LQB-118 compound inhibits migration and induces cell death in glioblastoma cells

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Abstract. Glioblastoma (GBM) is the most frequent malignant brain tumor. It represents the most aggressive astrocytoma with an overall survival of 14 months. Despite improvements in surgery techniques, radio- and chemotherapy, most patients present treatment resistance, recurrence and disease progression. Therefore, development of effective alternative therapies is essential to overcome treatment failure. The purpose of the study was to evaluate the antitumoral activity of the synthetic compound LQB-118, *in vitro*. Monolayer and three-dimensional (3D) cell culture systems of human-derived GBM cell lines were used to evaluate the effect of LQB-118 on cell viability, cell death and migration. LQB-118 reduced cell viability as determined by MTT and trypan blue exclusion assays and promoted apoptosis in monolayer cell lines with an intrinsic temozolomide (TMZ)-resistance profile. In 3D culture models, LQB-118 reduced cell viability as evaluated by APH assay and inhibited cell migration while the TMZ resistance profile was maintained. Moreover, LQB-118 reduced p38 and AKT expression and phosphorylation, whereas it reduced only the phosphorylated ERK1/2 form. LQB-118 reduced p38 and NRF2 expression, an axis that is associated with TMZ resistance, revealing a mechanism to overcome resistance. LQB-118 also demonstrated an additional effect when combined with ionizing radiation and cisplatin. In conclusion, the present data demonstrated that LQB-118 maintained its effectiveness in a 3D cell conformation, which shares more similarities with the tumor mass. LQB-118 is a promising agent for GBM treatment as monotherapy and associated with radiotherapy or cisplatin. Its effect is associated with inhibition of GBM-related survival signaling pathways.

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Introduction

Glioblastoma (GBM) is the most malignant primary brain tumor in humans. It is highly aggressive and heterogeneous, remaining a major therapeutic challenge, since patients have a mean overall survival (OS) of only 14 months and a progression-free survival (PFS) time of 7-10 months (1). First-line postsurgical therapy for GBM consists of temozolomide (TMZ) combined with regional fractionated radiotherapy followed by adjuvant TMZ (2,3). The introduction of TMZ as first-line treatment enhanced the quality of life and OS of patients (2). However, TMZ resistance has emerged and there is no standard of care treatment for recurrence (3). The use of chemotherapy for relapse cases yields response rates below 15% (1,4). New therapeutic strategies to overcome treatment failure and improve the OS of GBM patients are required. In this context, the synthetic pterocarpanquinones are promising compounds (5). Among them, LQB-118 has been revealed to have antitumor activity in myeloid leukemia cells, promoting cell death regardless of their resistance mechanisms (5-8). Likewise, LQB-118 cytotoxicity was demonstrated in prostate cancer cells resistant to androgen-based therapy, *in vitro* and *in vivo* (9,10). The basic chemical property of the compound may involve reduction of the paranaphtoquinone moiety in the mitochondria, producing ROS or a reaction product that acts like an alkylating agent (5). A comprehensive toxicology study demonstrated good tolerability of LQB-118 as confirmed by the absence of clinical, biochemical, or hematological parameter changes (11). Furthermore, the dose that induced subacute toxicity was 5 times higher than the therapeutic dose used to treat prostate xenografts in nude mice (11,12). LQB-118 also induced reactive oxygen species (ROS) formation and apop-

totic cell death by the intrinsic and endoplasmic reticulum stress pathways (10,12). The compound regulated NF-κB, FOXO3a and FOXM1 transcription factors without toxicity to mouse bone marrow-derived cells (8,13). MAPKs and Akt pathways are regulators of the aforementioned transcriptional factors and have been linked to GBM heterogeneity, invasiveness and treatment resistance (14-16). Therefore, understanding LQB-118 effects and mechanism of action has the potential to improve therapeutic strategies. The present study evaluated the antitumor activity of LQB-118 as a monotherapy and combined with radiotherapy or chemotherapy in GBM monolayer and spheroid models.

Materials and methods

Cell lines. Human GBM cell lines, U251-MG, T98G and A172 were kindly provided by Dr Vivaldo Moura-Neto. Cells were maintained in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM-F12; Gibco®; Thermo Fisher Scientific, Inc.) supplemented with penicillin (100 UI/ml), streptomycin (0.1 mg/ml) and 10% fetal bovine serum (FBS) as a monolayer at 37˚C with 5% CO_2. Cells were tested and authenticated by DNA (STR) profiling and periodically tested for Mycoplasma. Cells seeded at $3x10^4$ cells/cm^2 were used for all 2D experiments. Cells were allowed to adhere to culture flasks overnight before drug treatments and experimental analysis.

Compound handling. The synthetic compound, LQB-118 was developed by the Laboratory of Bioorganic Chemistry at the Natural Products Research Institute (IPPN) of the Federal University of Rio de Janeiro (UFRJ) (5). LQB-118 and TMZ (ITF chemical, Brazil) were stocked in powder form while cisplatin was stocked as a solution, all at room temperature (RT). TMZ was diluted in dimethyl sulfoxide (DMSO; cat. no. D2650; Sigma-Aldrich®; Merck KGaA) immediately before use. LQB-118 was diluted in DMSO and stored at -20˚C for no longer than two weeks. DMSO was used as a vehicle control for all experiments.

MTT assay. Cells were incubated with different concentrations of TMZ (5.0, 25.0, 50.0, 100.0, 250.0 and 500.0 μM) or LQB-118 (3.0, 6.0, 9.0 and 12.0 μM) for 24, 48 and 72 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; cat. no. 20395; SERVA) was added 4 h before the end of incubation. The formazan crystals formed were eluted in DMSO and the absorbance was measured at 492 nm using a Beckman Coulter DTX800 multimode spectrophotometer. Optical density of control cells was considered as 100% of viability. Three independent experiments were performed with four replicates for each experimental condition.

Trypan blue exclusion assay. Cells were treated with TMZ (50.0 and 100.0 μM) or LQB-118 (6.0 and 12.0 μM) for 24 and 48 h. After treatment, the supernatant was collected, cells were washed with phosphate-buffered saline (PBS) and detached using trypsin 0,125% (cat. no. 27250018; GIBCO®; Thermo Fisher Scientific, Inc.). Trypan blue was added to all collected cells (floating and detached by trypsin cells). Cells stained blue (trypan blue-positive cells) and not stained blue (trypan blue-negative cells) were counted under an optical microscope. The percentage of trypan blue-negative cells (assumed as viable cells) was calculated relative to the control, which was considered 100%. Additionally, the amount of trypan blue-positive cells (assumed as dead cells) was calculated relative to the total number of cells present in each condition.

Annexin V/Propidium iodide (PI) assay. Cells were treated with TMZ (50.0 and 100.0 μM) or LQB-118 (6.0 and 12.0 μM) for 24 and 48 h. After treatment, the cells were washed with PBS and detached from culture flasks using trypsin 0,125% (Gibco®; Thermo Fisher Scientific, Inc.). Then, all collected cells (floating and detached by trypsin cells) were centrifuged (700 x g) and incubated with PBS supplemented with 2% bovine serum albumin (BSA) for 30 min at RT. After washing, the cells were incubated with Annexin V-Alexa Fluor® 488 conjugated (cat. no. A13201; Invitrogen™; Thermo Fisher Scientific, Inc.) for 15 min in the dark at RT. PI was added before event acquisition and used to differentiate non-apoptotic cell death (Annexin V^-/PI^+). The drug-induced apoptotic rate (Annexin V^+/PI^- and Annexin V^+/PI^+) was compared to the control (spontaneous apoptosis). Events (10,000) were acquired using Cyan ADP (Beckman Coulter) and data were analyzed using Summit 4.3 software (Beckman Coulter Inc.). Three independent experiments were performed.

