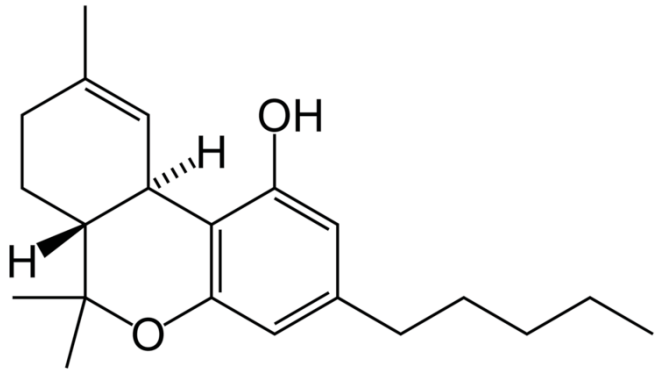
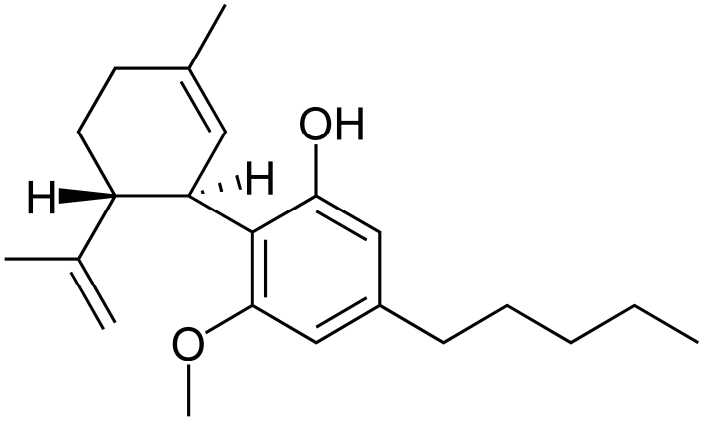
Torres et al. Supplementary Figure 1

A



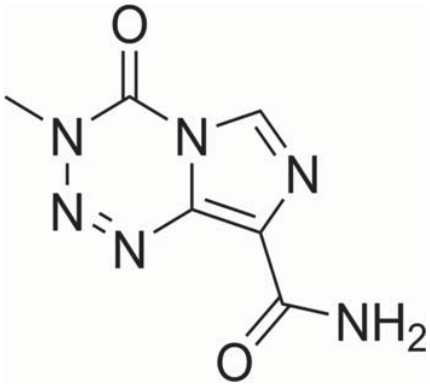
Δ^9-tetrahydrocannabinol (THC)

B



Cannabidiol (CBD)

C

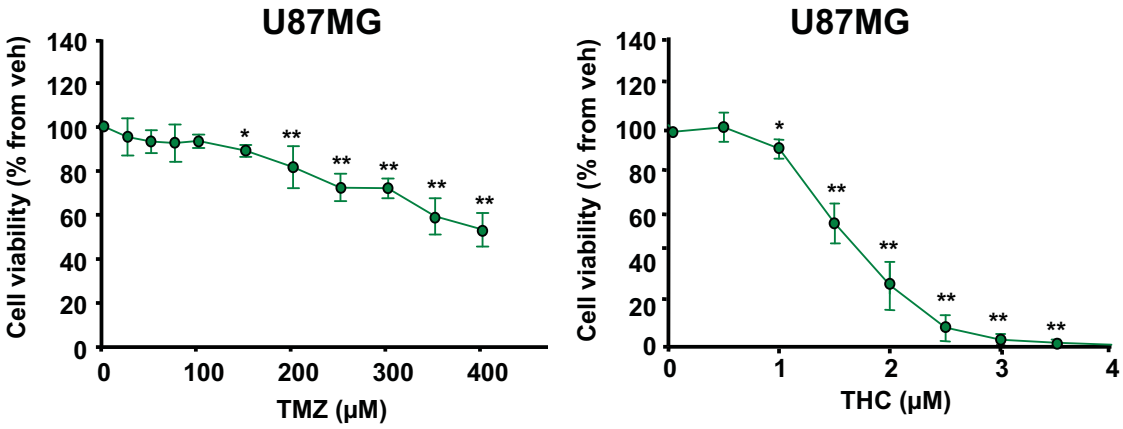


Temozolomide (TMZ)

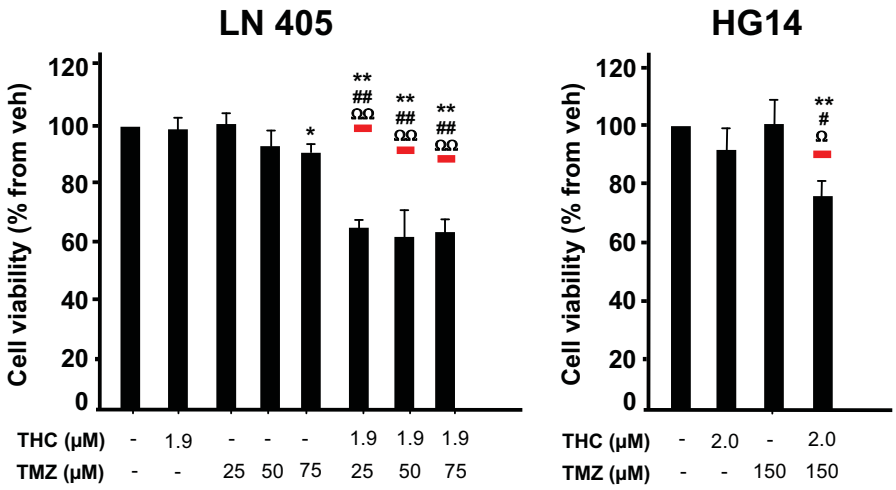
Supplementary Figure 1. Chemical structures of THC, CBD and TMZ.

