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EFFECT OF TEMOZOLAMIDE IN THE U-118 GLIOMA CELL LINE

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ABSTRACT

Glioblastomas (GBM) are the most lethal subtype of astrocytomas, with a survival rate of 12 months after diagnosis. The gold standard treatment of GBM, which comprises surgery followed by the combination of radiotherapy and chemotherapy with temozolomide (TMZ), increases the survival rate to 14.6 months. The success of TMZ seems to be limited by the occurrence of chemoresistance that allows glioma cells to escape from death signaling pathways. The mechanisms of TMZ action and of chemoresistance development are not completely understood. When TMZ started to be used the chemoresistance was associated to an increased activity of DNA repair enzymes, namely MGMT. However, recent studies pointed that the activity of survival signaling pathways such as PI3K/AKT, ERK 1/2 MAP kinase and autophagy also contributes to chemoresistence, but the interaction between those signaling pathways and TMZ has not been fully investigated. Therefore, our aim was to study the TMZ mechanism of action in the U-118 human glioma cell line and at the same time to investigate the possible mechanisms associated to the development of chemoresistance.

For that, U-118 cells were incubated with TMZ and the activity of the survival pathways PI3K/AKT, ERK 1/2 MAP kinase and autophagy as well as the proliferation and apoptosis ability were studied. Cell proliferation was evaluated by incorporation of bromodeoxyuridine. Apoptosis was studied by flow cytometry and also by fluorescence confocal microscopy. The expression of LC3, PI3/AKT and ERK1/2 was evaluated by western blot.

In U-118 cells, TMZ induced a significant reduction of the proliferation ability, but only a slight increase in the percentage of apoptotic cells. The evaluation of autophagy revealed that TMZ induced an increase in LC3 expression, the autophagy-associated protein, suggesting that U-118 cells may use autophagy to escape from cell

death. In addition, TMZ induced a slight reduction in the phosphorylation status of PI3K/AKT, but did not alter the phosphorylation status of ERK1/2 MAP kinase which suggests that these signaling pathways may also contribute to chemoresistance.

In order to confirm the contribution of autophagy, PI3K/AKT and ERK 1/2 MAP kinase to chemoresistance, U-118 cells were incubated with specific inhibitors of the pathways plus TMZ and the occurrence of apoptosis was evaluated by flow cytometry. The results showed that chemoresistance was partially overwhelmed when the cells were simultaneously treated with TMZ plus the inhibitors.

Therefore, our results indicated that the treatment of GBM with alkylating agents such as TMZ should be accompanied by the use of inhibitors of the survival pathways.

Keywords: glioblastoma, temozolamide, signaling pathways, AKT/PI3K, ERK1/2

RESUMO

Os glioblastomas (GBM) são considerados os astrocitomas mais letais, apresentando uma taxa de sobrevivência de 12 meses após o diagnóstico. O tratamento dos GBM assenta na cirurgia seguida de radioterapia e quimioterapia com temozolamida (TMZ). A TMZ é considerada o padrão de ouro no tratamento destes tumores desde que foi demonstrado que a taxa de sobrevivência dos doentes tratados com TMZ aumentava em média para 14.6 meses, após o diagnóstico. No entanto, o sucesso da TMZ é limitado pela ocorrência de quimioresistência que permite que as células activem mecanismos de fuga às alterações induzidas pela TMZ. Os mecanismos celulares e moleculares que contribuem para a quimioresistência não são bem conhecidos. Nos estudos iniciais pensou-se que a resistência estava associada à actividade das enzimas reparadoras do DNA, nomeadamente à MGMT. No entanto, estudos mais recentes demonstraram que as vias de sinalização associadas à sobrevivência celular como a PI3K/AKT, cinase ERK 1/2 MAP e autofagia pareciam também contribuir para a quimioresistência.

Os mecanismos de interação entre a TMZ e as vias de sinalização não foram ainda completamente estudados. Assim, o objectivo deste trabalho foi estudar o mecanismo de acção da TMZ na linha celular de glioma U-118 e ao mesmo tempo tentar identificar os mecanismos envolvidos na quimioresistência.