Western blot analysis. Cells were treated with TMZ (50.0 and 100.0 μM) or LQB-118 (6.0 and 12.0 μM) for 24 h. After detachment by trypsin and washing with PBS, the cells were lysed with Cell Extraction Buffer (cat. no. FNN0011; Invitrogen™; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Protein concentration was determined by Lowry method using a commercial Kit (DC™ Protein Assay; cat. no. 500-0116; Bio-Rad Laboratories, Inc.). Western blot analysis using 30 μg of protein was performed with 12% SDS-PAGE and transferred to Hybond-P membranes (cat. no. 29047575; GE Healthcare®) and immunoblotted. The membranes were blocked with nonfat milk 5% for 1 h, washed with Tris-buffered saline (TBS)-Tween 0.2%, and incubated with primary antibodies (Abs) diluted in nonfat milk overnight at 4˚C. The following day, the membranes were incubated with secondary antibodies for 1 h at RT. Primary antibodies used were anti-Akt (1:1,000 dilution; rabbit polyclonal antibody (pAb); cat. no. 9272), anti-p-Akt (1:1,000 dilution; phosphorylation at Ser473; rabbit pAb; cat. no. 9271), anti-p38 MAPK (1:1,000 dilution; rabbit pAb; cat. no. 9212), anti-p-p38 MAPK (1:1,000 dilution; phosphorylation site: T180/Y182; rabbit pAb; cat. no. 9211), anti-ERK1/2 (p44/42MAPK) (clone 137F5; 1:1,000 dilution; rabbit monoclonal antibody (mAb); cat. no. 4695; all from Cell Signaling Technology, Inc.), anti-p-ERK1/ERK2 (clone 15H10L7; 1:1,000 dilution; phosphorylation at Thr185, Tyr187; rabbit mAb; cat. no. 700012; ABfinity™; Invitrogen™; Thermo Fisher Scientific, Inc.), anti-NRF2 (clone C-20; 1:1,000 dilution; rabbit pAb; sc-722; Santa Cruz Biotechnology, Inc.), anti-pro-caspase-7 (clone MCH3101; 1:1,000 dilution; cat. no. MAB823; R&D Systems®), anti-PARP (1:1,000 dilution; rabbit pAb; cat. no. 9542 Cell Signaling Technology®, Inc.) and anti-HSC70 (1:1,000 dilution; mouse mAb (B-6); cat. no. sc-7298; Santa Cruz Biotechnology, Inc.). Secondary antibodies were anti-mouse IgG and anti-rabbit IgG-HRP

conjugated (1:20,000 dilution; Amersham ECL™ Western blotting Detection Reagents; GE Healthcare®; cat. nos. respectively, A9169 and A9044). ECL Prime Detection System (cat. no. RPN2236; Amersham Biosciences™; GE Healthcare) was applied for protein detection by C-Digit™ Blot Scanner, generating images in Image Studio Lite software v3.1 (LI-COR Biosciences®). Three independent experiments were performed and analyzed qualitatively. Protein expression was normalized by HSC70 expression.

Spheroid model three-dimensional (3D) culture. Multicellular tumor spheroids were formed from the cell lines U251-MG and A172 using the liquid-overlay technique (17). Cells (200 μl) (10^4 cells/ml) in DMEM-F12 medium supplemented with 10% of FBS were seeded in a 96-well flat bottom plate previously coated with 1.5% agarose type II (cat. no. A6877; Sigma-Aldrich®; Merck KGaA). The outer wells were filled with PBS to avoid evaporation. Cells were cultured for 4 days until tightly aggregated spheroids of ~300 μm of diameter were fully formed.

Acid phosphatase (APH) assay (3D culture). Cell viability in spheroids was assessed using the APH (cat. no. 71768; Sigma-Aldrich®; Merck KGaA) assay (18). Fully formed spheroids were treated with different concentrations of LQB-118 (3.0, 6.0, 9.0 and 12.0 μM) or TMZ (25.0, 50.0, 100.0 and 200.0 μM) for 72 h. Then, spheroids were transferred to a 96-well plate without an agarose coat and washed twice to remove the medium. Substrate solution containing nitrophenylphosphate (2 mg/ml) and Triton X-100 in citrate buffer (0.1M), diluted in PBS, was added and incubated for 90 min in an incubator at 37˚C . Then, NaOH (1M) was added and the absorbance measured at 405 nm using Beckman Coulter DTX800 multimode spectrophotometer. Optical densities were normalized using the absorbance of spheroids treated with DMSO as a control. The experiment was repeated at least 3 times with 8 replicates each.

Migration assay (3D culture). Spheroids were transferred to a 24-well plate coated with 0.1% gelatin and culture medium containing LQB-118 (1.0 and 3.0 μM) or TMZ (25.0, 50.0, 100.0 and 200.0 μM). Migration was assessed after 24, 48 and 72 h. To avoid cell proliferation, a medium containing only 2% FBS was used in the experiments, carried out in triplicate. Quantification of the migration index was performed manually using the software ImageJ v.1.5 (19) and the migration index was calculated using the following formula: (Area of the bigger halo)/(area of the spheroid at 0 h).

Drug interaction analysis. U251-MG and A172 cells were treated with LQB-118 (3.0 and 6.0 μM) combined with ionizing radiation (4 Gy), TMZ (50 and 100 μM) or cisplatin (CDDP, 3.0 and 30.0 nM) for 48 h. Cell viability and cell death were evaluated by MTT, trypan blue exclusion and Annexin V/PI assays as aforementioned. The significance of concurrent combination treatment by MTT was evaluated by combination index (CI) value calculated by CompuSyn software (ComboSyn, Inc; www.combosyn.com) derived from the Chou-Talalay method (20,21). Statistical analysis of trypan blue exclusion and Annexin V/PI were realized by GraphPad Prism software version 5.0 (GraphPad Software® Incorporated) as the limited number of conditions did not allow synergism analysis.

Statistical analysis. Statistical and graphical information was determined using the GraphPad Prism software version 5.0. One-way ANOVA test followed by Bonferroni post hoc test was used to compare treatment groups. Significant variance of ANOVA F-values ranged from 4.63 to 49.61. DMSO was used as a reference group to determine statistical significance and P-values were reported at 95% confidence intervals. A P-value of <0.05 was considered to indicate a statistically significant difference and denoted as $P<0.05$. Statistical significance in the figures was represented by $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. The results are presented as the mean of independent experiments ± standard error.

Results

LQB-118 reduces cell viability and induces apoptosis in three GBM cell lines. U251-MG, A172 and T98G cells were treated with different concentrations of LQB-118 to assess cell line sensibility. Initially, LQB-118 reduced cell viability by MTT of all cell lines (Fig. 1). LQB-118 6.0 μM significantly reduced U251-MG and A172 cell viability after 48 and 72 h, but not of T98G cells (Fig. 1A-C). Higher concentrations of LQB-118 (9.0 and 12.0 μM) reduced cell viability by almost 90% after 48 and 72 h of treatment in U251-MG and A172 (Fig. 1A and B). In T98G cells, LQB-118 9.0 and 12.0 μM reduced cell viability by 60 and 80%, respectively (Fig. 1C). Therefore, a concentration that had a moderate effect (6.0 μM) and one (12.0 μM) that demonstrated a high reduction of cell viability were selected to perform further experiments for 24 and 48 h of treatment. Corroborating the viability results, LQB-118 6.0 and 12.0 μM reduced cell viability by approximately 50 and 80%, respectively, as quantified by trypan blue exclusion assay (Fig. 1D). However, only 12.0 μM induced cell death (Fig. 1E). Furthermore, cell detachment from culture flasks was observed in all cell lines after treatment with 12.0 μM, but not in the controls and LQB-118 6.0 μM groups (Fig. S1). Apoptotic cell death induction by LQB-118 was evaluated by Annexin V/PI labeling (Fig. 2). In U251-MG and T98G cells, only 12.0 μM induced significant 60% Annexin V labeling after 48 h (Fig. 2A and C). A172 cells were sensitive to 9.0 and 12.0 μM, presenting 40 and 80% of Annexin V labeling after 48 h, respectively (Fig. 2B). The collective results demonstrated the antitumoral activity of LQB-118 against three GBM-derived cell lines.