Torres et al. Supplementary Figure 2

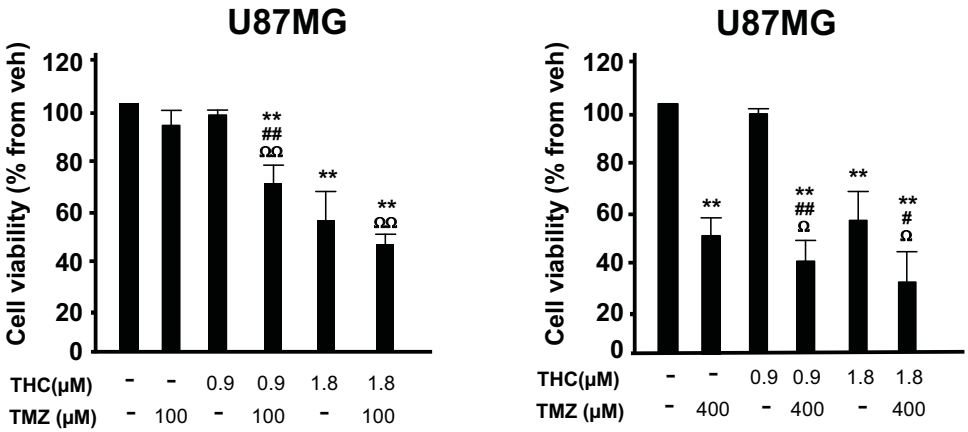
A



B



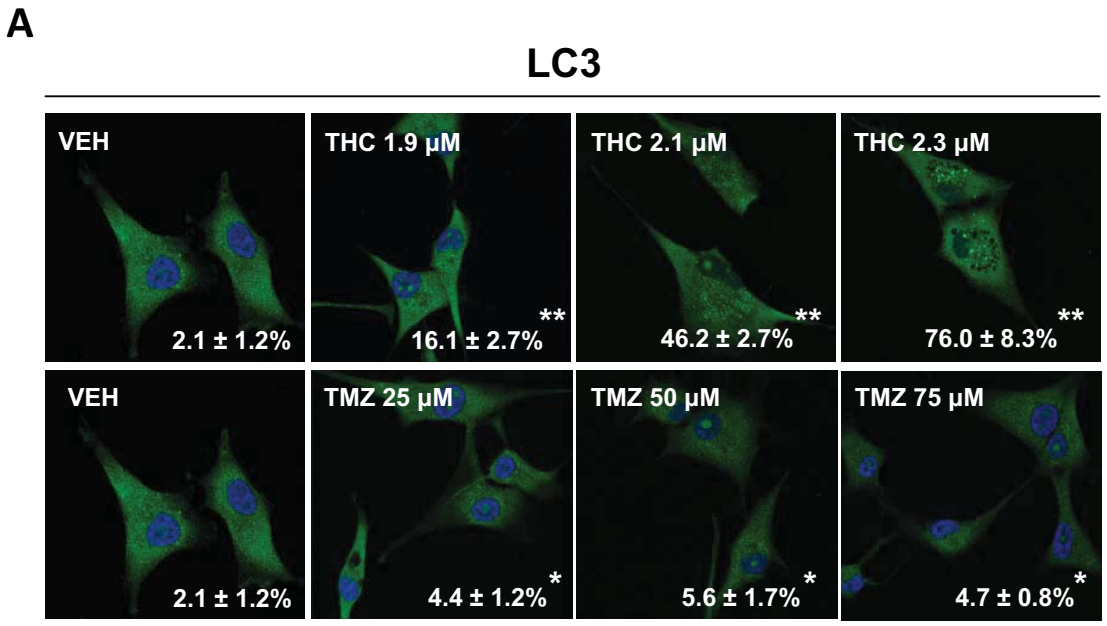
C

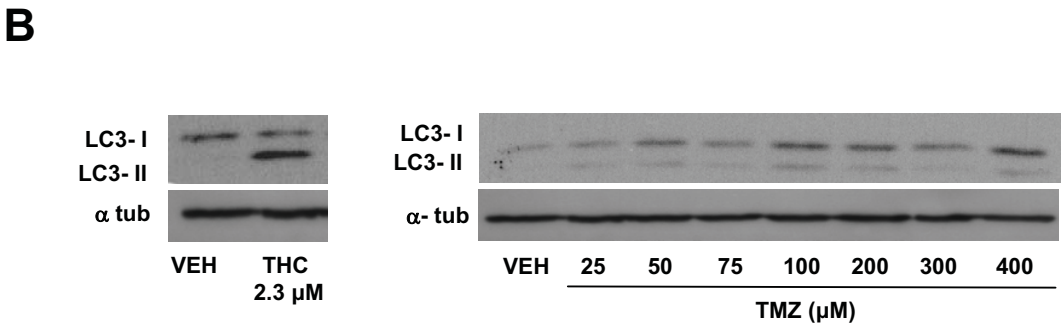


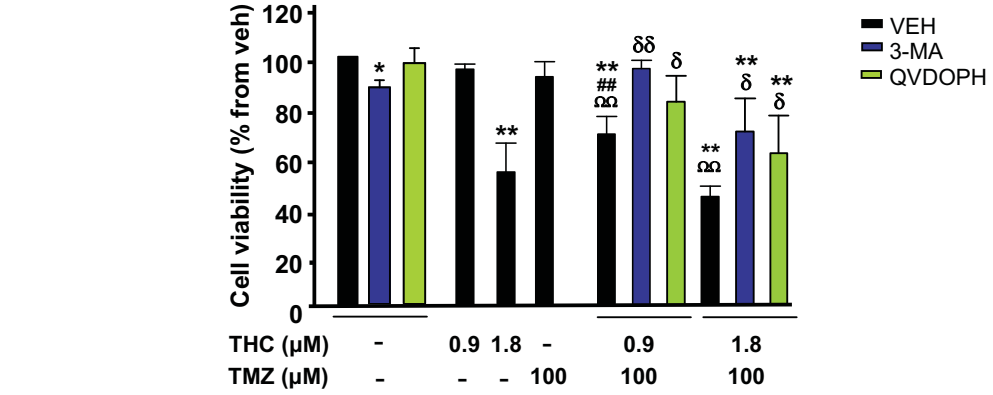
Supplementary Figure 2. THC enhances TMZ-induced glioma cell death

(A) Effect of TMZ (left panel) and THC (right panel) on the viability (72 h) of U87MG cells (n = 3; ** $p < 0.01$ from vehicle-treated cells). (B) Effect of THC, TMZ and THC+TMZ on the viability (72 h) of LN405 (human glioma cell line) and HG14 (primary culture of human glioma cells) as determined by the MTT test (mean ± s.d.; n = 6 for LN405 and n = 4 for HG14 cells; ** $p < 0.01$ from vehicle-treated cells; $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ from THC-treated cells; $^{\Omega}p < 0.05$ or $^{\Omega\Omega}p < 0.01$ from TMZ-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 TMZ at each concentration of these agents. (C) Effect of THC and TMZ on the viability (72 h) of U87MG cells (n = 3; ** $p < 0.01$ from vehicle-treated cells; $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ from THC-treated cells; $^{\Omega}p < 0.05$ or $^{\Omega\Omega}p < 0.01$ from TMZ-treated cells).

