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Tamoxifen in combination with temozolomide induce a synergistic inhibition of PKC-pan in GBM cell lines

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ABSTRACT

Background: Glioblastoma (GBM) is a highly proliferative, angiogenic grade IV astrocytoma that develops resistance to the alkylating agents used in chemotherapy, such as temozolomide (TMZ), which is considered the *gold standard*. The mean survival time for GBM patients is approximately 12 months, increasing to 14.6 months after TMZ treatment. The resistance of GBM to chemotherapy seems to be associated to genetic alterations and to the constitutive activation of several signaling pathways. Therefore, the combination of different drugs with different mechanisms of action may contribute to circumvent the chemoresistance of glioma cells. Here we describe the potential synergistic behavior of the therapeutic combination of tamoxifen (TMX), a known inhibitor of PKC, and TMZ in GBM.
Methods: We used two GBM cell lines incubated in absence and presence of TMX and/or TMZ and measured cell viability, proliferation, apoptosis, cell cycle, migration ability, cytoskeletal organization and the phosphorylated amount of the p-PKC-pan.
Results: The combination of low doses of TMX with increasing doses of TMZ shows an increased antiproliferative and apoptotic effect compared to the effect with TMX alone.
Conclusions: The combination of TMX and TMZ seems to potentiate the effect of each other. These alterations seem to be associated to a decrease in the phosphorylation status of PKC.
General significance: We emphasize that TMX is an inhibitor of the p-PKC-pan and that these combination is more effective in the reduction of proliferation and in the increase of apoptosis than each drug alone, which presents a new therapeutic strategy in GBM treatment.

1. Introduction

Glioblastoma (GBM) is the most common and the most aggressive type of primary brain tumor, comprising almost 50% of all diagnosed glioma [1–3]. Glioma cells show a very high rate of proliferation, are highly resistant to apoptosis and have an increased ability to migrate, characteristics sustained by the constitutive activation of several signaling pathways, cytokine and chemokine production [4] and catepsin activity [5], among others. The local migration of glioma cells turns impossible the complete resection of the tumor and consequently also contributes to the reduced mean survival time of GBM patients. Without treatment, the mean survival time of GBM patients is 9 to 12 months, which improved when patients began to receive chemotherapy with temozolomide (TMZ). Since then, the mean survival rate has increased to 14.6 months, and the percentage of patients that survive for 2 years has increased by 17% compared to patients not treated with TMZ [6]. TMZ is the leading compound in a new class of chemotherapeutic alkylating agents that enter the cerebrospinal fluid and do not require hepatic metabolism for activation [7]. This alkylating agent induces

Abbreviations: GBM, glioblastoma; MGMT, O6-methylguanine-DNA methyltransferase; TMX, tamoxifen; TMZ, temozolomide; PKC, protein kinase C; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PGP, (P-glycoprotein); SERM, non-steroidal selective estrogen receptor modulator

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the formation of O^6-methylguanine in DNA, which mispairs with thymine during the DNA replication, leading to activation of the apoptotic pathways. Taking these characteristics into consideration, it was expected that TMZ would increase very significantly the survival of GBM patients [8–10]. According to previous studies, the limited success of TMZ in GBM treatment appears to be related to the activity of O^6-methylguanine-DNA methyltransferase (MGMT) and to the occurrence of gene mutations that cause permanent activation of several survival signaling pathways, such as the AKT, ERK1/2 MAP kinase and also protein kinase C (PKC) [11–13].

PKC is a family of serine/threonine-specific protein kinases that are involved in the development of many tumors, due to their ability to regulate signaling pathways involved in cellular transformation, proliferation, survival and migration. Nevertheless, some studies have reported that several PKC isoforms may also act as tumor suppressors since they can activate pro-apoptotic pathways [14]. Because of the variability of cell functions controlled by PKC isoforms, the contribution of this kinase family to the development of GBM is poorly understood, and its contribution to glioma cell proliferation, apoptosis and migration is not fully elucidated [14–16]. One of the PKC inhibitors is tamoxifen (TMX), which has an anti-angiogenic effect, low toxicity, minimal side effects, low cost and the capability to cross the blood-brain barrier [9,17–20]. Previous studies reported in several types of tumor cells that TMX induces apoptosis and reduces tumor cell migration in a PKC-dependent manner [21–23]. In glioma cell lines, the results with TMX were controversial and the clinical trials reported that TMX increased the survival of GBM but in a reduced percentage of patients [9,24–26].

Taking into account that the activity of PKC in glioma cells is increased, the effect of TMX in glioma cells was not fully understood, the effect of TMZ in GBM patients is reduced and combination therapies directed to different targets have now become common, we hypothesized that the combination of TMX and TMZ could be a promising therapeutic approach in the GBM treatment [27].

Thereby, in this study, the main aim was to investigate the therapeutic effect of the combination of TMX and TMZ in the human GBM cell lines, U87 and U118, through the evaluation of the effect on the survival, proliferation, apoptosis and migration ability.

2. Materials and methods

2.1. Reagents

DMEM, fetal bovine serum (FBS) and propidium iodide [28] were supplied by Invitrogen (Paisley, UK). Protease and phosphatase inhibitors were supplied by Roche (Indianapolis, IN, USA). Antibodies for Phospho-AKT (p-AKT) and total AKT (t-AKT), Phospho-ERK1/2 (p-ERK 1/2) and total ERK1/2 (t-ERK 1/2) and p-PKC pan (the antibody detects endogenous levels of PKC α, β I, β II, δ, ε, η and θ isoforms only when phosphorylated at a carboxy-terminal residue homologous to serine 660 of PKC β II) were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-actin antibody was purchased from Boehringer Mannheim (Germany). The phosphatase linked anti-mouse and anti-rabbit antibodies and the substrate for the phosphatase were obtained from GE Healthcare (UK). PVDF membranes were purchased from Millipore (Billerica, MA, USA). TMZ and TMX and the other chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA). TMZ and TMX were dissolved in dimethylsulfoxide (DMSO) at a stock concentration of 0.133 M and 3 mM, respectively. These stocks were aliquoted and diluted with culture medium according to the concentration used. The 5-ethynyl-2′-deoxyuridine (EdU) kit to detect cell proliferation was purchased from Invitrogen. The annexin V was purchased from BD-Pharmingen (BioLegends, San Diego, California) and the PI/RNAse for cell cycle analysis was purchased from Immunostep (Salamanca, Spain).

2.2. Cell line culture conditions

The U87 and U118 GBM cell lines were purchased from the American Tissue Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.5 mg/ml glucose, 0.1 mg/ml penicillin, 0.14 mg/ml streptomycin and 10% inactivated FBS. The cultured cells were maintained at 37 °C in an atmosphere containing 95% air and 5% CO_2. Cells were subcultured every 48 h by lifting them with a cell scraper. The cells were then centrifuged and resuspended in fresh DMEM. For the experiments, unsynchronized cells were treated with different concentrations of TMZ (200, 250 and 350 μM), taking into account the doses of TMZ used in clinical practice (150 mg/m^2 once per day for 5 days) and/or with two concentrations below the IC50 of TMX (5 and 7 μM) for 48 h [29,30]. The curve for the calculation of the drug concentration necessary to inhibit cell proliferation by 50% was fitted using GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA).