Para tal, incubaram-se células U-118 com TMZ e avaliou-se a actividade das vias de sinalização PI3K/AKT, ERK 1/2 MAP cinase e autofagia bem como a taxa de proliferação e de apoptose. A proliferação celular foi determinada utilizando a bromodeoxiuridina. A percentagem de células apoptóticas foi avaliada por citometria de fluxo e por microscopia confocal de fluorescência de modo a determinar a percentagem de células em sub G0/G1 e o grau de condensação de cromatina, respectivamente. A

expressão de LC3, PI3/AKT e de ERK1/2 foi avaliada por "western blot".

Verificou-se que nas células U-118 a TMZ induziu uma redução significativa da proliferação mas que apenas induziu um ligeiro aumento na percentagem de células apoptóticas. Verificou-se também que a TMZ induziu um aumento significativo na expressão de LC3, o que parece indicar que a TMZ induz autofagia e consequentemente pode contribuir para que as células U-118 sejam resistentes ao tratamento com TMZ. Os resultados obtidos demonstraram também que a TMZ induz uma ligeira redução na fosforilação da PI3K/AKT mas que não altera o estado de fosforilação da cinase ERK1/2 MAP. A manutenção da actividade destas vias de sinalização pode também contribuir para a quimioresistência. Para confirmar que a autofagia, a PI3K/AKT e a cinase ERK 1/2 MAP podem contribuir para quimioresistência, células U-118 foram incubadas com inibidores específicos destas vias de sinalização e a percentagem de células apoptóticas foi determinada por citometria de fluxo. Os resultados demonstraram que na presença destes inibidores a percentagem de células apoptóticas aumentava quando comparada com a percentagem de células apoptóticas obtida apenas na presença de TMZ.

Assim, os resultados obtidos neste trabalho parecem indicar que o tratamento de células de glioma deve ser um tratamento combinado envolvendo não só agentes alquilantes como a TMZ mas também inibidores específicos das vias de sinalização envolvidas na sobrevivência e morte celular.

Palavras Chave: glioblastoma, temozolamida, vias de sinalização, AKT/PI3K, ERK1/2

The work presented in this thesis was developed between 2008 and 2011 and was published in two different articles. In the first article it was done a revision of the biology of glioblastoma and in the second one, part of the results that were used in this thesis was published.

- Anália Carmo, Inês Crespo and Maria Celeste Lopes (2011). Genetics and Biology of Glioblastoma Multiforme, Molecular Targets of CNS Tumors, Miklos Garami (Ed.), ISBN: 978-953-307-736-9, InTech, http://www.intechopen.com/articles/show/title/genetics-and-biology-of-glioblastoma-multiforme

- Carmo, H. Carvalheiro, I. Crespo, I. Nunes, M. C. Lopes (2011) Effect of temozolomide on the U-118 glioma cell line. Oncol Lett 2,6: 1165-1170. http://www.spandidos-publications.com/ol/2/6/1165

LIST OF ABBREVIATIONS

AKT	Serine/threonine kinase, also named protein kinase B
ATCC	American Tissue Culture Collection
CBTRUS	Central Brain Tumor Registry of the United States
JNK	c-jun NH2-terminal kinase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK 1/2	Extracellular signal regulated kinase 1/2
GBM	Glioblastoma multiforme
LC3	Light chain 3
MAPK	Mitogen-activated protein kinases
MGMT	O^6-methylguanine-DNA methyltransferase
MMR	Mismatch Repair System
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NP-40	Nonyl phenoxypolyethoxylethanol
PBS	Phosphate buffered saline
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PVDF	Polyvinylidene difluoride
PTEN	Phosphatase and tensin homolog
RIPA	Radioimmunoprecipitation assay buffer

SDS	Sodium lauryl sulfate
TBST	Tris-buffered saline and tween 20
TMZ	Temozolomide
WHO	World Health Organization

FIGURES INDEX

Figure 1. Evaluation of U-118 cells viability in the presence of TMZ**.** U-118 cells were incubated for 48h with different concentrations of TMZ (5,10, 20, 80, 100, 300, 500 and 1000µM) and viability was evaluated by the MTT assay. **A.** Cell viability in the presence of TMZ. **B.** Determination of IC50 for TMZ. The IC50 was 145.2 ± 1.1µM. All values are mean ± SE of three independent experiments, each of them performed in triplicate .*p<0,01 as compared to control.