Torres et al. Supplementary Figure 3



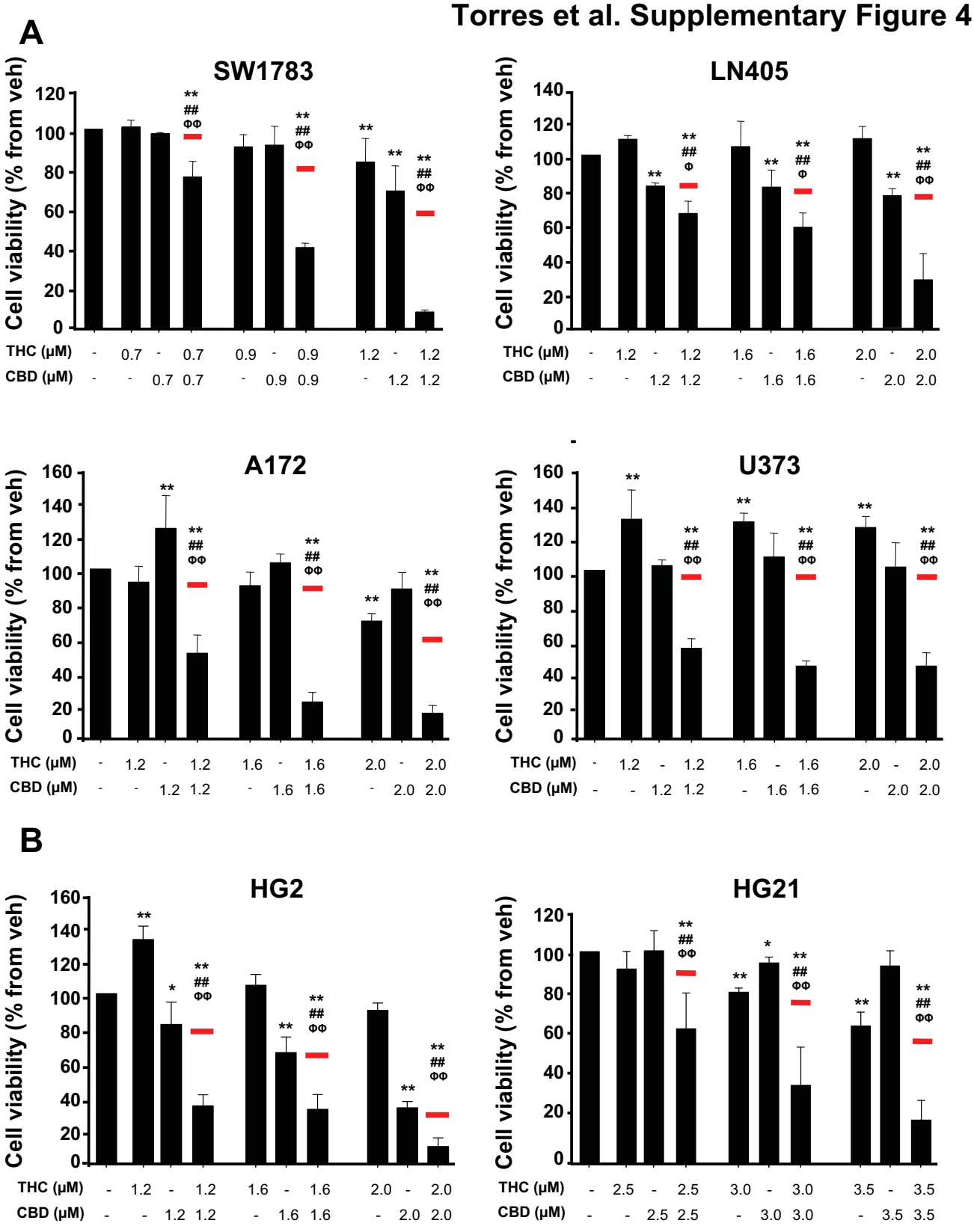




Supplementary Figure 3. Autophagy and apoptosis are involved in the mechanism of THC+TMZ-induced cell death

(A) Effect of THC and TMZ (24 h) on LC3 immunostaining of U87MG cells. Values in the lower right corner of each photomicrograph correspond to the percentage of cells with LC3 dots relative to the total number of cells (mean ± s.d;; n = 3; representative photomicrographs of each condition are shown; ** $p < 0.01$ or * $p < 0.05$ from vehicle-treated cells. (B) Effect of THC and TMZ on LC3 lipidation. A representative experiment of 3 is shown. (C) Effect of 3MA (5 mM) and QVDPOH (15 μM) on the viability (72 h) of U87MG cells treated with THC, TMZ or THC+TMZ (n = 6; mean ± s.d, ** $p < 0.01$ from vehicle-treated cells; $^{\#\#}$ $p < 0.01$ from THC-treated cells; $^{\Omega\Omega}$ $p < 0.01$ from TMZ-treated cells and $^{\delta}$ $p < 0.05$ or $^{\delta\delta}$ $p < 0.01$ fom THC+TMZ-treated cells). Additional controls are omitted for clarity.

Torres et al. Supplementary Figure 4

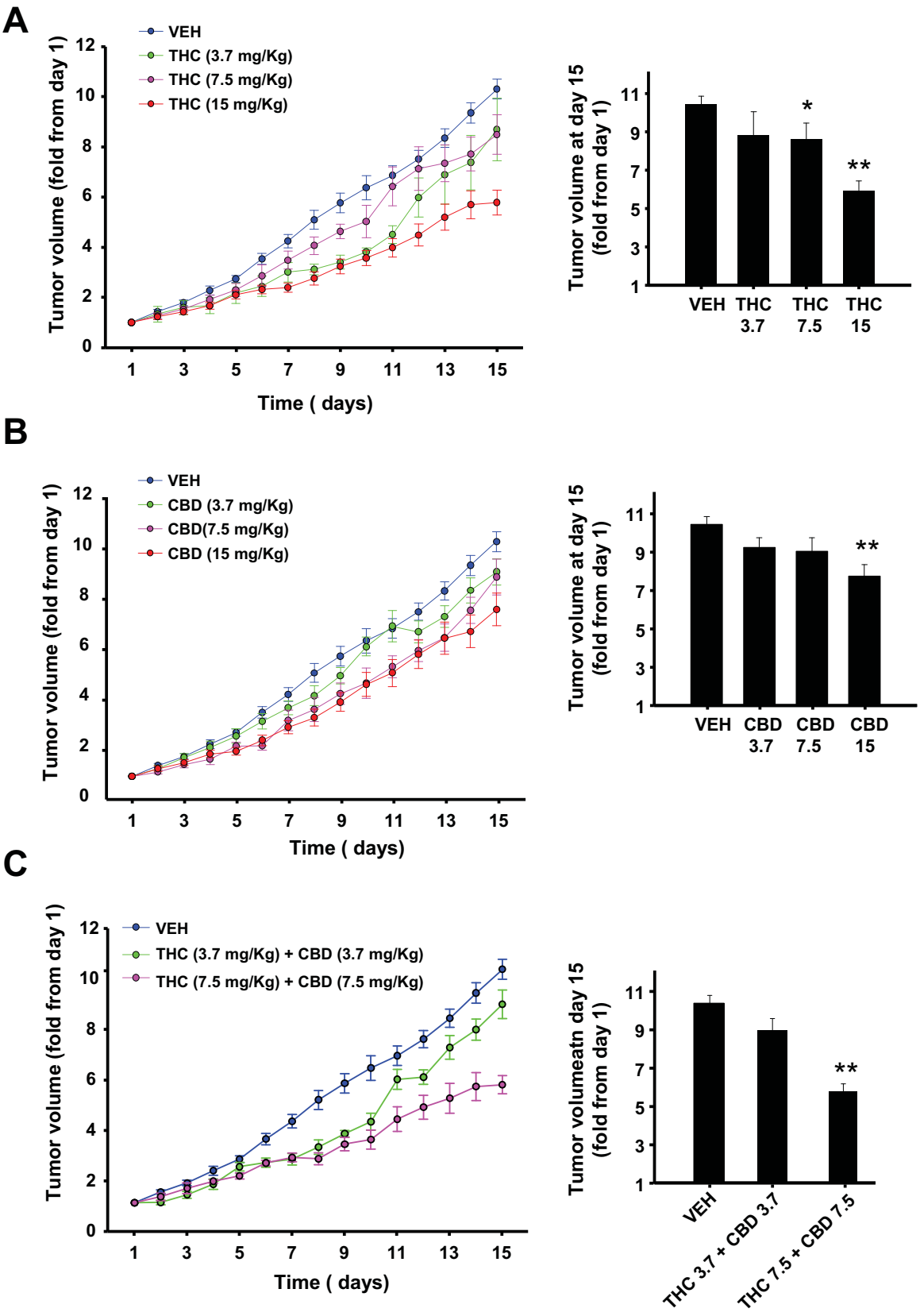


Supplementary Figure 4. Effect of THC and CBD on the viability of human glioma cell lines and primary cultures of human glioma cells

Effect of THC and CBD on the viability (72 h) of several human glioma cell lines (A) and primary cultures of human glioma cells (B) as determined by the MTT test (mean ± s.d.; n = 6 for SW1783, LN405, A172 and U373 and n = 4 for HG2 and HG21 cells; ** $p < 0.01$ from vehicle-treated cells; $^{\#\#}$ $p < 0.01$ from THC-treated cells; $^{\Phi\Phi}$ $p < 0.01$ or $^{\Phi}$ $p < 0.05$ 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.