2.3. Cell viability using MTT assay

Metabolically active cells were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction colorimetric assay, as previously reported [31]. Briefly, cells were plated in 6 multi-well plates and were incubated for 48 h with TMZ and TMX at different concentrations, alone or in combination, for 48 h. After 48 h of incubation, MTT (5 mg/ml) was added to each well at a final concentration of 0.5 mg/ml and left for 45 min. The blue formazan crystals were dissolved by adding 200 μL of acidified isopropanol (0.04 N HCl). The solubilized products were transferred to 96-well plates, and the absorbance was read in a microplate reader at 570 nm, using 620 nm as the reference wavelength. Cytotoxicity was expressed as the percentage of cells surviving in relation to untreated cells. The drug concentration required to inhibit growth by 50% (IC50) was estimated with GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA).

2.4. Study of protein expression by western blot

Cells were incubated with TMZ and/or TMX for 48 h and centrifuged at 1,500 rpm for 10 min at 4 °C. The supernatant was discarded; the cells were resuspended in RIPA buffer (50 mM Tris–HCl at pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 2 mM EDTA, supplemented with protease and phosphatase inhibitors and DTT), and sonicated. The protein content of each sample was assessed and then the proteins were denatured. For that, buffer (Tris 0.5 mM, pH 6.8; 50% glycerol, 10% SDS, 10% 2β-mercaptoethanol and blue bromophenol) was added to each sample at a 1:1 ratio [32]. The protein extracts were then boiled at 95 °C for 5 min before use. For the western blotting assay, 30 μg of protein was separated on a 12% SDS–PAGE and then transferred to a PVDF membrane. The PVDF membrane was incubated with a solution of 5% non-fat milk in TBST for 1 h at room temperature and incubated overnight at 4 °C with the primary antibody against p-AKT (1:1000), p-ERK1/2 (1:1000), or p-PKC (1:1000), diluted in TBST with 1% milk supplemented with azide. Immunocomplexes were detected with anti-rabbit antibody (1:1000) and conjugated with alkaline phosphatase, and an enhanced chemifluorescence detection reagent was then used. Finally, the protein expression was quantified using the software *Image Quant TL for Windows* (version 2005; Amersham Biosciences, Piscataway, NJ, USA) with the expression of β-actin as a loading control for p-PKC, as well as total AKT and total ERK1/2 as a loading control for p-AKT, p-ERK1/2, respectively.

2.5. Cell cycle analysis by flow cytometry

The cell cycle analysis was performed by flow cytometry, using the detection kit PI/RNAse (Immunostep). For that, cells were incubated

for 48 h with TMZ and/or TMX collected and washed with PBS, and the pellet was resuspended in cold 70% ethanol, during vortex agitation, being incubated during 30 min on ice. After incubation period, cells were washed again and resuspended in PI/RNAse solution. Then 20,000 events were acquired, and cells were evaluated through *CellQuestTM* and data analyzed by *modfif LTMM software*. The results are expressed by the percentage of cells in each phase of cell cycle with a mean ± SEM of at least three independent experiments.

2.6. Cell proliferation using EdU assay

Cells were plated in six multi-well plates with different TMZ and/or TMX concentrations, for 48 h. The effect of TMZ and TMX on the proliferation rate was assayed using the EdU kit. Briefly, at the end of the incubation period, EdU labeling solution was added to each well at a final concentration of 100 μM. The cells were incubated for 60 min at 37 °C in a humidified atmosphere (5% CO_2). The cells were washed with PBS and incubated for 30 min at 4 °C with a fixative solution of ethanol 70% on ice. Then the cells were incubated for 10–20 min at RT with HCL-denaturation solution. Finally, the cells were incubated with anti-EdU-antibody working solution for 30 min at 37 °C in a humidified atmosphere (5% CO_2). The incorporation of EdU was analyzed by flow cytometry and data were analyzed with *modfif LTMM software*.

2.7. Cell apoptosis by flow cytometry

Cells were collected after 48 h of incubation with TMZ and/or TMX, washed with PBS, resuspended in binding buffer and incubated with AV (BD Pharmingen) and IP (BioLegends) for 15 min in the dark. After incubation time, cells were diluted in binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$) and analyzed using a FACScalibur flow cytometer. The experiments were performed in triplicated and the analysis of the results was performed using the Paint-a-GateTM program.

2.8. Staining with Hoechst 33258 for nuclei morphology evaluation (Supplementary data)

The Hoechst assay was performed as previously described [13]. Briefly, the cells were plated in six multi-well plates and incubated with TMZ and/or TMX for 48 h. Next, the cells were washed in a PBS solution, detached and centrifuged at 1,500 rpm for 10 min. Cells were then incubated for 15 min with a 4% solution of paraformaldehyde in 1% PBS, centrifuged at 1,500 rpm for 10 min, washed with PBS, and incubated with 5 μg/ml Hoechst 33258 solution for 5 min at room temperature. Then the cells were washed and resuspended in PBS and mounted on glass slides using anti-fade mounting medium. The images were captured under a Zeiss LSM 510 Meta confocal microscope at a magnification of 40x, and viewed on a *Zeiss LSM image browser* (Version 4.2.0.121; Carl Zeiss Inc., Germany).

2.9. Evaluation of cell migration ability

Cell migration was studied according to the method described by Liang et al. [33]. Briefly, cells were plated on 12-well plates and when the cultures were confluent, TMZ and/or TMX were added at different concentrations, for 48 h. Next, the cell monolayer was scraped in a straight line with a p200 pipette tip, debris was removed by washing the cells with culture medium, and then new culture medium was added. The plate was then placed under the phase-contrast microscope and an image from each well was acquired. Cells were then photographed at 0, 2, 4 and 6 h after the addition of drugs. The images in Fig. 7 were obtained before the addition of drugs and 6 h after the addition. *ImageJ software* (National Institute of Health, Bethesda, MD, USA) was used to record the coordinates for each scratch location using a computer-controlled stage and the mean scratch width at 6 h was calculated to the original scratch width (0 h). Each experiment was repeated three times.

2.10. Analysis of F-actin filament organization

F-actin filament organization was studied according to protocols described by Romão LF et al. [34]. Briefly, U87 and U118 cells were plated on round coverslips and then incubated with TMZ and/or TMX for 48 h. For detection of actin filaments, cells were fixed with 2.5% paraformaldehyde/PBS for 20 min, permeabilized with 0.1% Triton X-100/PBS for 3 min and then incubated for 30 min with the Alexa Fluor 568 phalloidin staining solution (5 U/ml) in PBS containing 1% BSA. Nuclei were stained with DAPI for 2 min. The coverslips were mounted on glass slides and inspected under a Zeiss LSM 510 Meta confocal microscope at a magnification of 40x, using a filter set with an excitation filter of 568 nm and a barrier filter of 585 nm, and viewed on a Zeiss LSM image browser (Version 4.2.0.121; Carl Zeiss Inc., Germany). The circularity parameter was analyzed using *ImageJ software* (National Institute of Health, Bethesda, MD, USA).

2.11. Statistical analysis

Statistical analysis was performed on GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). All values are expressed as mean ± SEM. After the confirmation of the assumption of normality and homogeneity of variance across groups, the groups were compared by nested design, including analysis of variance and post-hoc comparison with correction of α error according to Bonferroni probabilities to compensate for multiple comparisons. Statistical significance within groups was assessed by a one-way ANOVA and a Dunnet's test, with a significance threshold of $p \leq 0.05$.