Figure 2 – Evaluation of U-118 proliferation in the presence of TMZ. U-118 cells were incubated for 48 h in the presence of different concentrations of TMZ. Proliferation rate was evaluated by measuring the incorporation of BrdUrd using a flow cytometer. All values were mean±SEM of triplicate analysis, each one performed in triplicate. *p<0.05 as compared to control.

Figure 3 - Cell cycle analysis of U-118 cells treated with TMZ (20µM, 100µM and 250µM), for 48h. A. Histograms of the U-118 cells. B Percentage of cells in each cell cycle phase. The results were obtained through the analysis of histograms and are representative of three different experiments performed in triplicate. A total of 10.000 events were analyzed for each experiment. *p<0.05 as compared to control.

Figure 4 – Analysis of nuclear morphology by confocal microscopy. U-118 cells incubated with different concentrations of TMZ for 48h were stained with

Hoechst 33258. Magnification: 63x. The images are representatives of the results obtained.

Figure 5 –LC3 expression in the U-118 cells. After the incubation of U-118 cells with TMZ, protein extracts were prepared and used for Western blot analysis with anti-LC3 antibody. Loading control was performed with an antibody for β-actin. The image is representative of three independent experiments.

Figure 6 - AKT phosphorylation status. After the incubation of U-118 cells with TMZ, protein extracts were prepared and used for Western blot analysis with anti-p-AKT antibody. Loading control was performed with an antibody for total-AKT. The image is representative of three independent experiments.

Figure 7 – ERK1/2 phosphorylation status. After the incubation of U-118 cells with TMZ, protein extracts were prepared and used for Western blot analysis with anti-p-ERK1/2 antibody. Loading control was performed with an antibody for total ERK1/2. The image is representative of three independent experiments.

Figure 8 - Cell cycle analysis of U-118 cells. Cells were treated with TMZ 250µM, wortmannin (1mM), rapamycin (150nM) and U-0126 (15mM), for 48h. The results were obtained through the analysis of histograms and are representative of three experiments performed in triplicate. A total of 10.000 events were analyzed for each experiment. *$p<0.01$ as compared to cells incubated with control (250µM).

1. INTRODUCTION

Glioblastoma multiforme (GBM) is the most malignant and the most frequent primary brain tumour, comprising approximately 50% of the cerebral gliomas, with an incidence of 2-3 new cases per 100.000 adults/year [1-4]. There are two types of GBM: a) the primary or *de novo* GBM which develops in older patients and the secondary GBM that develops from a malignant transformation of a previously diagnosed low-grade tumour, in younger patients. Despite their often circumscribed appearance with imagiological studies, gliomas typically exhibit extensive infiltration of surrounding brain, making ablastic tumour resection impossible [3-9].

The best treatment currently available consists of cytoreductive surgery, followed by simultaneous radiation and chemotherapy and then chemotherapy alone with alkylating drugs such as carmustine or temozolomide (TMZ) [1, 7, 8]. TMZ is the most recent alkylating drug, being considered the gold standard of GBM treatment after a randomized study performed by Stupp [7, 8, 10]. The Stupp multicenter phase II study enrolled 287 patients and demonstrated that the concomitant use of radiotherapy plus TMZ in newly diagnosed GBM allowed a median survival of 14.6 months [7, 8]. Unfortunately, part of the patients treated with TMZ are chemoresistant and therefore the survival rate for them remains 9 / 12 months [7, 8, 10, 11].

TMZ mediates its cytotoxic effects via the formation of DNA O^6-methylguanine (O^6-meG) which, during DNA replication, pairs preferentially with tymidine (T). The postreplication mismatch repair system (MMR) recognizes the O^6-meG:T mispairs leading to cell death [11-13]. However, in addition to MMR cells also present the DNA repair protein O^6-alkylguanine-DNA-alkyltransferase (MGMT), that repairs the O^6-meG

DNA adducts, avoiding the induction of apoptosis. Previous studies reported that in several tumours, including brain tumours there is an upregulation of MGMT [10, 11, 14, 15]. In addition, several clinical trials revealed a relationship between low MGMT activity and better therapeutic response in patients with malignant gliomas on treatment with alkylating drugs, including TMZ [11, 16, 17]. Therefore, MGMT activity seems to contribute to the chemoresistance and also to the fact that several glioma cells lines upon treatment with TMZ arrest cell cycle in G2/M phase and very few undergo apoptosis [14, 15].