U87MG

Torres et al. Supplementary Figure 5



Supplementary Figure 5. Anti-tumoral action of different doses of THC and CBD

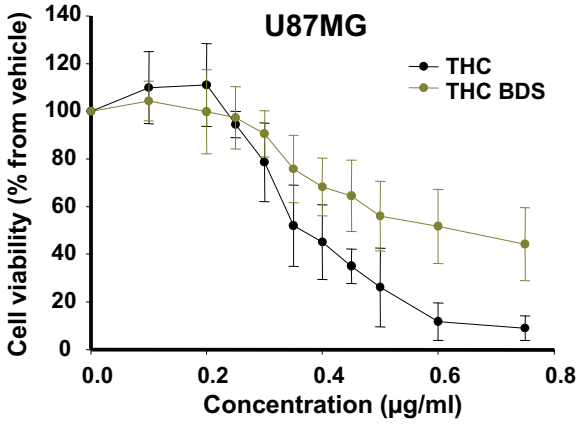
Effect of THC (A), CBD (B) or THC+CBD (C) on the growth of U87MG cell-derived tumor xenografts. Once the tumors reached 250 mm^3, THC, CDB or THC+CBD were daily administered for 14 days with a single peri-tumoral injection (n = 6-8 for each condition; mean ± S.E.M; In panels A, B and C, symbols of significance are omitted for clarity; see below for a description of the statistical differences for each treatment). Right panels, data correspond to the mean fold-increase in tumor growth ± S.E.M at the last day of the treatment. (n = 8; ** $p < 0.01$ or * $p < 0.05$ from vehicle-treated tumors.). Photographs of representative tumors at the last day of the treatment are shown.

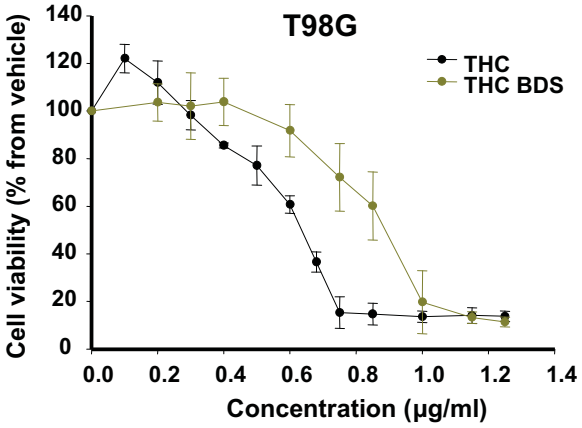
Legend to Supplementary Figure 5 (Statistical analyses)

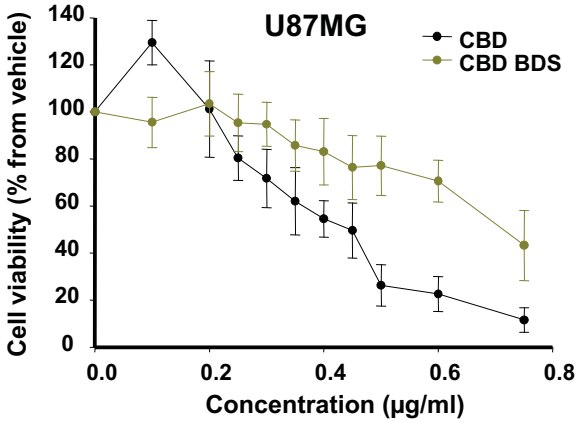
Panel A: THC (15 mg/kg)-treated tumors were significantly different from Veh-treated tumors at day 5 ($p < 0.05$), and from day 6 until the end of the treatment ($p < 0.01$). THC (7.5 mg/kg)-treated tumors were significantly different from Veh-treated-tumors at days 14 and 15 ($p < 0.05$). THC (3.7 mg/kg)-treated tumors were significantly different from Veh-treated tumors at days 6, 7 and 8 ($p < 0.05$) and from day 9 until day 11 ($p < 0.01$). Panel B: CBD (15 mg/kg)-treated tumors were significantly different from Veh-treated tumors at days 5 to 13 ($p < 0.05$) and at days 14 and 15 ($p < 0.01$). Panel C: THC (3.7 mg/kg) + CBD (3.7 mg/kg)-treated tumors were significantly different from Veh-treated tumors at days 6 and 12 ($p < 0.05$), and from day 7 until day 10 ($p < 0.01$). THC (7.5 mg/kg) + CBD (7.5 mg/kg)-treated tumors were significantly different from Veh-treated tumors at days 6 and 7 ($p < 0.05$), and from day 8 until the end of the treatment ($p < 0.01$).

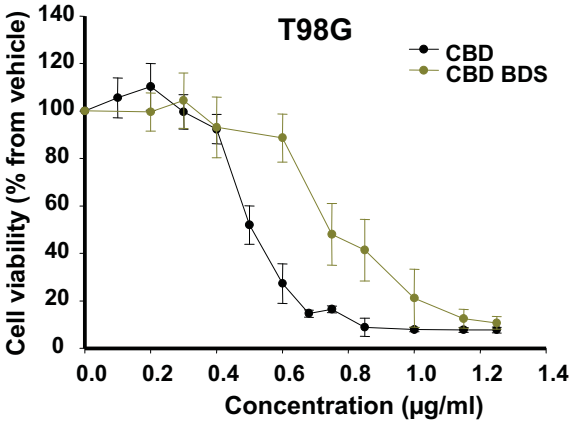
Torres et al. Supplementary Figure 6

A





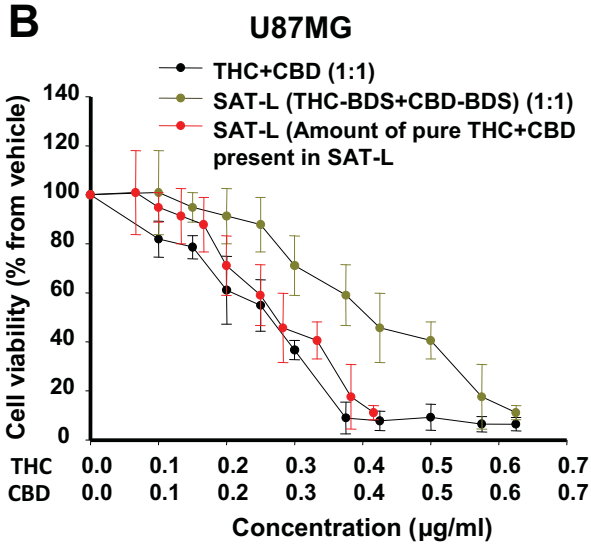


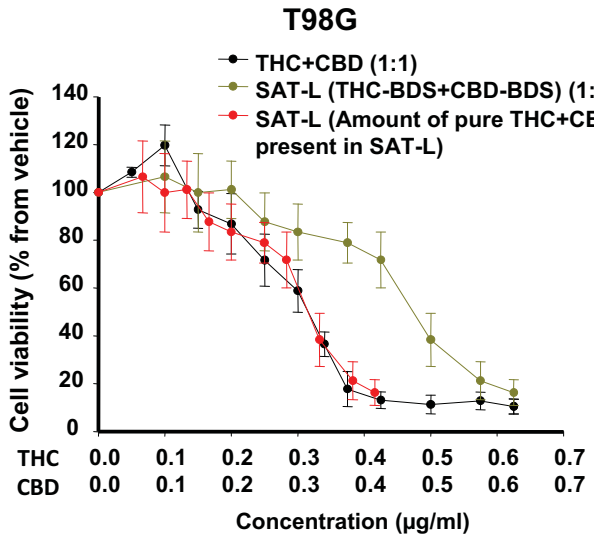


	IC50 µg/ml (Pure cannabinoid)	IC50 µg/ml (BDS)	IC50 µg/ml (Pure cannabinoid in BDS)
THC	0.37	0.64	0.43
CBD	0.47	0.72	0.47