3. Results

3.1. Evaluation of glioma cell viability in the presence of TMX and TMZ

Cell viability was assessed using the MTT assay according to the method described by Francisco V et al. [31]. U87 and U118 cells were incubated for 48 h with increasing TMX and/or TMZ concentrations (1, 2, 5, 7.5 and 10 μM). In the U87 cell line, TMZ did not induce a significant reduction in cells viability. In fact, the highest reduction in cell survival was 29.5% ± 6.7% achieved with the concentration of 500 μM, Fig. 1A. Regarding the effect of TMX in the U87 cell line, the analysis of the dose–response curve revealed that the IC50 value was 9.1 μM, Fig. 1A.

In the U118 cell line, TMZ had similar effects to those observed in the U87 cell line. The highest reduction in survival was 37.5% ± 5.0% detected when cells were also incubated with the concentration of 500 μM, Fig. 1B. The analysis of the dose–response curve revealed that the IC50 value of TMX was 7.3 μM.

When U87 (Fig. 1A) and U118 cells (Fig. 1B) were treated with the combination of TMX plus TMZ (50, 100, 150, 200, 250, 350 and 500 μM), the maximum survival reduction was 50% ± 2.9% and 90% ± 2.0%, respectively. These reductions were obtained when U87 and U118 cells were treated with TMX 7.5 μM plus TMZ 200 μM and TMX 7.5 μM plus TMZ 250 μM. Considering that NCCN guidelines version 2.2014 recommended for the treatment of central nervous system tumors the TMZ concentration of 350 μM and that the effect of different TMZ concentrations, on the reduction of cell survival, is not significantly different the TMZ concentrations used in this study were 250 and 350 μM.

3.2. Phosphorylation status of p-PKC-pan in glioma cells

The analysis of the p-PKC-pan expression, by western blot, showed that it was constitutively expressed in both cell lines. In order to determine the effect of TMX on the amount of p-PKC-pan, the protein

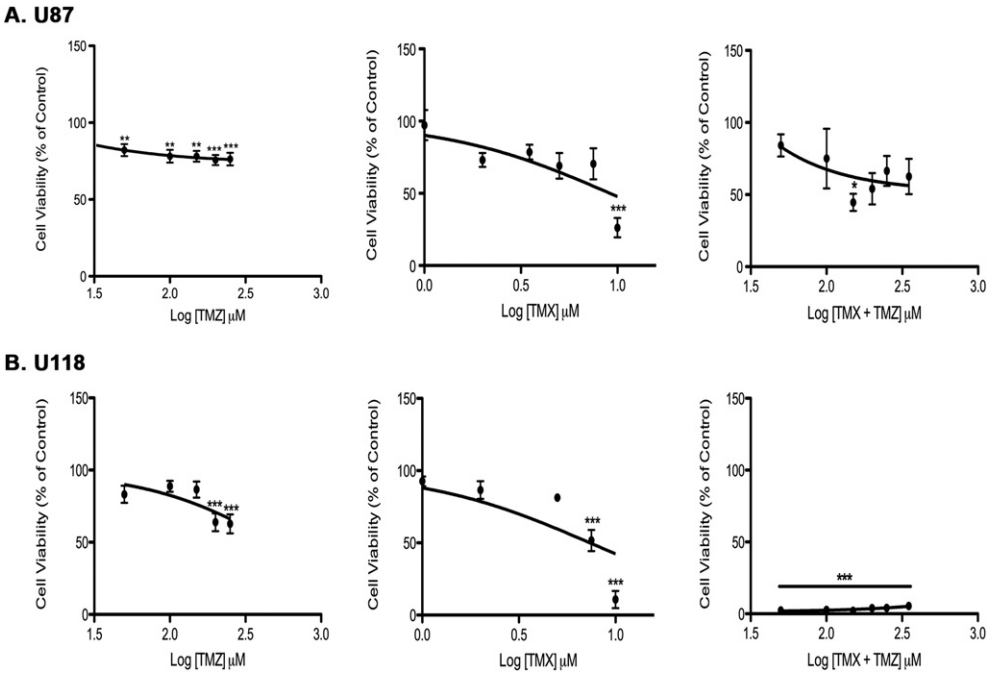

Fig. 1. Evaluation of TMX and TMZ effect on cell viability, evaluated through MTT assay. U87 (A) and U118 (B) cells were exposed to TMX at 1, 2, 5, 7.5 and 10 μM, respectively, and were exposed to TMZ at 50, 100, 150, 200, 250 and 350 μM, respectively. After 48 h of incubation, MTT assays were performed and cell growth and survival were determined by ELISA plate reading at 570 nm and reference 620 nm. The drug concentration required to inhibit growth by 50% (IC50), and the statistical analysis were estimated by GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). Each value represents the mean ± SEM from three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

expression in the presence of TMX or in association with TMZ was evaluated by western blot (Fig. 2). In U87 cells, the results showed that at 5 and 7 μM concentrations of TMX, the amount of p-PKC-pan was significantly reduced by 39.7% ± 4.3% and 39.1% ± 4.2%, respectively ($p < 0.05$ for both values) (Fig. 2A). This reduction was increased when cells were incubated with TMX plus TMZ. With the combination of TMX (7 μM) plus TMZ (350 μM), the amount of p-PKC-pan decreased 57% ± 6.1% as compared to control ($p < 0.05$), which represents an additional decrease of 17.8% compared to TMX alone (Fig. 2A).

In U118 cells, TMX also induced a significant reduction on the amount of p-PKC-pan but the decrease was less than that observed in the U87 cell line (Fig. 2B). In fact, in cells incubated with 5 and 7 μM concentrations of TMX, there was a reduction of 25.6% ± 3.4% and of 28.8% ± 3.6%, respectively, in the amount of p-PKC-pan ($p < 0.05$ for both values). However, when U118 cells were incubated with TMX (7 μM) plus TMZ (350 μM) the amount p-PKC-pan was reduced to 55.2 ± 6.9 as compared to that of the control cells ($p < 0.05$), which represents a decrease of 26.4% as compared to TMX alone.