However, more recent studies performed in gastric cancer, pituitary adenoma and glioma suggested that chemoresistence to TMZ could also be associated with mutations in genes that control the expression of protein involved in apoptosis, autophagy, proliferation and migration [18-20]. Among the signaling pathways involved in survival and proliferation particular attention was given to the protein kinase C, phosphoinositide 3-kinase PI3K and its main downstream effector the serine-threonine kinase (PI3K/AKT) and the mitogen-activated protein kinases such as the extracellular signal regulated kinase (ERK 1/2), c-jun NH2-terminal kinase (JNK) and the p38 mitogen-activated protein kinases (MAPK) [15, 18, 20-24].

The activity of these signaling pathways may explain the controversial results obtained in glioma cells treated in vitro with TMZ. In fact, there is no consensus regarding the effect of TMZ in glioma cells since some studies indicated that TMZ induces apoptosis but others showed that TMZ induces autophagy [14, 15, 25, 26].

Therefore, since the effect of TMZ in glioma cells and the contribution of the survival pathways to chemoresistance is not fully elucidated we incubated human glioma cells with TMZ and studied its effect in proliferation and apoptosis. In addition, we also investigated whether TMZ may alter the activation satus of autophagy, PI3K/AKT, and ERK1/2 signaling pathways.

2. MATERIALS AND METHODS

2.1 Reagents

DMEM, fetal bovine serum (FBS), propidium iodide (PI) and Hoechst were supplied by Invitrogen. The protease and phosphatase inhibitors were from Roche. Antibodies for Phospho-AKT, Phospho-ERK1/2 and total ERK1/2 were purchased from Cell Signalling Technology (MA, USA), the LC3 antibody was purchased from Affinity Bioreagents (Rockford, IL, 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 acquired from GE Healthcare (UK). PVDF membrane was purchased to Millipore (MA, USA). TMZ and the others chemicals were purchased to Sigma Chemicals (Saint Louis, MO, USA). TMZ was prepared according to Sigma instructions. For that, TMZ was dissolved in DMSO in a concentration stock of 0.133M. This stock was aliquoted and diluted with culture medium according the used concentration.

2.2 Cell line and cell culture conditions

The U-118 GBM cell line was purchased from ATCC (American Tissue Culture Collection), and maintained in Dulbecco's Modified Eagle Medium 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% of air and 5% of CO_2. The cells were subcultured every 48h by lifting them up with a cell scrapper, centrifuged and resuspended in fresh DMEM. For the experiments, unsynchronized cells were treated with different concentrations of TMZ (0, 5, 10, 20,

80, 100, 300, 500, 700 and 1000 µM) and also with 0.75% of DMSO, for 48 hours. After the incubation periods cells were prepared according each experiment.

2.3 Cell viability assay

The cytotoxic effect of TMZ on U-118 cells was determined by using the MTT assay as described previously [27]. Briefly, cells were plated in 48 multi-well plates according their growth rate, incubated with TMZ for 48h. At the end a solution of MTT (5mg/ml) was added to each well and incubated for an additional hour, in the incubator, at 37°C. The formazan salts were then dissolved in 300 µl of a solution of 4 mM HCl in isopropanol and the absorbance was read in an ELISA plate reader at 570 nm, with a reference wavelength of 620 nm.

2.4 Cell cycle analysis by flow cytometry

Cells were plated in 6 multi-well plates at different cell concentrations with different TMZ concentrations for 48h. At the end of the incubation period cells were centrifuged at 1500 rpm for 10 minutes, the culture medium was discarded, and the pellet was resuspended in a solution of 70% ethanol and maintained overnight. The cells were then centrifuged at 1500 rpm for 10 minutes, the pellet was resuspended and incubated for one hour, in the dark, at room temperature, in a solution of PBS containing 10 µl/ml PI and 10 µl/ml RNAse. The PI fluorescence was measured on a FACScan flow cytometer (BD FACSCalibur™) and the data was gated in order to exclude cell debris and aggregates. Data was analyzed on WinMDI for Windows.