Temozolomide has a minor cytotoxic effect in GBM cell lines. U251-MG, A172 and T98G sensibility to first line chemotherapy were evaluated by MTT. The highest TMZ concentration, 500 μM, reduced cell viability in U251-MG cells while no effect was observed in A172 and T98G cells (Fig. 3A-C). In the studied conditions, the cell lines demonstrated a resistance profile to TMZ while they were sensitive to LQB-118. The conventional dose schedule of TMZ reaches ~50 μM in plasma (22). Therefore, 50.0 and 100.0 μM were selected for further experiments in U251-MG cells since this cell line has been revealed to be tumorigenic in nude mice and resistant to TMZ by p38/NRF2 axis activation (15,23).

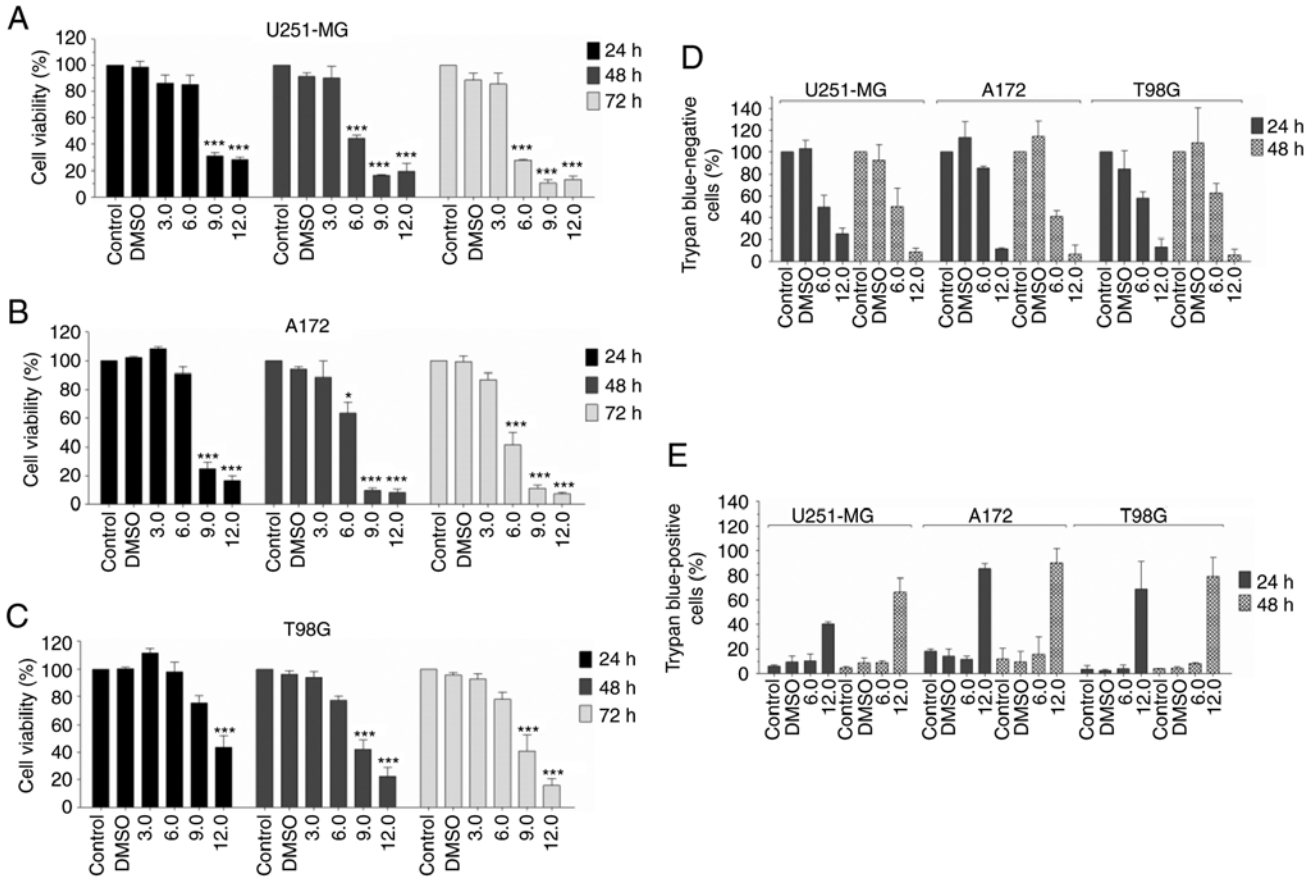

Figure 1. Effect of LQB-118 on GBM cell line viability. Percentage of (A) U251-MG, (B) A172 and (C) T98G viable cells after screening of increasing concentrations of LQB-118 evaluated by MTT. Mean of three independent experiments ± SEM. *P<0.05; ***P<0.001. (D) Percentage of trypan blue-negative cells relative to the control and (E) percentage of trypan blue-positive cells in each treatment condition. Mean of two independent experiments ± SD. GBM, glioblastoma.

TMZ did not induce cell detachment from culture flasks, nor cell death by trypan blue exclusion assay or Annexin V labeling, but induced a slight reduction in pro-caspase-7 (Fig. 3D-G). Furthermore, treatment with LQB-118 induced caspase-7 activation as suggested by the reduced expression of its pro-caspase form (Fig. 3G). LQB-118 also promoted PARP cleavage in U251-MG and A172 cells (Fig. 5). These results corroborated apoptosis induction by the compound LQB-118.

LQB-118 is cytotoxic and reduces cell migration in spheroids of GBM cell lines. A 3D cell culture system is a great tool for drug screening (24). LQB-118 antineoplastic activity was also evaluated in 3D cultures. LQB-118 concentrations of 9.0 and 12.0 μM significantly reduced U251-MG spheroid viability, while TMZ did not (Fig. 4A). Cell lines maintained the resistance to TMZ observed in the monolayer and LQB-118 maintained its cytotoxicity, overcoming TMZ resistance. GBM is highly infiltrative, therefore, the ability of LQB-118 to impair cell migration was evaluated in a 3D culture. Lower LQB-118 concentrations were used (1.0 and 3.0 μM) to guarantee that the effect observed was derived exclusively from migration inhibition and not an artefact of proliferation inhibition or death induction. The results revealed that LQB-118 significantly reduced the migratory potential of GBM cells while TMZ had no effect, corroborating the LQB-118 effectiveness in GBM cells (Fig. 4B and C). A similar profile was observed for A172 spheroids (Fig. 4D-F).

Cytotoxic effect of LQB-118 is associated with downregulation of survival pathways. The PI3K/Akt and MAPK pathways are markedly relevant in GBM invasion, progression and treatment resistance (14,25,26). Considering the effect of LQB-118 on cell migration and viability, and to further understand its mechanism, Akt, ERK and p38 pathways were investigated by western blotting. TMZ 50.0 and 100.0 μM did not regulate p38 and total Akt and phosphorylated protein levels, while it slightly reduced ERK phosphorylation in U251-MG cells (Fig. 5A). Conversely, 12.0 μM of LQB-118 reduced total protein expression and phosphorylated levels of p38 and Akt and ERK phosphorylation, while the levels of total ERK were not reduced (Fig. 5A). Furthermore, LQB-118 reduced NRF2 expression and concurrently inhibited MAPK and Akt pathways, reinforcing the potential of LQB-118 against this heterogeneous disease. As aforementioned for the 3D assays, a similar profile was observed for the A172 cell line (Fig. 5B).