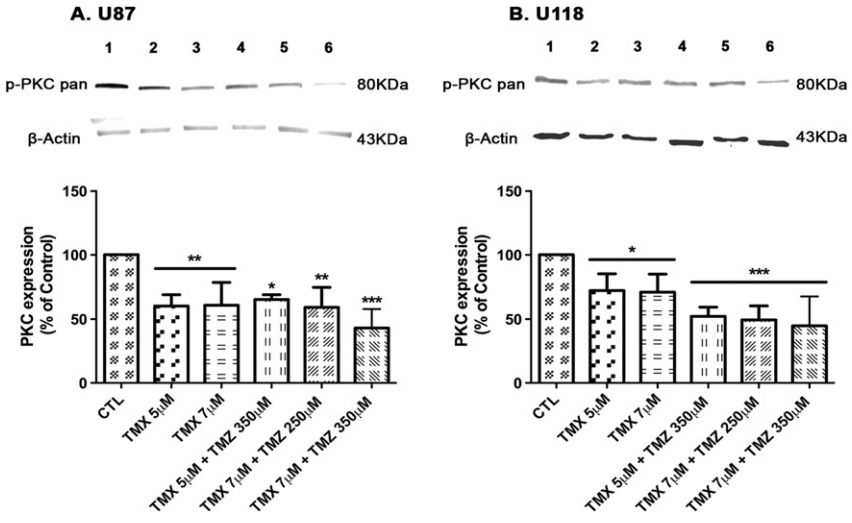

Fig. 2. Effects of TMX and/or TMZ on p-PKC pan expression. Following the incubation of U87 and U118 cells with TMX and/or TMZ for 48 h, the protein extracts were prepared and used for western blot analysis with anti-p-PKC pan antibody. Loading control was performed with an antibody for β-actin. In U87 (A) cells the p-PKC expression decreased when cells were treated with TMX at 5 μM and 7 μM about 39.7 % ± 4.3% and 39.1 % ± 4.2%, respectively. Also, p-PKC pan expression decreased when cells were treated with combination of TMZ at different concentrations until 56.9% ± 6.1% at TMX 7 μM combined with TMZ 350 μM. In U118 (B) cells, the p-PKC pan also decreased when cells were treated with TMX at 5 μM and 7 μM about 27.6% ± 3.4% and 28.9 % ± 3.6%, respectively. Also, p-PKC pan expression decreased when cells were treated with combination of TMZ at different concentrations until 55.2% ± 6.9% at TMX 7 μM combined with TMZ 350 μM. Statistical analysis was performed in GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). Each value represents the mean ± SEM from three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To determine if TMX affects the amount of the p-AKT and p-ERK 1/2, which are also involved in the proliferation, and survival of glioma cells, the amount of the phosphorylated kinases was evaluated by western blot in the presence of TMX and/or TMZ (Fig. 3). The analysis of the blots showed that in both cell lines the amount of p-AKT and p-ERK 1/2 was not altered by these chemotherapeutic drugs (Fig. 3).

3.3. Evaluation of glioma cell cycle in the presence of TMX and TMZ

U87 cells treated with the combination of TMX (7 μM) plus TMZ (350 μM) showed a decrease of 35.7% ± 12.2% ($p < 0.001$) of cells in the G0/G1 phase. This decreased was accompanied by a statistically significant increase of the percentage of cells in S and G2/M phases

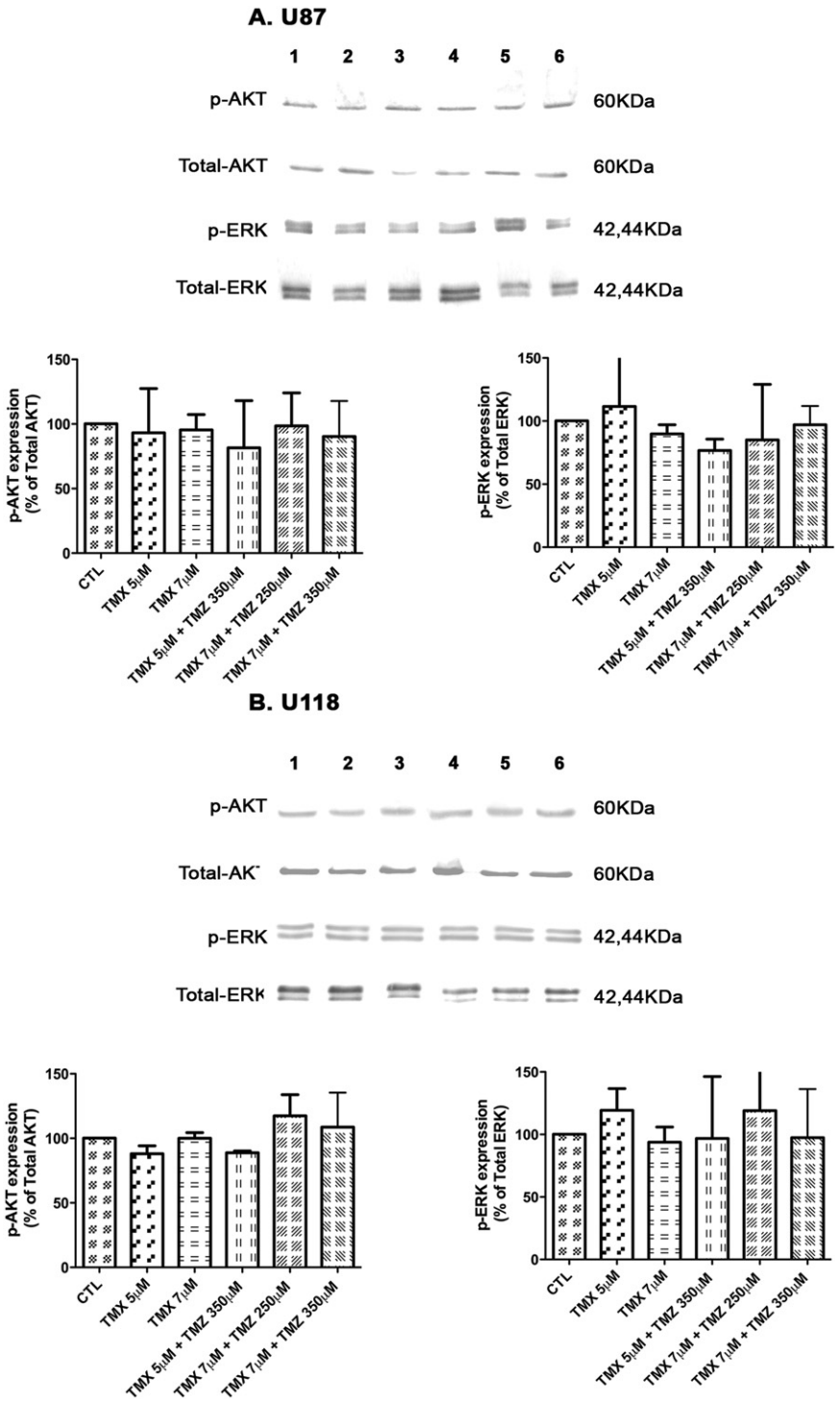

Fig. 3. Effects of TMX and TMZ on p-AKT and p-ERK 1/2 expression. Following the incubation of U87 (A) and U118 (B) cells with TMX and/or TMZ for 48 h, the protein extracts were prepared and used for western blot analysis with anti-p-AKT and p-ERK 1/2 antibody. Loading control was performed with an antibody for total AKT and total ERK 1/2, respectively. Statistical analysis was performed in GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). Each value represents the mean ± SEM from three independent experiments, $p > 0.05$.

comparing to control (untreated cells) and to TMX and TMZ alone (data not shown).

In the U118 cell line, the percentage of cells in each cycle phase was not affect by the TMX, or TMZ or by the combination of TMX plus TMZ as compared to control cells (Fig. 4B).

3.4. Evaluation of glioma cells proliferation in the presence of TMX and TMZ

The effect of TMX and/or TMZ on glioma cell proliferation was evaluated by flow cytometry quantification of the incorporated EdU. In U87 cells, the proliferation rate was not significantly altered by TMX alone or by the combination with TMZ as compared to the proliferation rate in control cells (Fig. 5A).

However, in U118 cells (Fig. 5B), the proliferation rate decreased 2.8% ± 0.3% and 7.9% ± 1.1% in cells incubated with 5 and 7 μM of TMX, respectively ($p > 0.05$). This decrease was more pronounced in cells treated with TMX plus TMZ. In fact, in cells incubated with TMX (7 μM) plus TMZ (350 μM) and in cells incubated with TMX (5 μM) plus TMZ (250 μM), the proliferation rate decreased 52.5% ± 7.0%, 59.2% ± 7.9% and 68.3% ± 9.2%, respectively ($p < 0.05$ for the three conditions), which represents a decrease of 60.4% compared to TMX alone (5 μM) and a decreased of 68.3% compared to control.