2.5 Staining of U-118 TMZ-treated cells with Hoescht

The cells were plated in 6 multi-well plates and incubated with TMZ, for 48h. After that, cells were washed in a PBS solution, centrifuged at 1500 rpm for 10 minutes and incubated for 15 minutes with a solution of methanol and acetone (1:1). Cells were then centrifuged at 1500 rpm for 10 minutes, washed with PBS and incubated with 5 µg/ml Hoescht 33258 solution for 5 minutes at room temperature. After the incubation time, cells were washed and resuspended in PBS and mounted with Vectashield on glass slides. The pictures were taken under a Zeiss LSM 510 Meta confocal microscope, and viewed on Zeiss LSM Image Browser (Version 4.2.0.121, Carl Zeiss Inc., Germany). Magnification was 63x.

2.6 Preparation of protein extracts

The U-118 cells incubated with TMZ for 48h were centrifuged at 1500 rpm for 10 minutes at 4°C, the supernatant was discarded, cells were treated with RIPA buffer (50 mM Tris HCl at pH=8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with protease, phosphatase inhibitors and DTT, cell suspensions were then sonicated. The protein content of each sample was assessed through the bicinchoninic acid method. Finally, the denaturing buffer (Tris 0.5mM, pH 6.8; 50% glicerol, 10% SDS, 10% 2β-mercaptoethanol, blue bromophenol) was added to the samples in a 1:1 ratio. The protein extracts were then boiled at 96°C for 5 minutes and used.

2.7 Study of the effect of TMZ on the expression of LC3

For Western Blotting analysis of the LC3 expression, 70 µg of protein was separated on a 12% SDS-PAGE, and then transferred to a PVDF membrane. The PVDF membrane was blocked with a solution of 5% milk in TBST for 1h at room temperature and then

incubated overnight at 4°C with the primary antibody against LC3 diluted in TBST with 1% milk supplemented with azide. Bound antibody was detected with an alkaline phosphatase conjugated anti-rabbit, using enhanced chemifluorescence Western blotting detection reagents. The protein expression was quantitated using the ImageQuant TL for Windows (version 2005, Amersham Biosciences) having the expression of β-actin as loading control.

2.8 Study of the effect of TMZ on the PI3K/AKT and ERK1/2 activation

For Western Blotting analysis of the PI3K/AKT and ERK1/2 activation, 70 µg of protein was separated on a 12% SDS-PAGE, and then transferred to a PVDF membrane. The PVDF membrane was blocked with a solution of 5% milk in TBST for 1h at room temperature and then incubated overnight at 4°C with a rabbit polyclonal primary antibody against phosphor-AKT (p-AKT) or phospho-ERK1/2 (p-ERK1/2) diluted in TBST with 1% milk supplemented with azide. Bound antibody was detected with an alkaline phosphatase conjugated anti-rabbit, using enhanced chemifluorescence western blotting detection reagents. The protein expression was quantitated using the ImageQuant TL for Windows (version 2005, Amersham Biosciences) having the expression of total AKT and total ERK1/2 as loading control.

2.9 Statistical analysis

Statistical analysis was performed on GraphPad Prism 5 for Windows (version 5.00; GraphPad Software, Inc., San Diego. CA, USA). Statistical significance within groups was assessed by a t test and between groups by a two-way ANOVA, with a significance threshold of $p \leq 0.05$.

3. RESULTS

3.1 Effect of TMZ on U-118 cells viability

The results showed that TMZ reduced U-118 cells viability and also that this effect is dependent on the TMZ concentration. Reduction in viability was statistically significant from 10 µM ($p<0.01$) (figure 1A). In the presence of 10 µM there was a 12% reduction in cells viability as compared to the viability in control cells. The percentage of non-viable cells increased with the concentration of TMZ in a non-linear way, reaching 84,3% in the presence of 1000 µM. The half maximal inhibitory concentration (IC_{50}) was 145.2 ± 1.1µM, figure 1B. Taking in consideration the value of $IC_{50,}$ in the next experiments the maximum TMZ concentration that was used was 250 µM.