	IC50 µg/ml (Pure cannabinoid)	IC50 µg/ml (BDS)	IC50 µg/ml (Pure cannabinoid in BDS)
THC	0.62	0.90	0.60
CBD	0.49	0.79	0.53

B



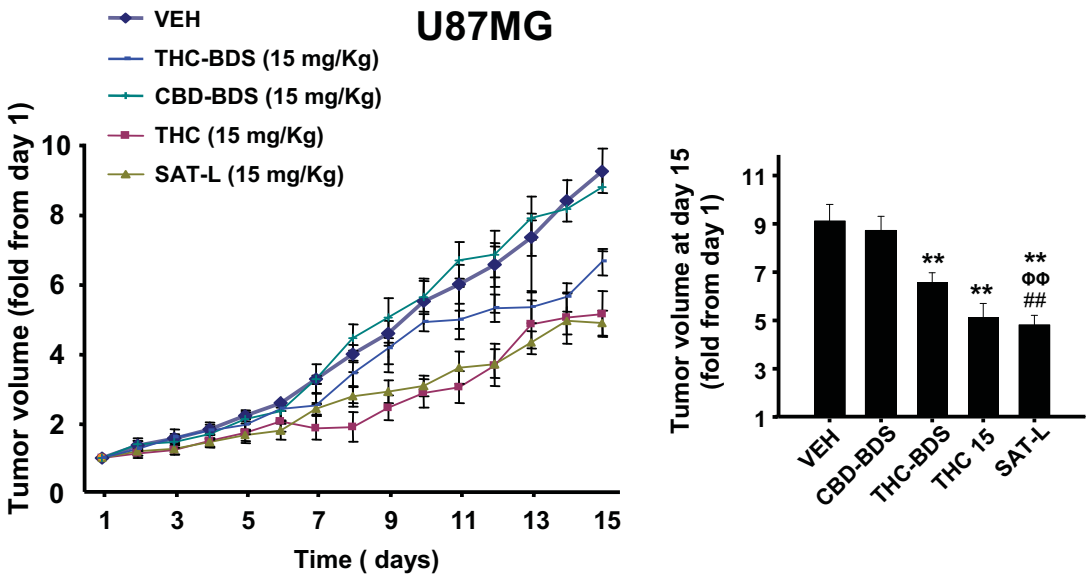


Supplementary Figure 6. Effect of pure cannabinoids and their corresponding botanical drug substances on the viability of human glioma cells

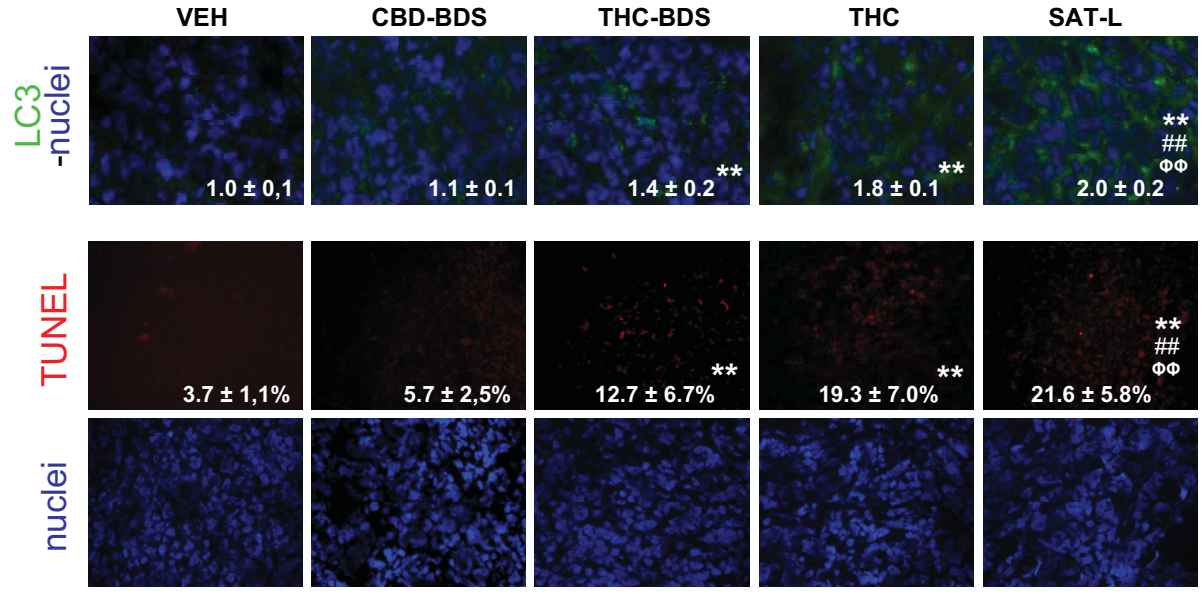
(A) Effect of THC and THC-BDS (upper panels), CBD and CBD-BDS (lower panels) on the viability (72 h) of U87MG and T98G cells (mean ± s.d.; n = 6; the IC_{50} values for each pure cannabinoid, their respective BDSs or the amount of pure cannabinoid present in each BDS are included in the table. (B) Effect of THC+CBD and Sativex-like (SAT-L; THC-BDS+CBD-BDS) on the viability (72 h) of U87MG and T98G cells (mean ± s.d.; n = 6).