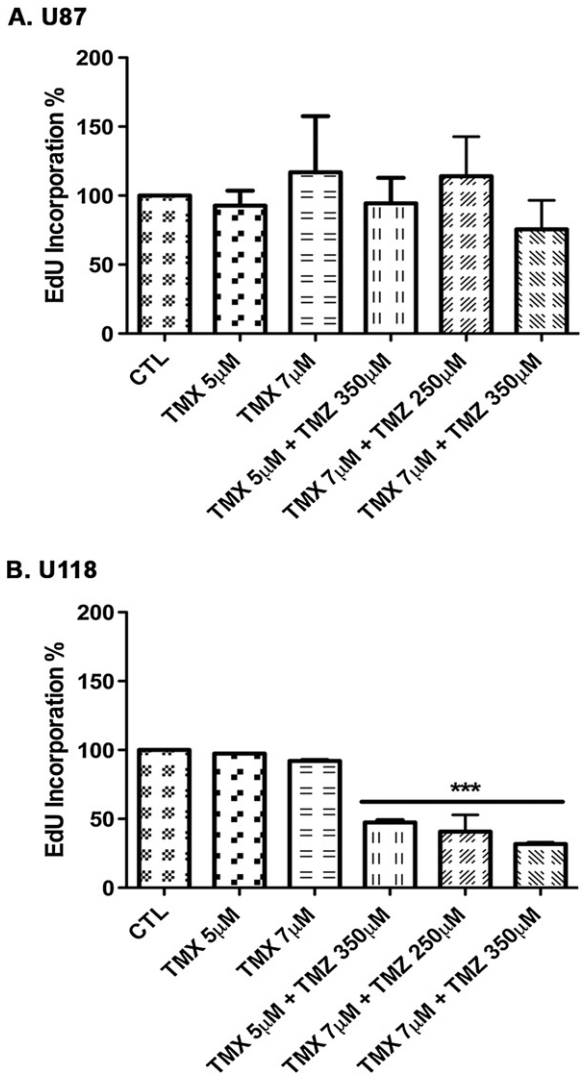

Fig. 4. Effects of TMZ and TMX on glioma cell proliferation. U87 (A) and U118 (B) cells were incubated for 48 h in the presence of different concentrations of TMZ and/or TMX. The proliferation rate was evaluated by measuring the incorporation of EdU. Statistical analysis was performed in GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). Each value represents the mean ± SEM from three independent experiments, $^{***}p < 0.001$.

3.5. Evaluation of apoptosis in glioma cells in the presence of TMX and TMZ

The ability of TMX and TMZ to induce apoptosis was analyzed by flow cytometry using annexin V/propidium iodide incorporation, as shown in Table 1. In U87 cells incubated with TMX alone, there was not a significant increase in the percentage of apoptotic cells. However, when cells were incubated with TMX plus TMZ the percentage of apoptotic cells significantly increased. This increase reached a maximum of 2.6% ± 4.0% in cells incubated with TMX (7 μM) plus TMZ (350 μM) as compared to control cells ($p < 0.05$) (Table 1). The percentage of necrotic cells was not affected by TMX alone, nor by the combination TMX plus TMZ.

In U118 cells, TMX alone or in combination with TMZ did not significantly alter the percentage of apoptotic cells. Nevertheless, in U118 cells incubated with TMX plus TMZ, there was a significant reduction of viable cells which was accompanied by an increase in the percentage of late apoptotic cells and by a statistically significant increase in the percentage of the necrotic cells which reached a maximum of 26.7 ± 5.6 in cells incubated with 7 μM TMX and 350 μM TMZ, as compared to control ($p < 0.05$) (Table 1).

3.6. Evaluation of nuclei morphology in glioma cells in the presence of TMX and TMZ

In order to study the nuclei morphology of glioma cells incubated with TMX alone or in combination with TMZ, nuclei from both cell lines was stained with Hoechst 33258. The results indicated that in both cell lines, after the incubation with the chemotherapy drugs alone or in combination, there was an increased number of cells with chromatin condensation, and of pycnotic nuclei with irregular contours (Supplementary data).

3.7. Study of cell migration in glioma cells treated with TMZ and/or TMX

To evaluate whether TMZ and/or TMX could regulate the motility of glioma cells, a scratch assay was used according to the method described by Liang et al. [33]. The results indicated that 6 h after the scratch, U87 and U118 control cells had the ability to move and to fill the scratch.

U87 cells incubated with TMX alone or in combination with TMZ showed a significantly decrease cell motility (Fig. 6A). The most significant decrease in motility was observed in the presence of 7 μM TMX plus 250 μM TMZ (41.3% ± 3.7%) compared to control cells ($p < 0.05$).

In U118 cells, there was also a significant reduction of the motility with TMX alone and in combination with TMZ. We noticed a decreased in the scratch closure capability of 25.8% ± 4.4% in cells treated with 7 μM TMX alone compared to control cells that was maintained in the combined treatment ($p < 0.05$) (Fig. 6B).

3.8. Evaluation of f-actin filament organization in the presence of TMX and/or TMZ

In order to evaluate if glioma cells acquired a phenotype compatible with the reduction of migration, the effect of TMX and TMZ on the organization of the f-actin filaments was evaluated. For that, cells were stained using Alexa Fluor 568 phalloidin and the circularity parameter according the *ImageJ Software* was determined. Control U87 cells showed a very irregular organization of f-actin filaments which limited the analysis of the results in the presence of TMX and TMZ (Fig. 7A). However, in U118 cells it was possible to identify several alterations induced by the chemotherapeutic agents. In control cells, f-actin was condensed in the cell body, and the cells were rounded (Fig. 7), this organization pattern became slightly disrupted in cells treated with