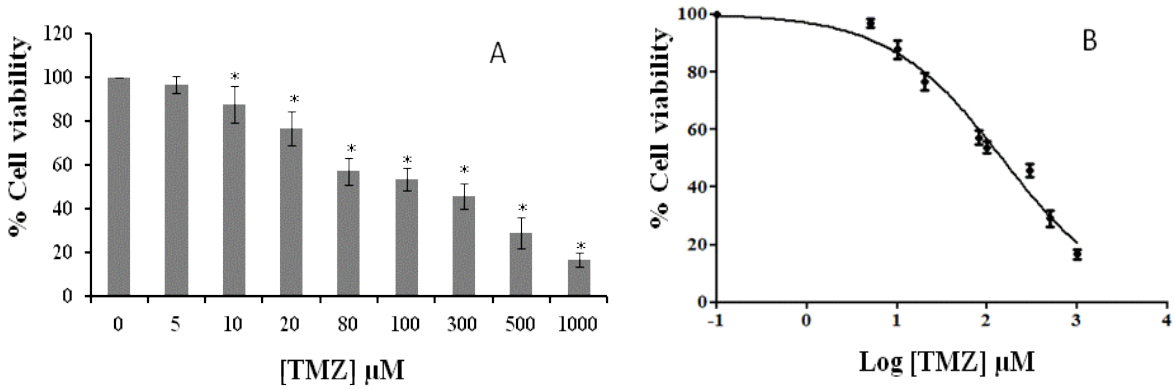


Figure 1. Evaluation of U-118 cells viability in the presence of TMZ**.** U-118 cells were incubated for 48h with different concentrations of TMZ (5,10, 20, 80, 100, 300, 500 and 1000µM) and viability was evaluated by the MTT assay. **A.** Cell viability in the presence of TMZ. **B.** Determination of IC50 for TMZ. The IC50 was 145.2 ± 1.1µM. All values are mean ± SE of three independent experiments, each of them performed in triplicate .*p<0,01 as compared to control.

3.2 Effect of TMZ on U-118 cells proliferation

In order to determine if the reduction in percentage of viable cells was due to a reduction in the proliferation rate, U-118 cells were incubated with TMZ in the presence of BrdUrd. The results showed that TMZ reduced the proliferation rate in a dose dependent manner, figure 2. In fact, in the presence of TMZ 10µM the proliferation rate was 86.7% ±4.98, in the presence of 100µM was 63.48% ±7 and in the presence of 250µM was 56.81% ±9.7, as compared to proliferation rate in control cells.