Torres et al. Supplementary Figure 7

A



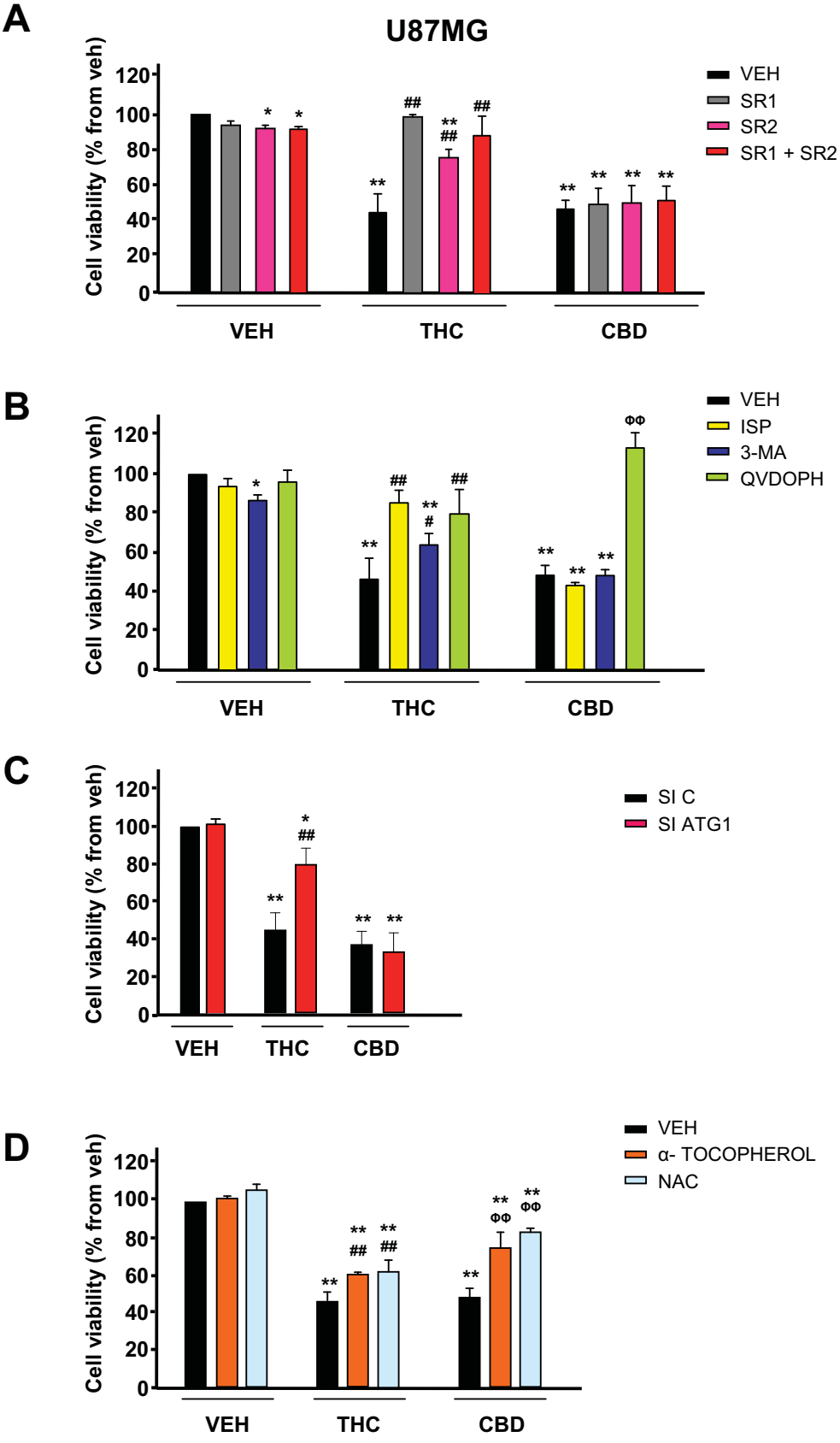
B



Supplementary Figure 7. Combined administration of THC-BDS and CBD-BDS strongly reduces the growth of U87MG cell-derived tumor xenografts

(A) Effect of THC (15 mg/kg), THC-BDS (15 mg/kg - containing 10 mg/kg THC), CBD-BDS (15 mg/kg - containing 10 mg/kg CBD) or Sativex-like [SAT-L; THC-BDS (7.5 mg/kg) + CBD-BDS (7.5 mg/kg)] on the growth of U87MG cell-derived tumor xenografts (n = 6-8 for each condition; mean ± S.E.M; in left panel A symbols of significance are omitted for clarity) [THC (15 mg/kg)-treated tumors were significantly different from Veh-treated tumors on days 7 to 10 ($p < 0.05$), and from day 11 until the end of the treatment ($p < 0.01$). THC-BDS-treated tumors were significantly different from Veh-treated tumors on day 13 ($p < 0.05$) and on days 14 and 15 ($p < 0.01$). SAT-L-treated tumors were significantly different from Veh-treated tumors on day 7 ($p < 0.05$) and from day 8 until the end of the treatment ($p < 0.01$)]. Right panels, data correspond to the mean fold-increase in tumor growth ± S.E.M at the last day of the treatment. [n = 8; ** $p < 0.01$ from vehicle-treated tumors; $^{\#\#}$ $p < 0.01$ from THC-BDS (15 mg/kg)-treated tumors; and $^{\Phi\Phi}$ $p < 0.01$ from CBD-BDS (15 mg/kg)-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 ± s.d.; arrows point cells with LC3 dots) or to the percentage of TUNEL-positive cells relative to the total number of nuclei in each section ± s.d. [10 sections of 3 different tumors from each condition were analyzed; ** $p < 0.01$ from vehicle-treated tumors; $^{\#\#}$ $p<0.01$ or $^{\#}$ $p < 0.05$ from THC-BDS treated tumors; $^{\Phi\Phi}$ $p<0.01$ or $^{\Phi}$ $p < 0.05$ from CBD-BDS treated tumors].

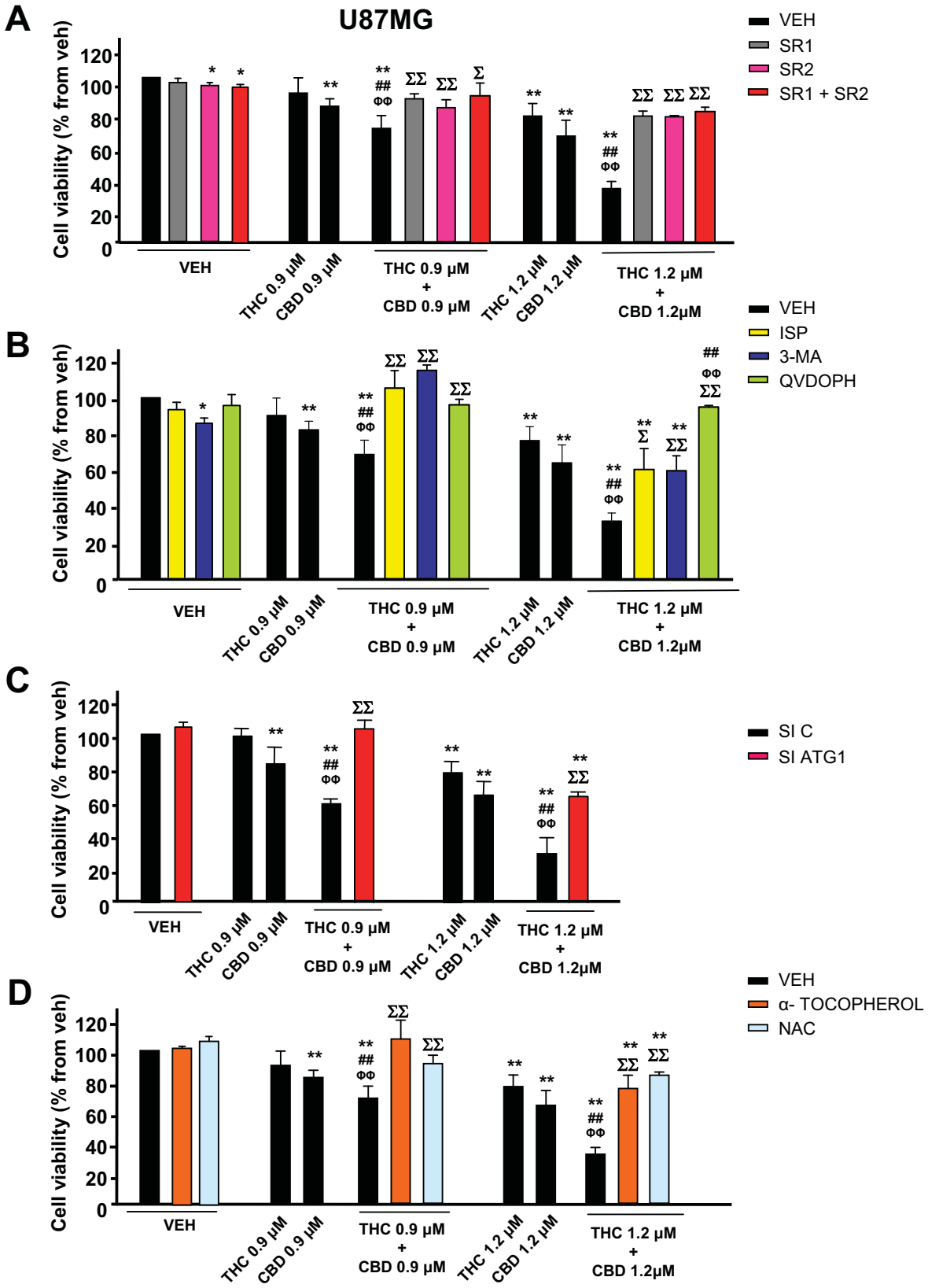
Torres et al. Supplementary Figure 8



Supplementary Figure 8. Effect of SR1, SR2, ISP-1, 3-MA and QVDPOH on THC or CBD-induced cell death