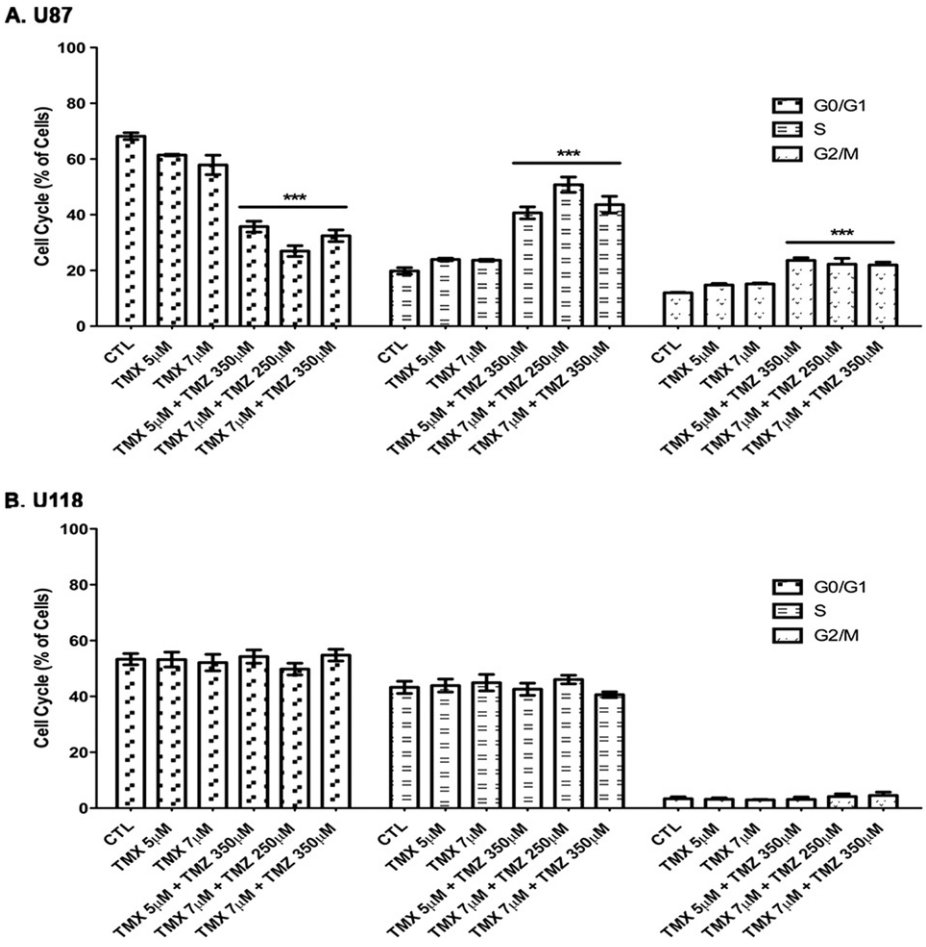

Fig. 5. Effects of TMX and TMZ on cell cycle analysis by flow cytometry. U87 (A) and U118 (B) cells were treated with TMZ (200, 250 and 350 μM) and/or TMX (5 and 7 μM) for 48 h. Cell cycle analysis was determined by gating Sub-G0/G1, S and G2/M on the PI-area signal. A total of 10,000 events were analyzed for each experiment in a BD FACS Canto II (BD Biosciences). Statistical analysis was performed in GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). Each value represents the mean ± SEM from three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

TMX alone and was replaced by a spindle-like pattern (Fig. 7B). In cells incubated simultaneously with TMX and TMZ, most of the cells showed f-actin organized in a spindle-pattern, with longer and thicker spindles.

4. Discussion

TMZ is considered the *gold standard* in GBM treatment, despite of its reduced ability to significantly increase the survival of GBM patients. The antitumor activity of TMZ is based on its ability to introduce DNA adducts and to activate apoptosis. Previous studies reported that the methylation status of the MGMT promoter has prognostic value [10,35,36]. However, other studies pointed that the TMZ-chemoresistance was also associated to gene mutations and to the constitutive activation of signaling pathways that control survival and proliferation, such as PKC. PKC is one of the most enigmatic signaling pathways since in tumor cells, according to the isoform, it may promote or inhibit

Table 1
Effects of TMX and TMZ on cell apoptosis by annexin V/PI double-staining assay.

	Live cells (%)	Apoptosis (%)	Necrosis (%)	Late apoptosis (%)
U87				
Control	92.8 ± 0.6	1.4 ± 0.2	1.8 ± 0.4	4.0 ± 0.5
TMX 5 μM	90.1 ± 1.8	2.8 ± 0.8	2.5 ± 0.8	4.6 ± 0.9
TMX 7 μM	90.8 ± 1.1	2.6 ± 0.5	2.1 ± 0.1	4.4 ± 0.6
TMX 5 μM + TMZ 350 μM	90.5 ± 0.8	3.4 ± 0.6*	1.5 ± 0.2	4.6 ± 0.7
TMX 7 μM + TMZ 250 μM	88.1 ± 0.9*	3.8 ± 0.6**	2.1 ± 0.4	6.0 ± 0.6*
TMX 7 μM + TMZ 350 μM	88.2 ± 1.9*	3.9 ± 0.8**	2.3 ± 0.3	5.5 ± 1.0
U118				
Control	85.8 ± 5.7	0.3 ± 0.2	11.1 ± 4.1	2.8 ± 1.7
TMX 5 μM	81.9 ± 7.8	1.7 ± 1.3	11.2 ± 2.2	5.2 ± 4.4
TMX 7 μM	77.3 ± 11.6	1.4 ± 0.5	15.9 ± 7.2	5.4 ± 3.9
TMX 5 μM + TMZ 350 μM	57.9 ± 6.4*	1.3 ± 0.1	31.8 ± 3.2**	9.1 ± 3.0
TMX 7 μM + TMZ 250 μM	60.3 ± 9.2*	1.1 ± 0.0	28.7 ± 4.0*	10.5 ± 6.5*
TMX 7 μM + TMZ 350 μM	49.8 ± 6.6**	1.2 ± 0.6	37.8 ± 5.9***	11.5 ± 3.4**