Synergic effect of LQB-118 and cisplatin reduces viability and increases apoptosis. GBM patients present resistance, disease progression and recurrence. Salvage therapies are not effective and LQB-118 downregulates radio- and chemoresistance-associated pathways. Therefore, its effect in association with DNA damage inducers, ionizing radiation, CDDP and TMZ was evaluated, in order to obtain a possible less toxic and more effective treatment approach. First, LQB-118 6.0

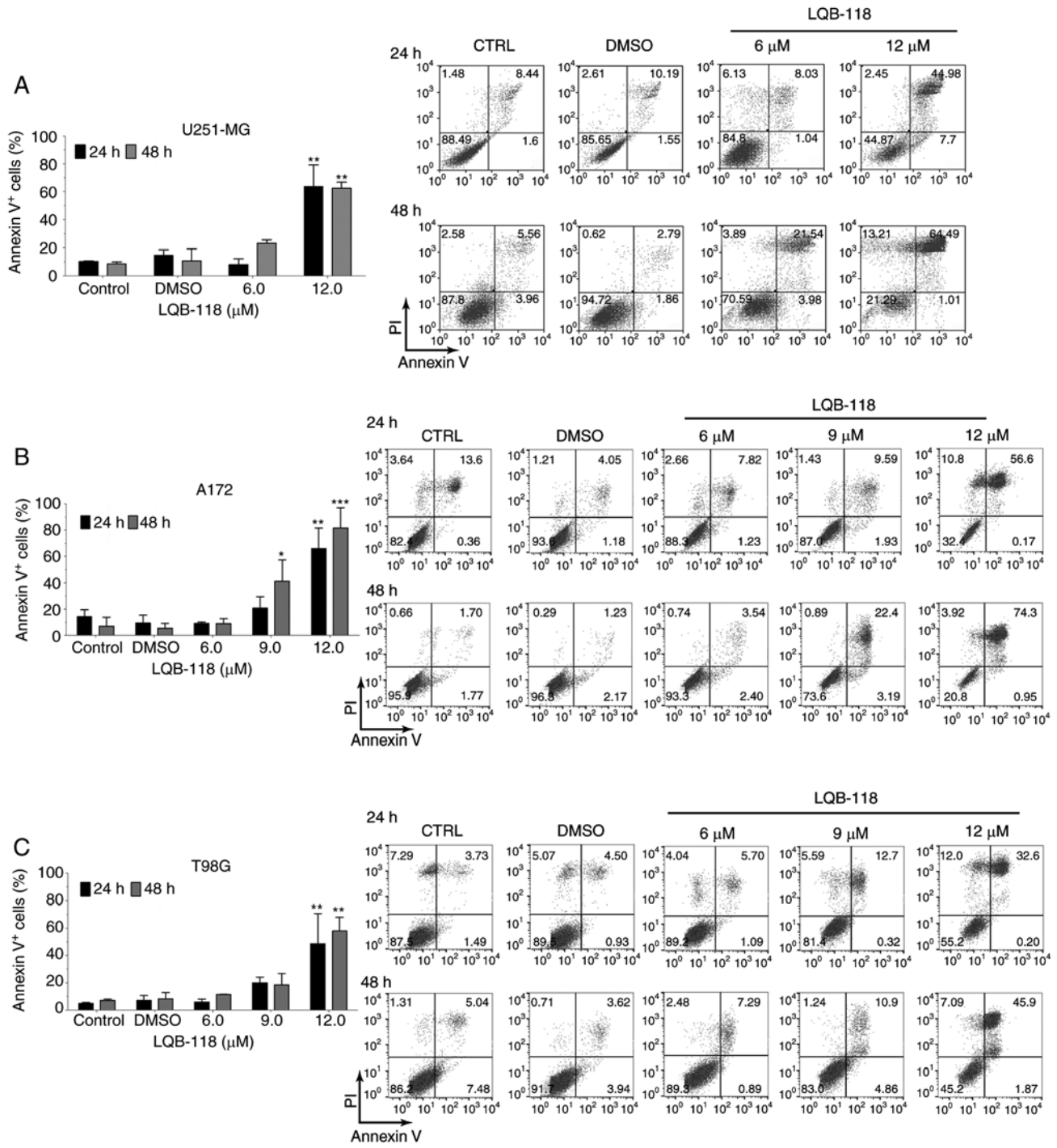

Figure 2. Effect of LQB-118 on GBM cell line apoptosis. Percentage of Annexin V-positive cells (Annexin V$^+$/PI$^-$ + AnnexinV$^+$/PI$^+$) after LQB-118 treatment in (A) U251-MG, (B) A172 and (C) T98G cells evaluated by flow cytometry. Representative dot plots (in the right column) and graphic bars (in the left column) with the mean of three independent experiments ± SD. One-way ANOVA and Bonferroni post hoc test: *P<0.05; **P<0.01; ***P<0.001. ANOVA F-values were 49.51, 25.38 and 24.12 after treatment for 48 h in U251-MG, A172 and T98G cells, respectively. GBM, glioblastoma.

or TMZ 50.0 μM where concurrently used with ionizing radiation. In U251-MG cells, 6.0 μM of LQB-118 combined with 4 Gy significantly reduced cell viability by ~40% as determined by trypan blue exclusion assay in comparison to isolated treatments, while TMZ 50.0 μM with 4 Gy reduced cell viability by ~27% (Fig. 6A). However, this effect was not observed by Annexin V labeling for both treatment strategies (Fig. 6B), demonstrating that LQB-118 and TMZ have similar results when combined with radiotherapy. In A172 cells, 4 Gy of ionizing radiation alone reduced cell viability and no additional effect was observed after combination with LQB-118 or TMZ in these cells (Fig. 6C and D).

Subsequently, LQB-118 was concurrently treated with CDDP or TMZ (Fig. 7). Previous data from our group demonstrated that cisplatin 3.0 μM had no effect while 30.0 μM reduced cell viability by 50% as determined by MTT (unpublished data). Therefore, these concentrations were selected for combination experiments. In U251-MG cells, LQB-118 (3.0

