaling route triggered by CBD may facilitate the stimulation of autophagy by THC. In any case, our data support that the stimulation of this cellular process plays an important role in THC + CBD-induced cell death. Of potential therapeutic relevance, coadministration of TMZ and submaximal doses of THC + CBD or of the SAT-L mixture but not of CBD alone (data not shown) strongly reduced the growth of either U87MG or T98G xenografts. Moreover, the use of lower doses of THC + CBD is enough for enhancing TMZ antitumoral action and overcoming the resistance of glioma xenografts to this chemotherapeutic agent. Altogether, these observations strongly support that the triple combination of TMZ, THC, and CBD or of TMZ and the SAT-L mixture could efficiently reduce the growth of gliomas.

Cannabinoids, a new family of potential anticancer compounds, are devoid of the strong side effects associated with other chemotherapeutic agents (10, 46). Thus, no signs of toxicity were observed in patients enrolled in a pilot clinical trial for the treatment of GBM with THC (17) or in tumor-bearing animals treated intracranially, peritumorally, or intraperitoneally with THC (refs. 14, 18; data not shown). Moreover, no overt toxic effects have been reported in other clinical trials with cannabinoids (including Sativex) in cancer patients for various applications (e.g., inhibition of nausea, vomiting, and pain), using different routes of administration (e.g., oral, oromucosal; refs. 11, 47). These characteristics, together with their remarkable anticancer activity, make cannabinoids excellent candidate drugs for combination with other antineoplastic agents. Results presented here specifically show that the coadministration of TMZ with THC and with THC + CBD exerts a strong antitumoral action in glioma xenografts. Moreover, our results show that the combination of a

SAT-L extract and TMZ is equally effective as the combination of THC and TMZ in reducing the growth of these tumors. Taken together, our observations support that the administration of cannabinoids, and in particular of Sativex, which is currently used for palliative applications in patients with cancer and multiple sclerosis, alone or in combination with TMZ, could be of potential clinical interest for the management of GBM.

Disclosure of Potential Conflicts of Interests

We declare that GW Pharmaceuticals and Schering-Plough funded part of the research included in this article. Part of the data obtained in this study were included in two patent applications presented by GW Pharmaceuticals. S. Torres, M. Lorente, F. Rodríguez-Fornés, M. Guzmán, and G. Velasco are co-authors of these patents.

Acknowledgments

We thank Horacio Zimman and Carmen Moreno, from Hospital Clínico San Carlos, as well as Leyre Urigüen, from Universidad del Pais Vasco, for their kind collaboration in the processing and delivery of glioma samples; Dolores Hernán, Ana Isabel Torres, and Esther Gil for their contribution to the development of experiments with microparticles, and other members of our laboratory for their continuous support.

Grant Support

This work was supported by grants from Spanish Ministry of Science and Innovation (MICINN; PS09/01401; HF2005/0021, FR2009-0052, and IT2009-0053 to G. Velasco; SAF2006/00918 to M. Guzman), Santander-Complutense (PR34/07-15856 to G. Velasco), Comunidad de Madrid (S-SAL/0261/2006 and 950344 to M.G.), GW Pharmaceuticals (G. Velasco and M. Guzman), and Schering-Plough (G. Velasco). S. Torres was a recipient of a research training contract from Comunidad de Madrid; M. Lorente was sequentially a recipient of a "Juan de la Cierva" contract, a postdoctoral contract from Spanish Ministry of Education and Science and a postdoctoral contract from Comunidad de Madrid. F. Rodríguez-Fornés was a recipient of a fellowship from Schering-Plough and GW Pharmaceuticals; M. Hernández-Tiedra was a recipient of a fellowship from MEC and of a research training contract from Comunidad de Madrid; S. Hernández-Tiedra has a technician contract from MICINN and Fondo Social Europeo.

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Received July 22, 2010; revised October 12, 2010; accepted October 19, 2010; published online January 10, 2011.