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

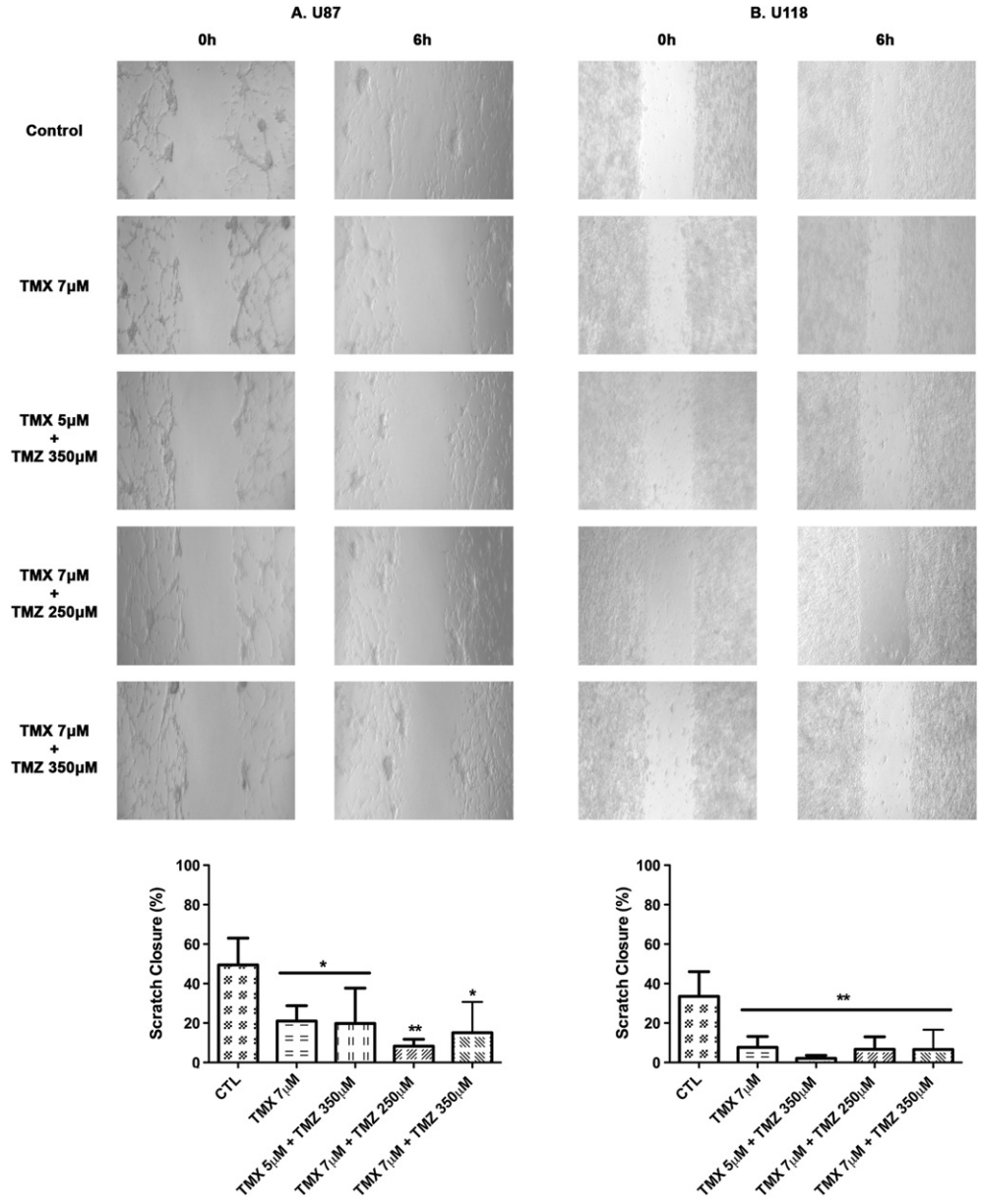

Fig. 6. Effects of TMX and TMZ on cell migration capability. The images represent photographs of U87 (A) and U118 (B) cells from scratch assay. The scratch closure was measured at various time points (T_0, 2, 4 and T_6) after the scratch was made in the culture dish. The figure represents the results at 0 h (T_0) and 6 h (T_6) for each condition. Confluent monolayers of control conditions, tamoxifen-treated cells with 7 μM, tamoxifen 5 μM in combination with temozolomide 350 μM and tamoxifen 7 μM in combination with temozolomide 250 μM and 350 μM were analyzed. Magnification is 10×. Statistical analysis was performed in GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). Each value represents the mean ± SEM from three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$.

apoptosis and cell survival [14,37]. Previous studies agree that GBMs are characterized by an increased expression and activity of PKC. However, there is no consensus regarding the PKC contribution to the aggressiveness of glioma cells. This controversy is associated to the existence of the isoforms that play different roles and also to the difficulty to obtain PKC inhibitors [14,18,24,38]. In fact, due to the homology of the different isoforms, the developing of specific PKC inhibitor has proven to be difficult.

Tamoxifen is one of PKC inhibitors used in *in vitro* studies with glioma cells and also in clinical trials with GBM patients. The *in vitro* effect of TMX was not completely elucidated, and the results of the *in vivo* studies were disappointing since treatment of patients with recurrent malignant glioma with low doses of TMX did not significantly increase the survival rate of the patients [15,18]. However, clinical trials using high doses of tamoxifen alone or in combination with other cytotoxic agents, have yielded better results [12,23,28,39–41]. Since TMX when used in high doses is a non-selective PKC inhibitor and is associated with an increased toxicity, the effect of the combination of low doses of TMX with TMZ on the proliferation, survival and migration of two different GBM cell lines was evaluated in this study [37].

In accordance with previous studies, our study showed that U87 and U118 cells constitutively express the p-PKC-pan. TMX induced a significantly reduction of the p-PKC-pan in both cell lines, confirming that TMX is an inhibitor of the p-PKC. The results also indicated that the expression of p-PKC in U87 cells is more susceptible to TMX than the p-PKC in the U-118 cells. In fact, in the U-87 cell line, TMX 7 μM induced a reduction of 39.1% in the amount of p-PKC-pan, while in the U118 cells it just induced a reduction of 28.8%. When cells were incubated with the combination of TMX plus TMZ, the amount of the phosphorylated p-PKC-pan was reduced to approximately 55% in both cell lines, indicating that the drug combination is more effective than the drugs alone. However, in the presence of TMX plus TMZ the amount of p-PKC was more significantly reduced in the U118 than in the U87 cells,

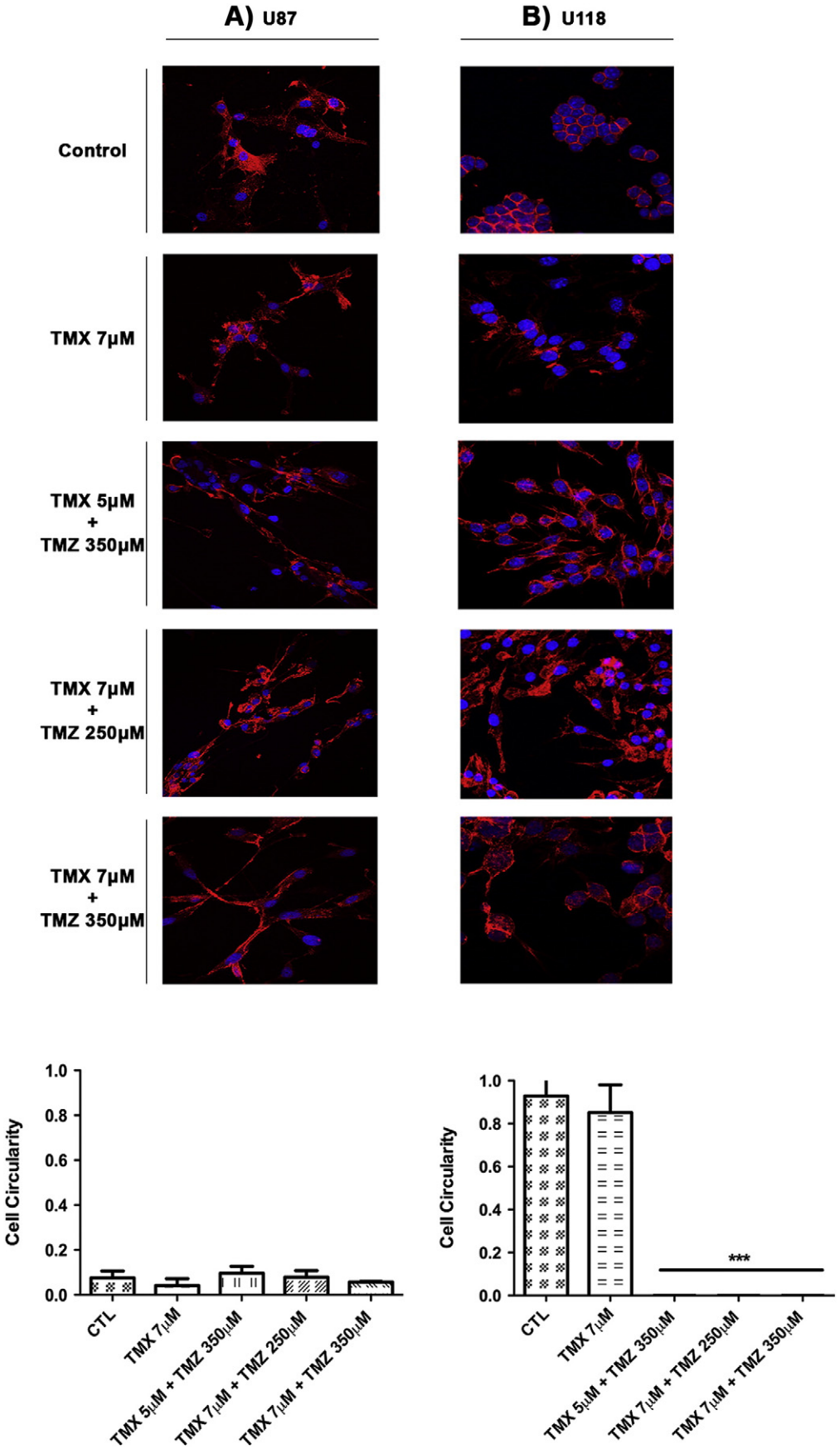

Fig. 7. Effects of TMX and TMZ on the organization of f-actin filaments. U87 (A) and U118 (B) cells were incubated for 48 h in the presence of TMZ and/or TMX. Cells were then fixed and f-actin was stained with Alexa Fluor 555 phalloidin. Nuclei were counterstained with DAPI. Red represents f-actin staining and blue represents cell nuclei. Magnification is 10×. Statistical analysis was performed in GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). Each value represents the mean ± SEM from three independent experiments, $^{***}p < 0.001$.

suggesting that the U118 cell line is more susceptible to TMZ than the U87 cells.