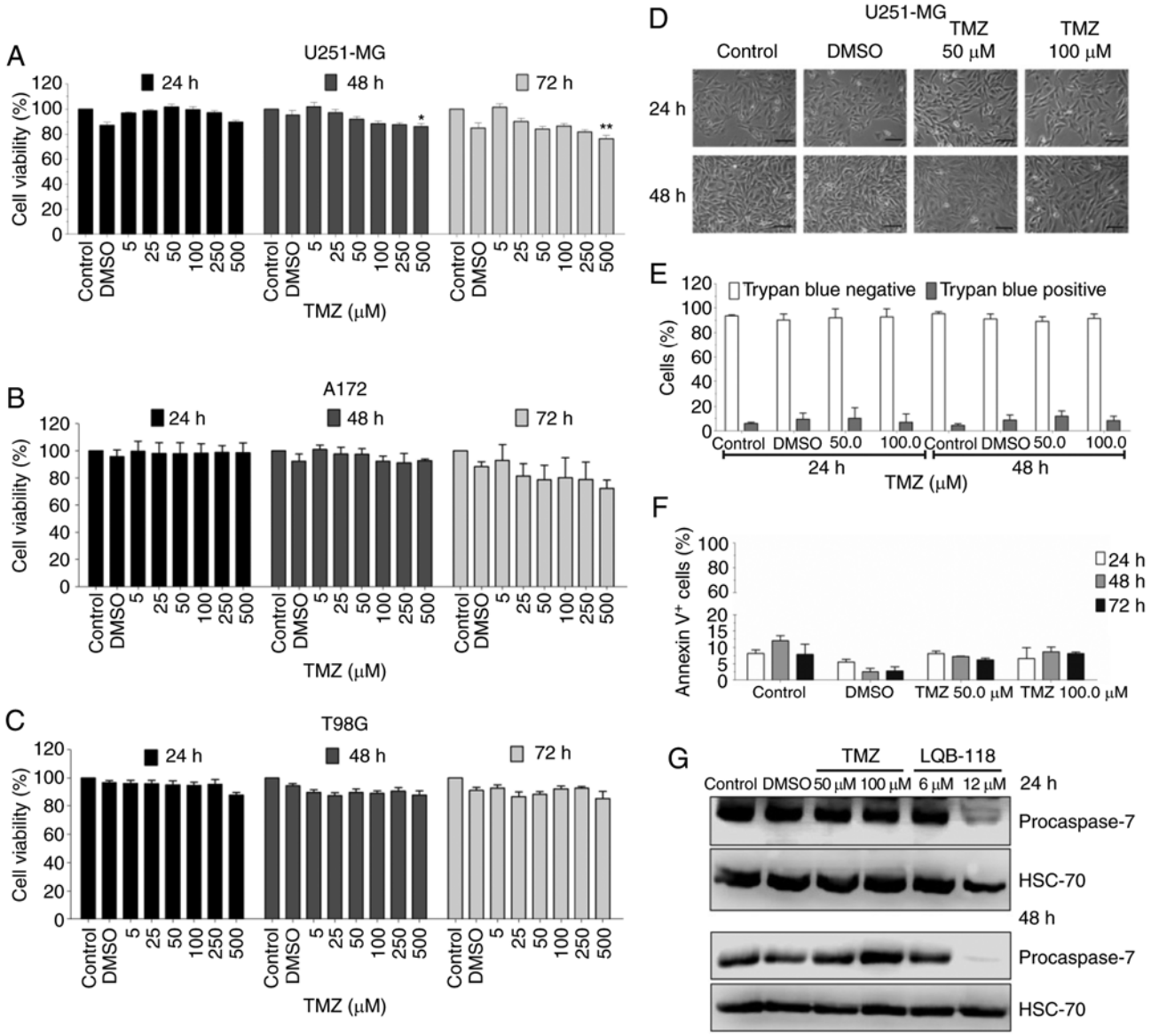

Figure 3. Effect of TMZ on GBM cell viability and cell death. Percentage of (A) U251-MG, (B) A172 and (C) T98G viable cells after treatment with increasing concentrations of TMZ evaluated by MTT. Mean of three independent experiments ± SEM. *P<0.05; **P<0.01. (D) Contrast phase photomicrography, magnified 10 times, of U251-MG cells after treatment with TMZ. Scale bar, 100 μm (E) Percentage of trypan blue-negative cells (cell viability) and trypan blue-positive cells (cell death) in each treatment condition. (F) Percentage of U251-MG Annexin V-positive cells (Annexin V^{+}/PI^{-}+ Annexin V^{+}/PI^{+}) after TMZ treatment evaluated by flow cytometry. (G) Procaspase-7 expression evaluated after U251-MG cells were treated with LQB-118 or TMZ. Images represent two independent experiments. TMZ, temozolomide; GBM, glioblastoma.

and 6.0 μM) demonstrated a synergic effect with CDPP (3.0 and 30.0 μM) and TMZ (50.0 and 100.0 μM) as revealed by MTT after 48 h (Fig. 7A and B; Table SI). The combination of LQB-118 3.0 μM with CDDP 3.0 μM significantly enhanced cell death as demonstrated by trypan blue exclusion and Annexin V/PI assays (Fig. 7C and D). Only 20% of U251-MG cells were viable after combined treatment (data not shown). In A172 cells, LQB-118 3.0 μM demonstrated an additive effect when associated with CDDP 3.0 and 30.0 μM (CI=1.01 and CI=0.95, respectively) (Fig. 7E) and an antagonist effect when treated with TMZ (Fig. 7F; Table SI). No significant enhancement in cell death was observed after treatment with CDPP (Fig. 7G) neither with TMZ (Fig. 7H). However, the combination of LQB-118 (3.0 μM) with CDDP (3.0 μM) reduced cell viability by ~24% as determined by trypan blue exclusion assay (data not shown). These data indicated that LQB-118 combined with CDDP has a notable effect on cell proliferation, revealing LQB-118 as a potential alternative for a subset of patients with disease recurrence.

Discussion

GBM is one of the most aggressive tumors and has a five-year survival rate of only 5.1% (27). TMZ can improve OS, however the majority of patients cannot complete treatment due to toxicity and resistance has become a clinical problem (2,28,29). Despite the therapeutic advances made in recent decades, patients relapse and progress to death. In the context of new drug development to overcome resistance and improve treatment, the present study evaluated the anti-