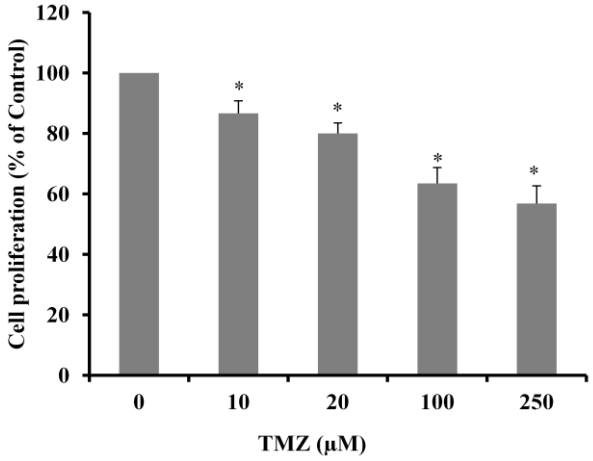


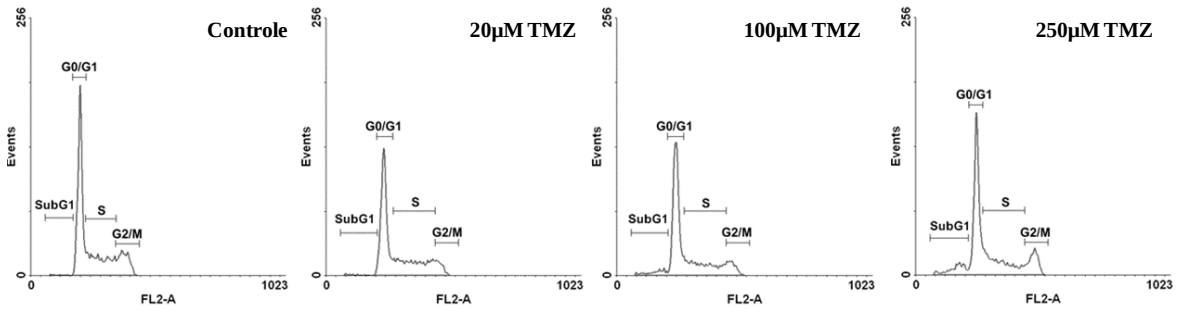
Figure 2 – Evaluation of U-118 proliferation in the presence of TMZ. U-118 cells were incubated for 48 h in the presence of different concentrations of TMZ. Proliferation rate was evaluated by measuring the incorporation of BrdUrd using a flow cytometer. All values were mean±SEM of triplicate analysis, each one performed in triplicate. *$p<0.05$ as compared to control.

3.3 Effect of TMZ on cell cycle of U-118 cells

To determine if the reduced proliferation was associated with cell cycle alterations, U-118 cells were permeabilized, incubated with PI and cell cycle was

analysed using a flow cytometer. Cell cycle of untreated cells was characterized by a long and well defined G0/G1 peak, with a slightly prominent G2/M peak and a very low sub G0/G1 fraction. When cells were incubated with 20 µM of TMZ there was a slight reduction in the percentage of cells in G0/G1 and in the S phases and a slight increase in the percentage of cells in the G2/M phase. This tendency was maintained when the TMZ concentration increased and was accompanied by an increase in the percentage of cells in subG0/G1, considered apoptotic cells. In fact, when cells were incubated with 100µM of TMZ the percentage of cells in G0/G1 decrease 5.2% and with 250µM of TMZ the percentage of cells in G0/G1 decrease 15%. The percentage of cells in the S phase only reduced 2% and 3% in the presence of 100 µM and 250 µM of TMZ, respectively. Regarding the G2/M, there was only a slight increase of approximately 2.5% independently of the TMZ concentration. The percentage of cells in subG0/G1 phase showed an increase of 4.6% in the presence of TMZ 100 µM and of 15.36% in the presence of TMZ 250 µM, indicating that the percentage of apoptotic cells was TMZ-concentration dependent.

A



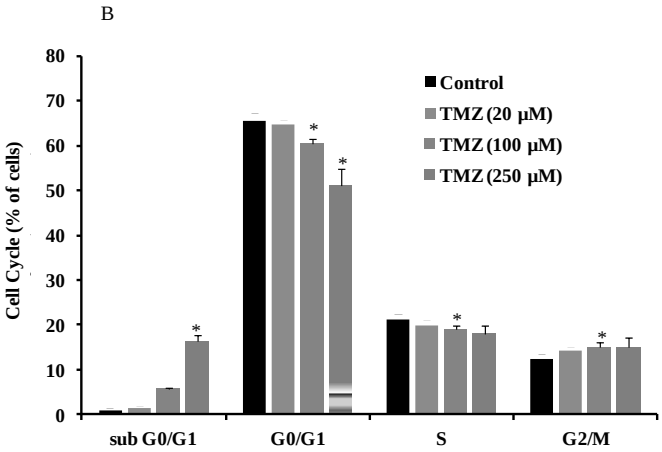


Figure 3 - Cell cycle analysis of U-118 cells treated with TMZ (20µM, 100µM and 250µM), for 48h. A. Histograms of the U-118 cells. B. Percentage of cells in each cell cycle phase. The results were obtained through the analysis of histograms and are representative of three different experiments performed in triplicate. A total of 10.000 events were analyzed for each experiment. *$p<0.05$ as compared to control.

3.4 Effect of TMZ in nuclei morphology

In order to evaluate if TMZ induced morphological alterations compatible with the feature of apoptosis, U-118 cells were stained with Hoechst 33258 and analysed under a confocal microscope. When cells were incubated with TMZ it was possible to detect in some cells chromatin condensation, irregular nuclei contour, and pycnotic nuclei confirming the results obtained from cell cycle analysis, figure 4. These alterations were TMZ-concentration dependent.