References

1. Nieder C, Adam M, Molls M, Grosu AL. Therapeutic options for recurrent high-grade glioma in adult patients: recent advances. Crit Rev Oncol Hematol 2006;60:181–93.
2. Wong ML, Kaye AH, Hovens CM. Targeting malignant glioma survival signalling to improve clinical outcomes. J Clin Neurosci 2007;14:301–8.
3. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. Acta neuropathol 2007;114:97-109.
4. Purow B, Schiff D. Advances in the genetics of glioblastoma: are we reaching critical mass? Nat Rev Neurol 2009;5:419–26.
5. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17:98–110.
6. Gaoni Y, Mechoulam R. Isolation, structure and partial synthesis of an active constituent of hashish. J Am Chem Soc 1964;86:1646–7.
7. Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev 2002;54:161–202.
8. Fernandez-Ruiz J, Romero J, Velasco G, Tolon R, Ramos J, Guzman M. Cannabinoid CB2 receptor: a new target for controlling neural cell survival? Trends Pharmacol Sci 2007;28:39–45.
9. Di Marzo V. The endocannabinoid system: its general strategy of action, tools for its pharmacological manipulation and potential therapeutic exploitation. Pharmacol Res 2009;60:77–84.
10. Pertwee RG. Emerging strategies for exploiting cannabinoid receptor agonists as medicines. Br J Pharmacol 2009;156:397–411.
11. Guzman M. Cannabinoids: potential anticancer agents. Nat Rev Cancer 2003;3:745–55.
12. Sarfaraz S, Adhami VM, Syed DN, Afaq F, Mukhtar H. Cannabinoids for cancer treatment: progress and promise. Cancer Res 2008;68:339–42.
13. Carracedo A, Lorente M, Egia A, Blazquez C, Garcia S, Giroux V, et al. The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. Cancer Cell 2006;9:301–12.
14. Galve-Roperh I, Sanchez C, Cortes ML, Gómez del Pulgar T, Izquierdo M, Guzman M. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nat Med 2000;6:313–9.
15. Salazar M, Carracedo A, Salanueva IJ, Hernandez-Tiedra S, Lorente M, Egia A, et al. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. J Clin Invest 2009;119:1359–72.
16. Velasco G, Carracedo A, Blazquez C, Lorente M, Aguado T, Haro A, et al. Cannabinoids and gliomas. Mol Neurobiol 2007;36:60–7.
17. Guzman M, Duarte MJ, Blazquez C, Ravina J, Rosa MC, Galve-Roperh I, et al. A pilot clinical study of Delta9-tetrahydrocannabinol in patients with recurrent glioblastoma multiforme. Br J Cancer 2006;95:197–203.
18. Carracedo A, Gironella M, Lorente M, Garcia S, Guzman M, Velasco G, et al. Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes. Cancer Res 2006;66:6748–55.
19. Salazar M, Carracedo A, Salanueva IJ, Hernandez-Tiedra S, Egia A, Lorente M, et al. TRB3 links ER stress to autophagy in cannabinoid anti-tumoral action. Autophagy 2009;5:1048–9.
20. Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol Sci 2009;30:515–27.
21. Massi P, Vaccani A, Ceruti S, Colombo A, Abbracchio MP, Parolaro D. Antitumor effects of cannabidiol, a nonpsychoactive cannabinoid, on human glioma cell lines. J Pharmacol Exp Ther 2004;308:838–45.
22. Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, et al. Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. J Pharmacol Exp Ther 2006;318:1375–87.
23. Massi P, Vaccani A, Bianchessi S, Costa B, Macchi P, Parolaro D. The non-psychoactive cannabidiol triggers caspase activation and oxidative stress in human glioma cells. Cell Mol Life Sci 2006;63:2057–66.
24. McAllister SD, Christian RT, Horowitz MP, Garcia A, Desprez PY. Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells. Mol Cancer Ther 2007;6:2921–7.
25. Massi P, Valenti M, Vaccani A, Gasperi V, Perletti G, Marras E, et al. 5-Lipoxygenase and anandamide hydrolase (FAAH) mediate the anti-tumor activity of cannabidiol, a non-psychoactive cannabinoid. J Neurochem 2008;104:1091–100.
26. Marcu JP, Christian RT, Lau D, Zielinski AJ, Horowitz MP, Lee J, et al. Cannabidiol enhances the inhibitory effects of delta9-tetrahydrocannabinol on human glioblastoma cell proliferation and survival. Mol Cancer Ther 2010;9:180–9.
27. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58:621–81.
28. Tallarida RJ. Interactions between drugs and occupied receptors. Pharmacol Ther 2007;113:197–209.

29. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 2004;11:448–57.
30. Milano V, Piao Y, LaFortune T, de Groot J. Dasatinib-induced autophagy is enhanced in combination with temozolomide in glioma. Mol Cancer Ther 2009;8:394–406.
31. Lefranc F, Facchini V, Kiss R. Proautophagic drugs: a novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. Oncologist 2007;12:1395–403.
32. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 2008;4:151–75.
33. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008;132:27–42.
34. Vaccani A, Massi P, Colombo A, Rubino T, Parolaro D. Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism. Br J Pharmacol 2005;144:1032–6.
35. Hegi ME, Liu L, Herman JG, Stupp R, Wick W, Weller M, et al. Correlation of O^6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. J Clin Oncol 2008;26:4189–99.
36. Hermisson M, Klumpp A, Wick W, Wischhusen J, Nagel G, Roos W, et al. O^6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. J Neurochem 2006;96:766–76.
37. Oesch S, Walter D, Wachtel M, Pretre K, Salazar M, Guzman M, et al. Cannabinoid receptor 1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma. Mol Cancer Ther 2009;8:1838–45.
38. Caffarel MM, Sarrio D, Palacios J, Guzman M, Sanchez C. Delta9-tetrahydrocannabinol inhibits cell cycle progression in human breast cancer cells through Cdc2 regulation. Cancer Res 2006;66:6615–21.
39. Casanova ML, Blazquez C, Martinez-Palacio J, Villanueva C, Fernandez-Acenero MJ, Huffman JW, et al. Inhibition of skin tumor growth and angiogenesis *in vivo* by activation of cannabinoid receptors. J Clin Invest 2003;111:43–50.
40. Blazquez C, Gonzalez-Feria L, Alvarez L, Haro A, Casanova ML, Guzman M. Cannabinoids inhibit the vascular endothelial growth factor pathway in gliomas. Cancer Res 2004;64:5617–23.
41. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ 2009;16:966–75.
42. Verfaillie T, Salazar M, Velasco G, Agostinis P. Linking ER stress to autophagy: potential implications for cancer therapy. Int J Cell Biol 2010;2010:930509.
43. Morselli E, Galluzzi L, Kepp O, Vicencio JM, Criollo A, Maiuri MC, et al. Anti- and pro-tumor functions of autophagy. Biochim Biophys Acta 2009;1793:1524–32.
44. Maiuri MC, Tasdemir E, Criollo A, Morselli E, Vicencio JM, Carnuccio R, et al. Control of autophagy by oncogenes and tumor suppressor genes. Cell Death Differ 2009;16:87–93.
45. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nat Rev Cancer 2007;7:961–7.
46. Mackie K. Cannabinoid receptors as therapeutic targets. Annu Rev Pharmacol Toxicol 2006;46:101–22.
47. Hall W, Christie M, Currow D. Cannabinoids and cancer: causation, remediation, and palliation. Lancet Oncol 2005;6:35–42.

Molecular Cancer Therapeutics

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Mol Cancer Ther 2011;10:90-103.