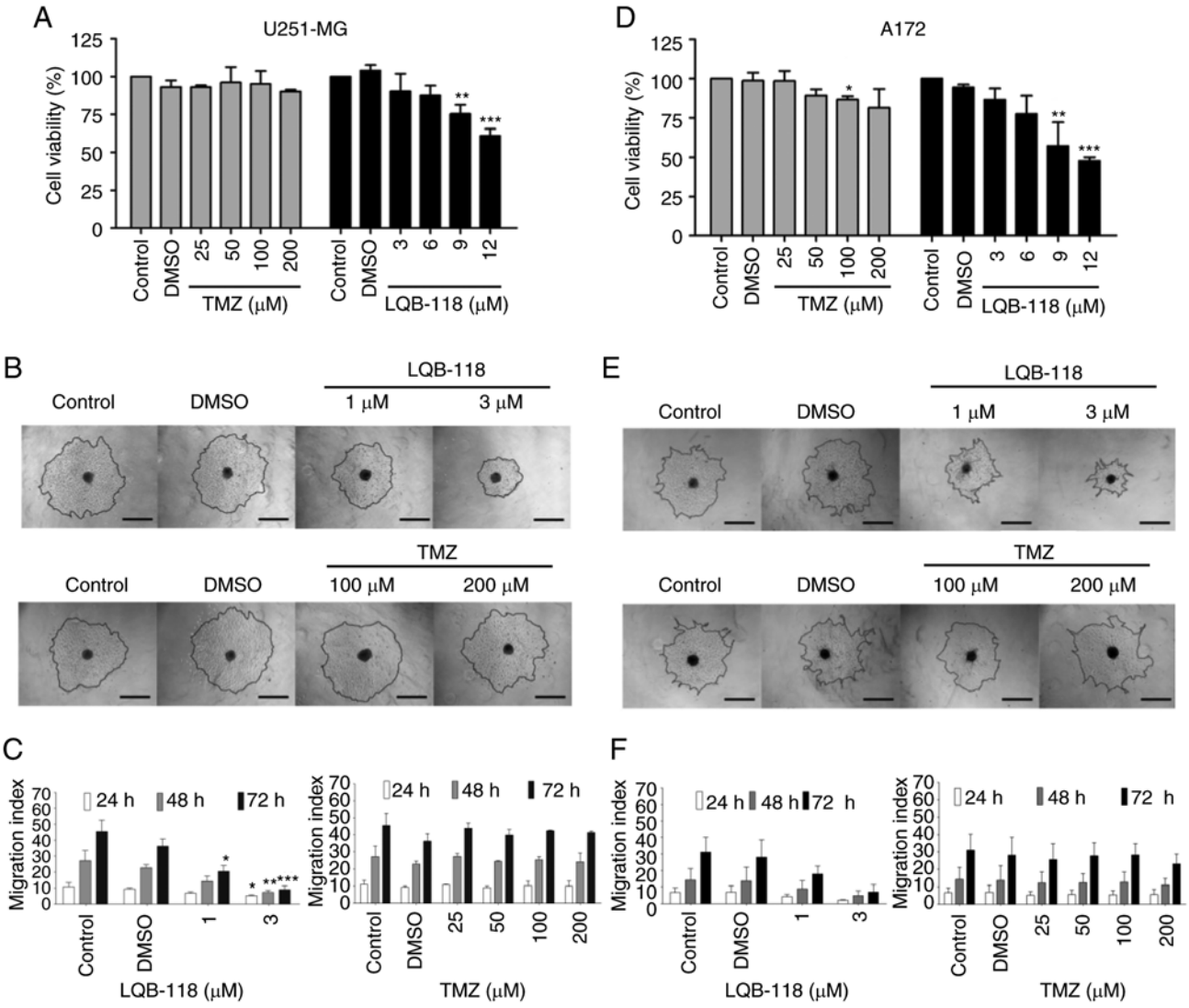

Figure 4. Effect of LQB-118 and TMZ on viability and the migration index of U251-MG and A172 spheroids. Percentage of viable cells in relation to the control after treatment with LQB-118 or TMZ evaluated by APH assay in (A) U251-MG and (D) A172 cells. ANOVA F-values were 18.76 and 18.44 for LQB-118 APH data and 0.45 and 4.63 for TMZ data of U251-MG and A172 cells, respectively. Representative figures of radial migration of spheroids after 72 h of treatment with LQB-118 and TMZ in (B) U251-MG and (E) A172 cells. At 72 h, ANOVA F-values were 34.85 and 5.98 for LQB-118 migration data and 2.06 and 0.32 for TMZ data of U251-MG and A172 cells, respectively. Graphic bars of the migration index after treatment with LQB-118 and TMZ in (C) U251-MG and (F) A172 cells. Mean of three experiments ± SEM. Scale bar, 300 μm. *P<0.05; **P<0.01, ***P<0.001. TMZ, temozolomide; APH, acid phosphatase.

tumoral effect of LQB-118, a synthetic compound. LQB-118 significantly reduced cell viability and induced high levels of apoptosis, while plasmatic concentrations of TMZ did not promote cell death, suggesting an intrinsic resistance of these cells to TMZ. GBM cell line response to TMZ is not uniform in literature mainly due to different treatment procedures and exposure times. In the present study, the same time-points were used for the compound to guarantee an unbiased comparison parameter. Under the studied conditions, LQB-118 induced cell death in different GBM cell lines while TMZ was not efficient in promoting cell death.

Accordingly, LQB-118 induced apoptosis in leukemia cell lines with multidrug resistance (MDR) phenotype (6,7,12) and in androgen-resistant prostate cancer cells (9), demonstrating its great potential to overcome MDR mechanisms in different tumor types. In addition, our group demonstrated the antineoplastic role of LQB-118 in peripheral blood samples obtained from leukemia patients (7), and LQB-118 reduced growth of prostate, melanoma and Erlich tumors, *in vitro* and *in vivo* (9,10,30). Moreover, LQB-118 presented no toxicity for bone marrow and spleen cells from healthy mice, primary and secondary organs of the immune system and activated human PBMC (peripheral blood mononuclear cells). These data demonstrate its selectivity for tumor cells and great potential for treatment of patients non-responsive to conventional therapy (8,30,31).

A preclinical study evaluated LQB-118 subacute toxicity and oral administration did not demonstrate clinical signs of toxicity (11). A dose, five times higher than the therapeutic dose induced liver focal necrosis, which was not accompanied by alterations in hepatic enzymes (11). A theoretical analysis of the pharmacokinetic properties of LQB-118 demonstrated it does not violate the Lipinski's rule of five (Ro5) and has a favorable profile, being more likely to progress to market (11). Furthermore, LQB-118 has 96% probability of crossing the blood brain barrier (BBB) and 100% probability of human

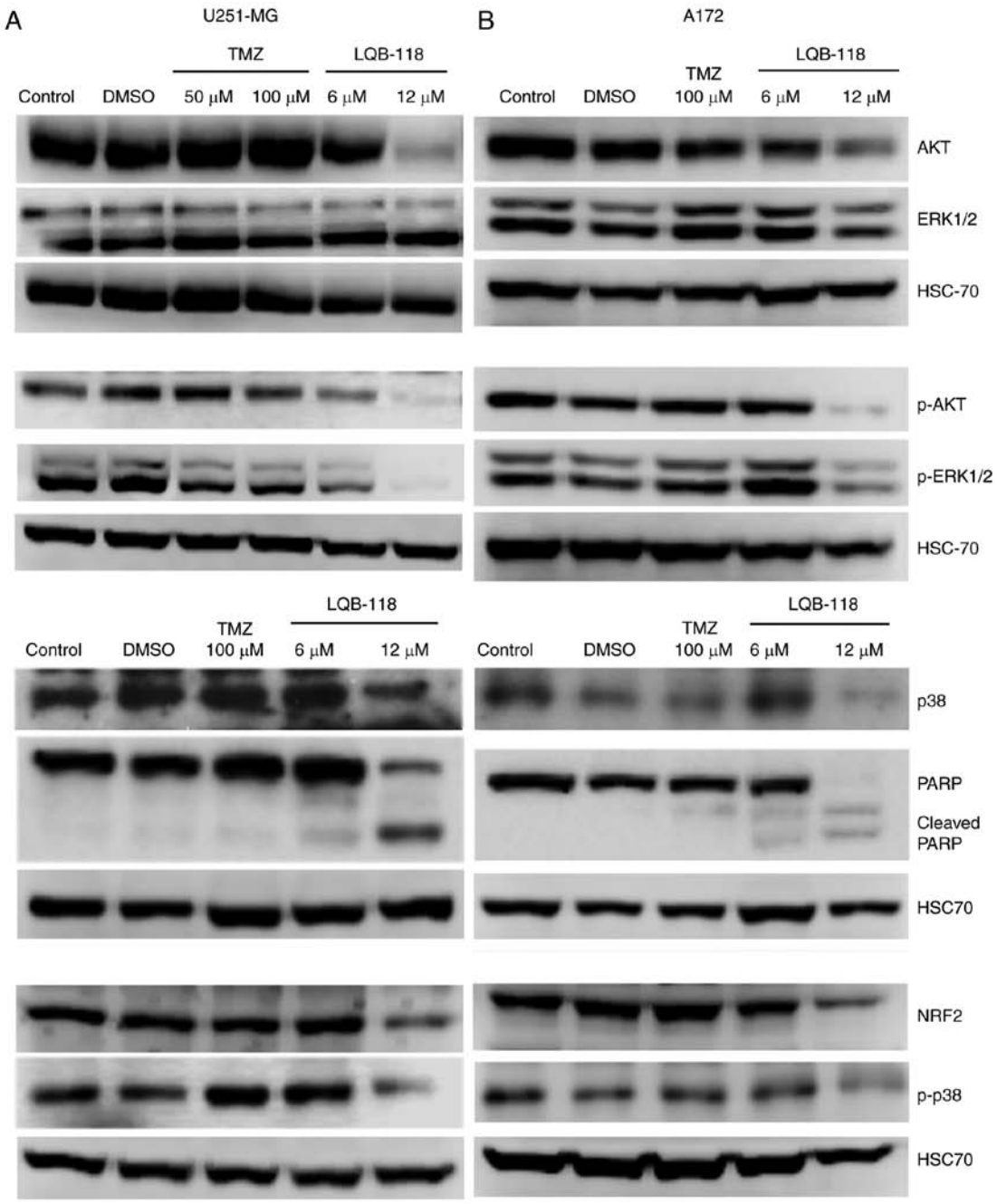

Figure 5. Effect of LQB-118 and TMZ on AKT and MAPK signaling pathways. Akt, p-Akt, ERK1/2, p-ERK1/2, p38, p-p38, NRF2 and PARP expression levels were analyzed after LQB-118 and TMZ treatment for 24 h in a monolayer culture of (A) U251-MG and (B) A172 cell lines as determined by western blotting. Images represent three independent experiments. TMZ, temozolomide.