(A and B) Effect of SR141716 (SR1; 1 μM), SR144528 (SR2; 1 μM) or SR1+SR2 (1 μM +1 μM) (panel A) or ISP-1 (ISP; 1 μM), 3MA (5 mM) and QVDPOH (15 μM) (panel B) on the viability of U87MG cells treated with THC (2 μM,) or CBD (2 μM) (mean ± s.d; n = 6; ** $p < 0.01$ from vehicle-treated cells; $^{\#\#}$ $p < 0.01$ from THC-treated cells; $^{\Phi\Phi}$ $p < 0.01$ from CBD-treated cells). (C) Effect of THC or CBD on the viability (72 h) of U87MG cells transfected with control (siC) or Atg1-selective (siAtg1) siRNA. (n = 6; mean ± s.d, ** $p < 0.01$ from siC-transfected, vehicle-treated cells; $^{\#\#}$ $p < 0.01$ from siC-transfected, THC-treated cells; $^{\Omega\Omega}$ $p < 0.01$ from siC-transfected, 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). (D) Effect of α-tocopherol (10 μM) or N-acetylcysteine (3 mM) on the viability of U87MG cells treated with THC (2 μM,) or CBD (2 μM) (mean ± s.d; n = 6; ** $p < 0.01$ from vehicle-treated cells; $^{\#\#}$ $p < 0.01$ from THC-treated cells; $^{\Phi\Phi}$ $p < 0.01$ from CBD-treated cells).

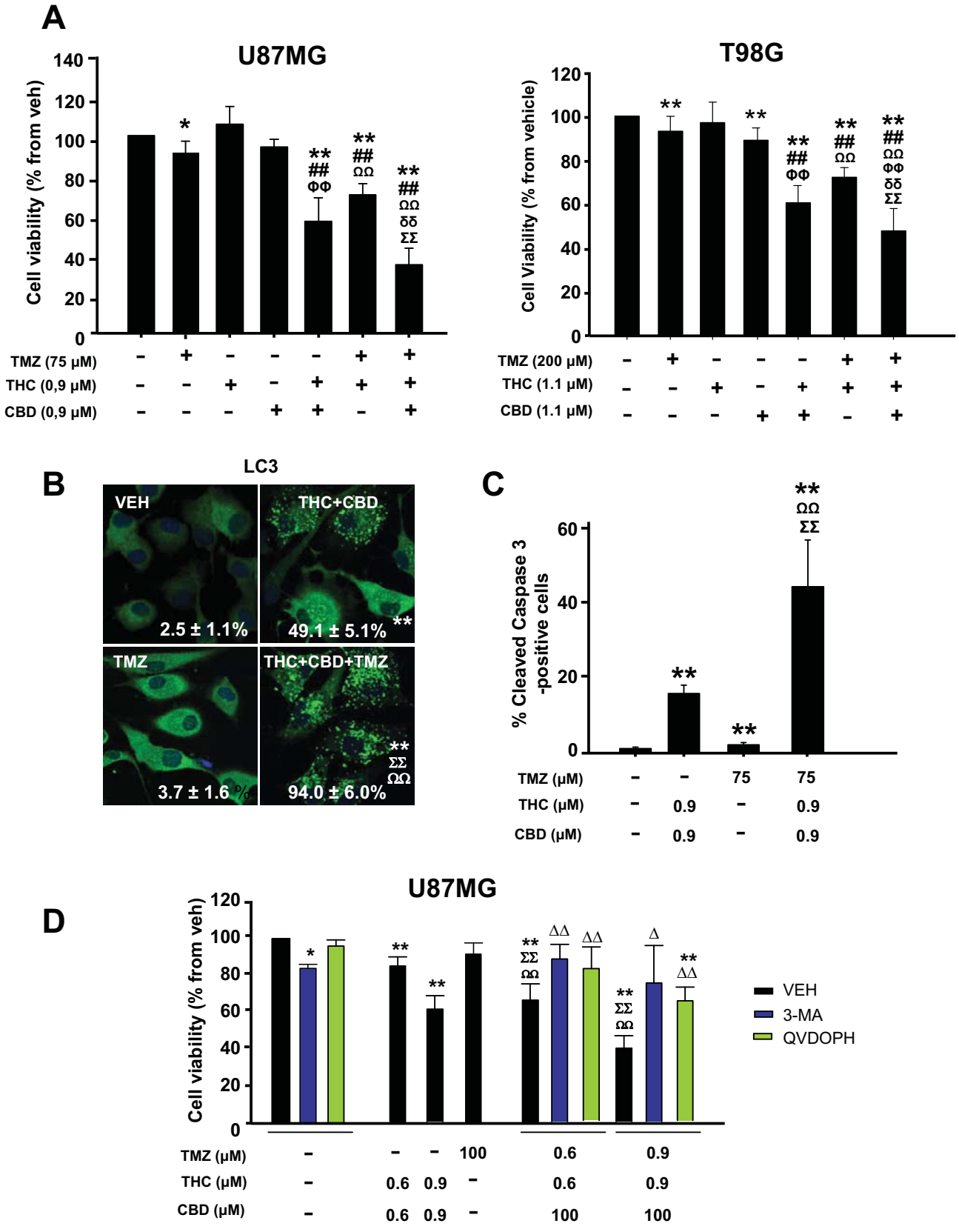
Torres et al. Supplementary Figure 9



Supplementary Figure 9. Effect of SR1, SR2, ISP-1, 3-MA and QVDPOH on THC+CBD-induced cell death