The study of cell viability in the presence of TMX also put in evidence the distinction in susceptibility of the U87 and U118 cell lines to TMX. In the U118 cell line, the IC50 was 7.3 μM, and in the U87 cell line, the IC50 was 9.1 μM. The differences among cell susceptibility to TMX and TMZ could be due to genetic and molecular alterations that characterize glioma cells [42,43]. One of the molecular alterations that may explain the difference of susceptibility of the two cell lines is the constitutive expression of p-ERK1/2 and of PI3k/AKT, which according previous studies contribute to the proliferation and resistance to apoptosis of glioma cells. Another alteration that could also contribute to the differences of susceptibility is the expression and activity of P-glycoprotein (PGP). Previous studies indicated that in GBM there is an overexpression of PGP coded by the multidrug resistance 1 gene, which may prevent the accumulation of several chemotherapeutic drugs in glioma cells [44]. Considering that the increase of PGP expression was correlated with a poor response to TMX treatment in several types of cells and also that TMZ may downregulate the expression of glycoprotein P, it is possible that the differences among U118 cells and U87 could be due to PGP [21,44].

Considering that previous studies reported that PKC contributes to the increased proliferation, it was expected that the reduction of p-PKC by TMX was accompanied by a reduction in cell proliferation. However, the proliferation rate was not significantly affected by TMX as revealed by the EdU incorporation assay. In fact, the proliferation rate of U87 cells was not significantly affected by TMX or by TMX plus TMZ. In the U118 cells, the proliferation rate was slightly reduced. This observation could be in accordance to a previous study from Cameron et al. (2008), who sustained that while the activity of PKC is not less than a minimum, it acts as a pro-mitogenic signal [45]. In accordance to this hypothesis is the fact that the greatest reduction in U-87 cell proliferation was achieved in the presence of TMX (7 μM) plus TMZ (350 μM), which was also the condition that induced the greatest reduction in the phosphorylation status of p-PKC-pan.

Considering that TMX alone or in combination with TMZ induced a reduction in the p-PKC and in cell viability but did not affect the percentage of dividing cells, we evaluated the effect of these drugs in cell cycle. In the U87 cell line, TMX alone did not alter the percentage of cells in each cell cycle phase. However, when cells were incubated with TMX and TMZ, there was a significantly increase in the percentage of cells in the S and G2/M phases, indicating that the drug combination induced a cell cycle arrest and is in agreement with the results from the proliferation assay. Considering that there is an increased percentage of cells in the S phase, that the entry of cells in the S phase is associated to cyclin D, which inhibits mitochondrial function and that the MTT assay depends on the activity of mitochondria to reduce MTT, we may hypothesize that the cell cycle arrest could be associated to a reduction in mitochondrial activity, to a decreased ability of cells to reduce MTT, and consequently to the reduced cell viability detected in the MTT assay.

In the U118 cells, the percentage of cells in each cell cycle phase was not affected by TMX or by TMZ. Since the results from the proliferation assay indicated that the combination of TMX plus TMZ significantly decreased the percentage of the incorporated Edu, we hypothesize that not all U118 cells have the same susceptibility to TMZ. Some cells are resistant to TMX and TMZ and maintain the proliferation ability, contributing to the maintenance of the cell cycle characteristics but other cells die when incubated with TMX and TMZ explaining the decreased cell viability detected by MTT. The variability of susceptibility among cells from a cell line isolated from a human GBM was previously described, reflects the genetic and molecular alterations that characterize GBM and may contribute to the development of chemoresistance [46]. In accordance with this hypothesis the analysis of apoptosis and necrosis revealed that in the U118 cells the combination of TMX plus TMZ induced a significant increase in the percentage of necrotic cells.

In the U87 cells, TMX plus TMZ induced a slight increase in the percentage of apoptotic cells that we hypothesize may result from the cell cycle arrest. Furthermore, since damage cells activate the DNA repair systems, which could be associated to the synthesis of DNA, it is possible that the incorporated EdU is a consequence of the proliferation and also of the repair systems. Considering that cell cycle arrest in G2, after DNA damage, may activate a process known as cell-cycle adaptation, in which cells reactivate cyclin-dependent kinase 2 complexes and proceed with mitosis, despite the presence of unrepaired damaged DNA, it is possible that U87 cells activate this process justifying the maintenance of the proliferation rate and of the cell cycle [47,48].

Moreover considering that (1) cell cycle progression is dependent on P21, which plays a critical role in arresting the cell cycle in G1 and G2 after DNA damage [49]; (2) the PKC activation upregulates the P21 protein [50]; and (3) the treatment of U118 cells with TMX plus TMZ significantly reduced the p-PKC-pan expression in this study, it is possible that the reduction of p-PKC-pan is involved in down-regulation of P21 expression, and as a result, the cell cycle progresses normally in some cells. We are now evaluating the level of P21 protein in the presence of TMX and/or TMZ.

In addition to the effect of TMX and TMZ on proliferation and cell survival, our results also showed that these drugs reduced the motility of both cell lines, which was accompanied by a reorganization of the f-actin filaments well visible on U118 cell line (Fig. 7B). The most significant reduction in motility was achieved in the U87 in the presence of TMX (7 μM) plus TMZ (250 μM), which is the combination that induced the highest reduction in the amount of phosphorylated PKC-pan.

Taking altogether, our results emphasize that PKC may play a relevant role in the proliferation, survival and migration of glioma cells. However, its contribution is dependent on the characteristics of the glioma cells, which emphasizes the need to establish a personalized therapy. In fact, the comparison of U118 cell line with U87 cell line showed that both cells have a basal expression of p-PKC. However, the susceptibility of each cell line to TMX and TMZ is different. The U118 cells are more susceptible to TMX than U87 cells, suffering a more significant reduction in cell proliferation and a more significant increase in necrosis when cells were incubated with TMZ plus TMX. On the other hand, U87 cells showed a more significant increase in the percentage of apoptotic cells and a more significant reduction in the motility when cells were incubated with TMX plus TMZ. In conclusion, our results showed that the combination of TMX and TMZ reduces the amount of the phosphorylated PKC-pan and contributes to the reduction of the aggressive behavior of the glioma cells. Together, the results show that PKC could be considered a therapeutic target and emphasize the importance of the combined therapy in the treatment of GBM.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.12.022.

Conflict of interest

We do not have any conflicts of interest to declare.

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