intestine absorption (11). Corroborating the potential in crossing the BBB, LQB-118 is effective in cells overexpressing P-glycoprotein (Pgp) and multidrug resistance protein (MRP) with enhanced efflux pump activity (7,12). These proteins are one of the most important components of BBB, protecting the brain from xenobiotics in physiological conditions. In the tumor context, the identification of modifications in BBB developed the concept of blood-brain tumor barrier (BBTB). The BBTB is characterized by angiogenesis, which generates abnormal leaky vessels and BBTB disruption allowing tumor infiltration in parenchyma among other alterations (32). LQB-118 is a potential drug to overcome BBTB protection, promoting an effective treatment and sensitizing tumor cells to conventional therapy. Accordingly, using an *in vivo* model of glioblastoma would be relevant to further confirm the potential of LQB-118 against this lethal malignancy.

Cell-based assays are important tools for novel compound identification. However, most assays used to assess the biological activity of novel compounds rely on traditional two-dimensional (2D) cell culture, having experimental limitations. The 3D architecture creates an environment with oxygen and nutrient gradients that ultimately alters gene expression resembling the tumor gene profile and microenvironment (33-35). The 3D cell culture system often exhibits different sensitivity to treatment, being better predictors of drug response (36). In the present study, LQB-118 maintained

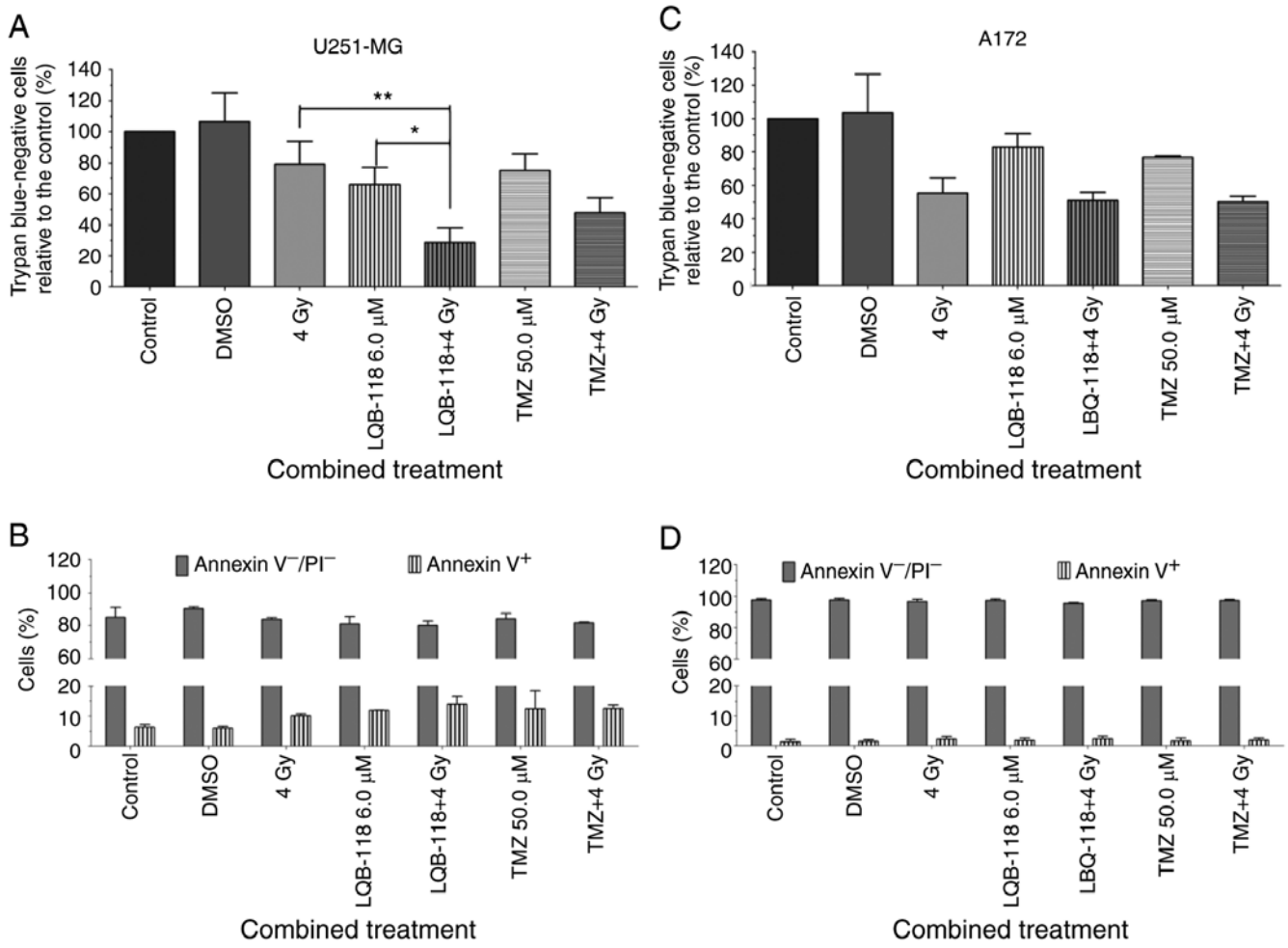

Figure 6. Effect of LQB-118 or TMZ combined with ionizing radiation on cell viability and cell death. (A) Percentage of U251-MG and (C) A172 trypan blue-negative cells relative to the control after treatment with LQB-118 or TMZ combined with ionizing radiation for 48 h. Percentage of (B) U251-MG and (D) A172 Annexin V/PI-negative cells (viable cells) and Annexin V-positive cells (Annexin V^+/PI^- + AnnexinV^+/PI^+) evaluated by flow cytometry. Mean of 3 independent experiments ± SD. TMZ, temozolomide.

the cytotoxic effect observed in 2D cultures when assessed in spheroids. Furthermore, low doses of LQB-118 that did not reduce cell viability neither induce cell death, where able to reduce the migration of spheroids as observed in a 3D conformation. These data support the effect of LQB-118 exclusively on cell migration in the concentrations of choice. Migration, observed in a 3D conformation, partially mimics the ability of cells to invade the parenchyma since the assay requires cells to move in a semisolid medium without any chemoattractive factor to induce it. Furthermore, GBM invades the surrounding parenchyma, but does not evade the central nervous system, reinforcing the importance to evaluate migration capacity more than invasion by conventional assays since this tumor does not systemically metastasize. Therefore, migration inhibition is an important mechanism to impair parenchyma infiltration by tumor cells, the main reason for disease recurrence after tumor resection.