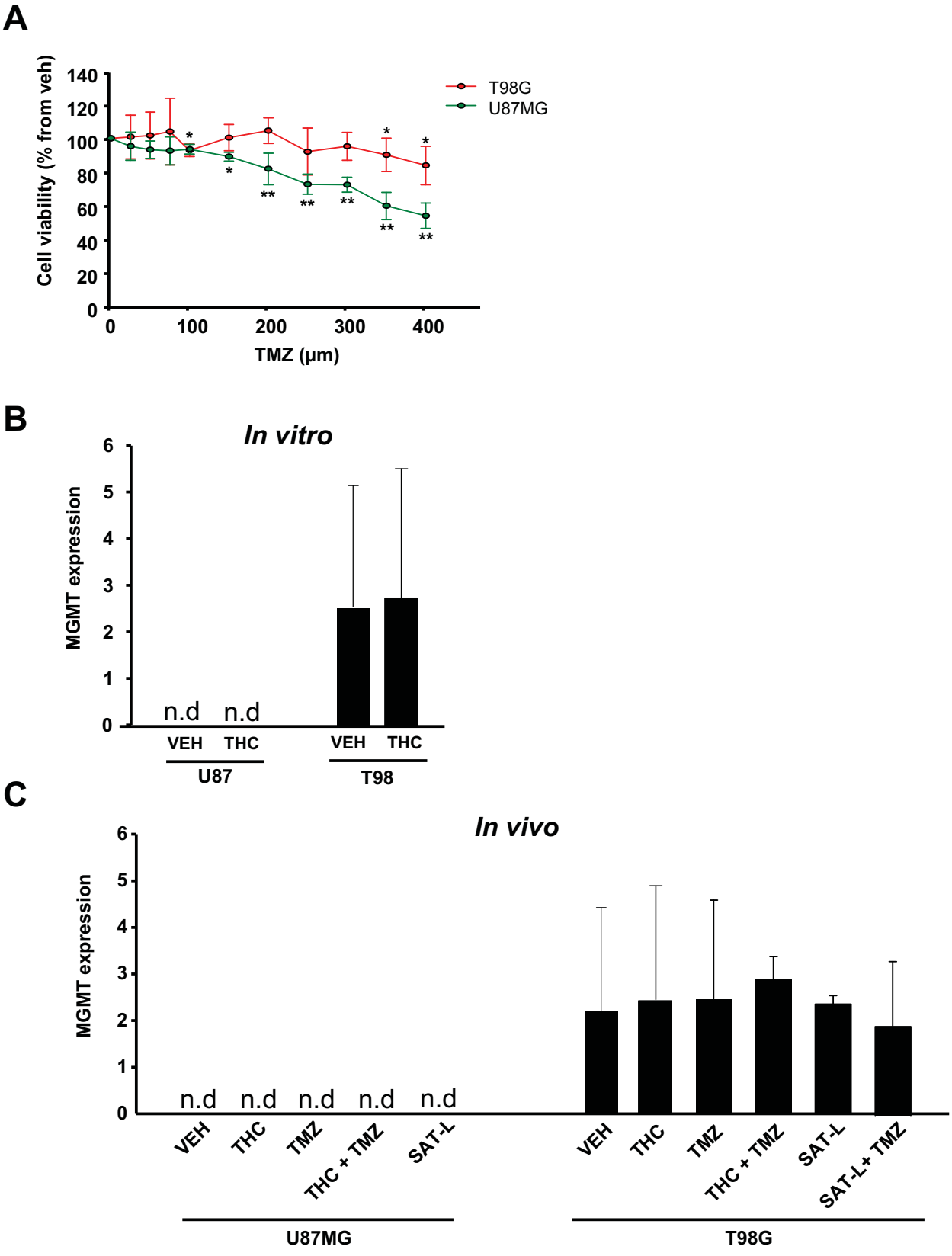
(A and B) Effect of SR141716 (SR1; 1 μM), SR144528 (SR2; 1 μM) or SR1+SR2 (1 μM +1 μM) (panel A) or ISP-1 (ISP; 1 μM), 3MA (5 mM) and QVDPOH (15 μM) (panel B) on the viability of U87MG cells treated with THC+CBD (mean ± s.d; n = 6; ** $p < 0.01$ from vehicle-treated cells; $^{\#\#}$ $p < 0.01$ from THC-treated cells; $^{\Phi\Phi}$ $p < 0.01$ from CBD-treated cells and $^{\Sigma\Sigma}$ $p < 0.01$ or $^{\Sigma}$ $p < 0.05$ from THC+CBD-treated cells). (C) Effect of THC, CBD and THC+CBD on the viability (72 h) of U87MG cells transfected with control (siC) or Atg1-selective (siAtg1) siRNA (n = 6; mean ± s.d, ** $p < 0.01$ 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). (D) Effect of α-tocopherol (2 μM) or N-acetylcysteine (3 mM) on the viability of U87MG cells treated with THC, CBD or THC + CBD (mean ± s.d; n = 6; ** $p < 0.01$ 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). (A-D). Additional controls are omitted for clarity

Torres et al. Supplementary Figure 10



Supplementary Figure 10. Combined administration of THC, CBD and TMZ strongly reduces the viability of human glioma cell lines. (A) Effect of THC, CBD, TMZ, THC+CBD, THC+TMZ and THC+CBD+TMZ on the viability (72 h) of U87MG (left panel) and T98G (right panel) cells as determined by the MTT test (n = 6; mean ± s.d; ** $p < 0.01$ from vehicle-treated cells; $^{\#\#}$ $p < 0.01$ from THC-treated cells; $^{\Omega\Omega}$ $p < 0.01$ from TMZ-treated cells, $^{\Phi\Phi}$ $p < 0.01$ from CBD-treated cells, $^{\Sigma\Sigma}$ $p < 0.01$ from THC+CBD-treated cells and $^{\delta\delta}$ $p < 0.01$ from THC+TMZ-treated cells). (B) Effect of THC+CBD (0.9 μM + 0.9 μM), TMZ (75 μM) and THC+CBD+TMZ (0.9 μM + 0.9 μM + 75 μM) (24 h) on LC3 immunostaining of U87MG cells. Values in the lower right corner of each photomicrograph correspond to the percentage of cells with LC3 dots relative to the total number of cells (mean ± s.d; n = 3; representative photomicrographs of each condition are shown; ** $p < 0.01$ from vehicle-treated cells; $^{\Omega\Omega}$ $p < 0.01$ from TMZ-treated cells and $^{\Sigma\Sigma}$ $p < 0.01$ from THC+CBD-treated cells). (C) Effect of THC+CBD, TMZ and THC+CBD+TMZ (24 h) 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 ± s.d; n = 3; ** $p < 0.01$ from vehicle-treated cells; $^{\Omega\Omega}$ $p < 0.01$ from TMZ-treated cells and $^{\Sigma\Sigma}$ $p < 0.01$ from THC+CBD-treated cells). (D) Effect of 3MA (5 mM) and QVDPOH (15 μM) on the viability of U87MG cells treated with THC+CBD, TMZ and THC+CBD+TMZ (mean ± s.d; n = 6; ** $p < 0.01$ or * $p < 0.05$ from vehicle-treated cells; $^{\Omega\Omega}$ $p < 0.01$ from TMZ-treated cells, $^{\Sigma\Sigma}$ $p < 0.01$ from THC+CBD-treated cells and $^{\Delta\Delta}$ $p < 0.01$ or $^{\Delta}$ $p < 0.05$ from THC+CBD+TMZ-treated cells). Additional controls are omitted for clarity.

Torres et al. Supplementary Figure 11



Supplementary Figure 11. Resistance to TMZ treatment correlates with higher MGMT expression

(A) Effect of TMZ on the viability (72 h) of U87MG and T98G cells (n = 3; ** $p < 0.01$ or * $p < 0.05$ from vehicle-treated cells). (B) MGMT mRNA levels as determined by real time quantitative PCR of U87MG and T98G cells treated with THC. Data correspond to the MGMT mRNA levels (n = 5; mean fold change ± s.d. relative to MGMT mRNA levels of vehicle-treated cells in one of the experiments). (C) Effect of the different treatments on MGMT mRNA levels (as determined by real-time quantitative PCR) of U87MG and T98G tumor xenografts. Data correspond to MGMT mRNA levels relative to one of the vehicle-treated tumors (mean ± s.d; n = 5 for each condition). n.d: non detected.