Previous literature has indicated an important role for mitochondrial metabolism in LQB-118 activity. The paranaphtoquinone moiety is reduced in the mitochondria and the resulting product can act like an alkylating agent or transfer electrons to molecular oxygen, producing ROS and inducing lipid peroxidation, *in vitro* (5,10,12). Considering that an MTT assay evaluates mitochondrial enzyme activity, this could explain the intensified effect of LQB-118 observed in GBM. Corroborating our findings of Akt and MAPK pathway down-regulation, our group demonstrated that FOXM1, FOXO3a and NF-κB transcriptional factors are regulated by LQB-118 in leukemic cell models (6,8,13). Therefore, LQB-118 may be regulating the transcription factors by PI3K/AKT and MAPK pathway inhibition or may be regulating these pathways indirectly by endoplasmic reticulum (ER) stress due to protein misfolding culminating with apoptosis.

GBM is a highly heterogeneous disease and p53, receptor tyrosine kinase (RTK), PI3K/PTEN and MAPK are core pathways in gliomagenesis (25,37). The selected cell lines present survival pathways constitutively activated at different levels, in which T98G and U251-MG cells are TP53 mutated whereas A172 is TP53 wild-type (23). Strategies that combine the inhibition of ERK and PI3K pathways, concurrently, have been demonstrated to be more efficient for glioma treatment (38,39). Recent literature studies have demonstrated that the p38/NRF2 axis is associated with TMZ resistance (15,40). PI3K/AKT and MAPK inhibition and NFR2 knockdown was revealed to sensitize GBM cells to radiation and TMZ treatment (15,38,40,41). The present study demonstrated that

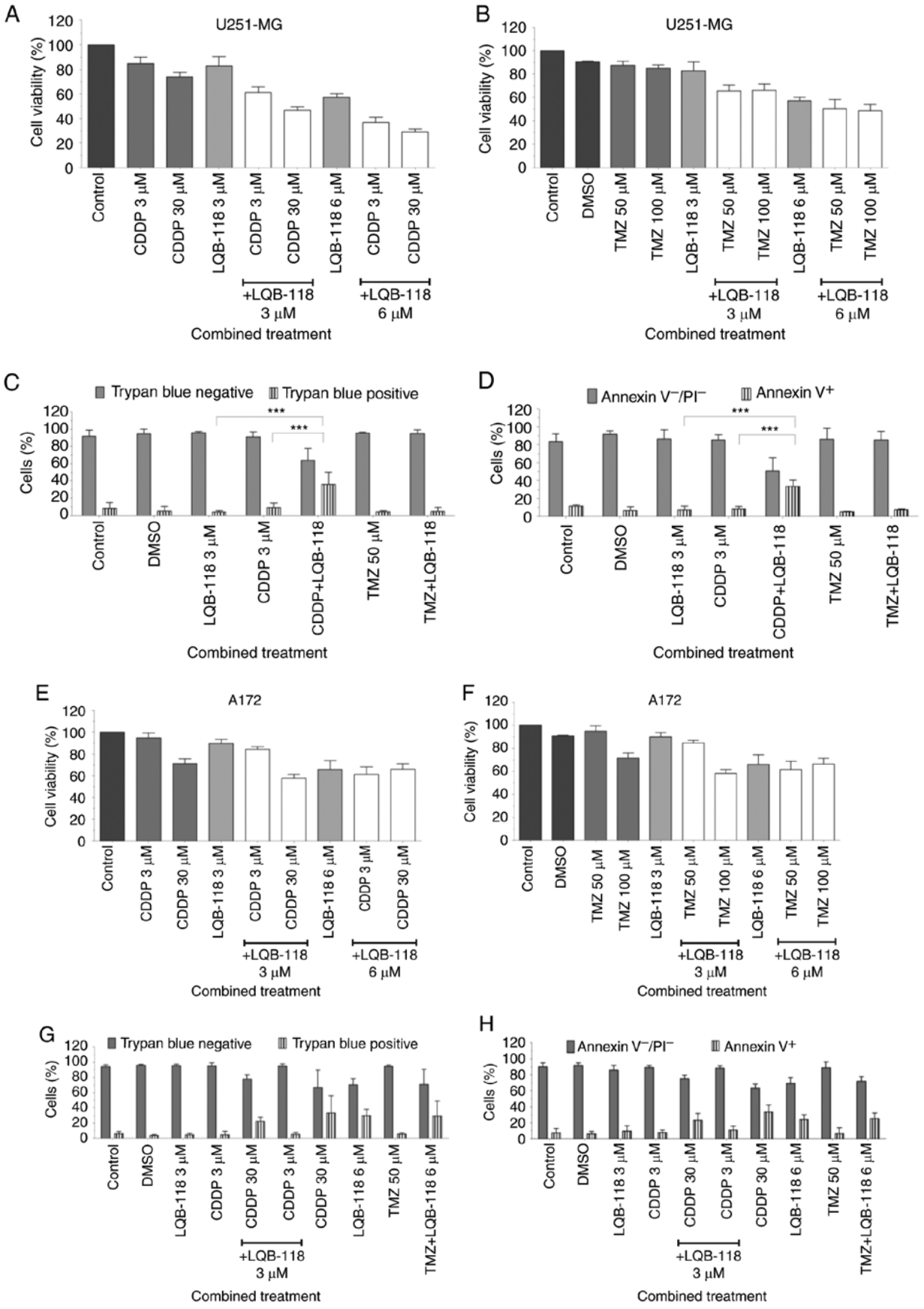

Figure 7. Effect of LQB-118 combined with chemotherapeutic agents on cell viability and cell death. Combined treatment of LQB-118 with CDDP or TMZ in U251-MG and A172 cells for 48 h. Percentage of U251-MG and A172 viable cells after treatment with LQB-118 combined with (A and E) CDDP and (B and F) TMZ as determined by MTT. Mean of three independent experiments ± SEM. Percentage of trypan blue-negative and -positive cells in each treatment condition of (C) U251-MG cells and (G) A172 cells. Percentage of Annexin V/PI-negative cells (viable cells) and Annexin V-positive cells (Annexin V^+/PI^- + Annexin V^+/PI^+) in (D) U251-MG and (H) A172 cells as determined by flow cytometry. Mean of three independent experiments ±SD. CDDP, cisplatin; TMZ, temozolomide.

LQB-118 concurrently inhibited ERK, Akt and p38 activation, followed by reduction of NRF2 levels. These data indicate a potential effect of LQB-118 to sensitize cells to TMZ and radiation, mainly because there is no standard of care for GBM relapse. Therefore, it was determined whether LQB-118 combined with ionizing radiation or chemotherapy would be more effective, *in vitro*. The results demonstrated that LQB-118 treatment used with radiation reduced cell viability, while combination with low doses of cisplatin significantly enhanced cell death. This effect was cell line-dependent, demonstrating the importance of understanding the molecular basis of the disease for treatment decision. These data demonstrated that LQB-118 has a potent cytotoxic effect as monotherapy as well as combined with other antitumor agents, mainly cisplatin.

Collectively, the present data provided consistent evidence of the effectiveness of LQB-118 against GBM cell lines in a monolayer and 3D conformation. The cytotoxic effect was associated with the inhibition of PI3K and MAPK pathways, well described resistance pathways mutated in 60% of GBM cases (25). This is the first study to demonstrate the effect of LQB-118 in GBM as monotherapy and in combination, revealing a potential therapeutic alternative for patients resistant to standard protocol.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

PSB was responsible for the study design, cell line monolayer experiments and manuscript writing. GHCG and FCCF performed western blotting and GMCL performed spheroid experiments. GPFL provided insights in the study design and revised it critically for important intellectual content. PRRC and CDN synthetized and provided LQB-118, revised the final version and agreed to be accountable for all aspects of the work. RCM was responsible for study conception and orientation. All authors critically revised and approved the final manuscript.

Ethics approval and consent to participate

The present study does not contain any studies with human participants or animals performed by any of the authors.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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