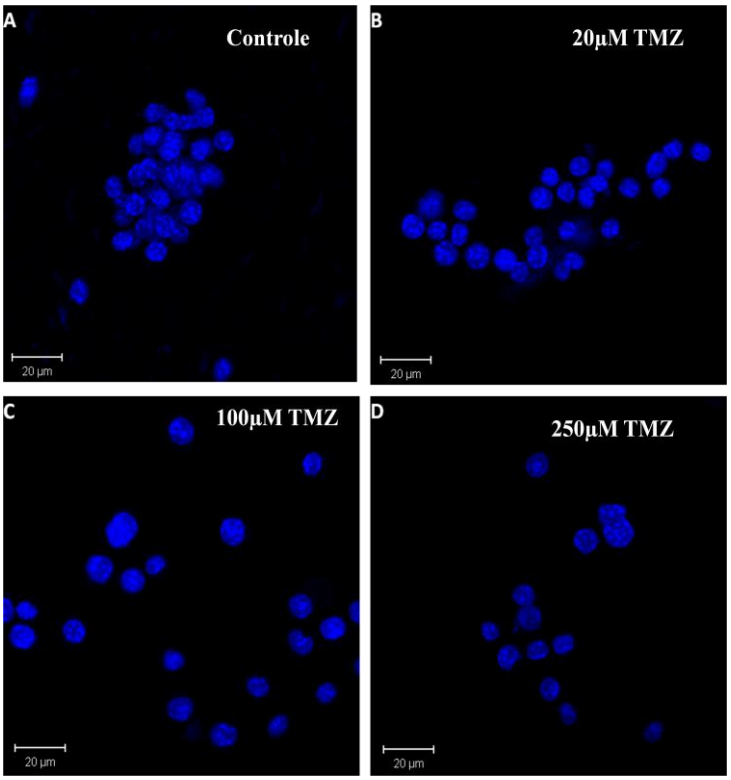


Figure 4 – Analysis of nuclear morphology by confocal microscopy. U-118 cells incubated with different concentrations of TMZ, for 48h, were stained with Hoechst 33258. Magnification: 63x. The images are representatives of the results obtained.

3.5 Expression of LC3 in the presence of TMZ

Since previous studies reported that tumour cells under chemotherapy may activate autophagy in order to survive it was investigated the effect of TMZ on the expression of LC3, one of the autophagosome-membrane proteins. The results showed that U-118 cells constitutively expressed the LC3 protein. However, in U-118 cells incubated with TMZ there was an increase in the LC3 expression, which reached the maximum in the presence of TMZ 250 µM, figure 5. Therefore, the results seem to indicate that TMZ induced autophagy in the U-118 cells.

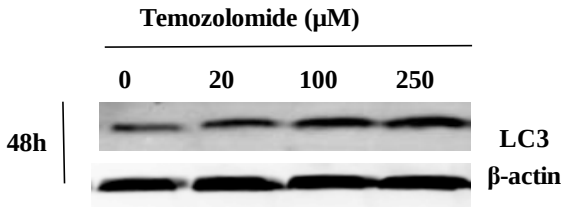


Figure 5 –LC3 expression in the U-118 cells. After the incubation of U-118 cells with TMZ, protein extracts were prepared and used for Western blot analysis with anti-LC3 antibody. Loading control was performed with an antibody for β-actin. The image is representative of three independent experiments.

3.6 Expression of p-PI3K/AKT and of p-ERK1/2 MAP kinase on the U-118 cells

Since the induction of apoptosis is dependent on the DNA repair systems and also on the activity of several signaling pathways we further evaluate the phosphorylation status of PI3K/AKT and ERK1/2 MAP kinase by Western Blot.

The results showed that the endogenous AKT was characterized by a basal phosphorylation of the Ser473, figure 6. When U-118 cells were incubated with TMZ there was a reduction on the level of p-AKT which was TMZ-concentration dependent. This reduction was not statistically significant.

The activation status of ERK1/2 MAP kinase was evaluated by determining the content of the p-ERK1/2 by Western Blotting. The results showed that U-118 cells present a basal activation of ERK 1 and 2, which was not significantly altered by TMZ, figure 7.

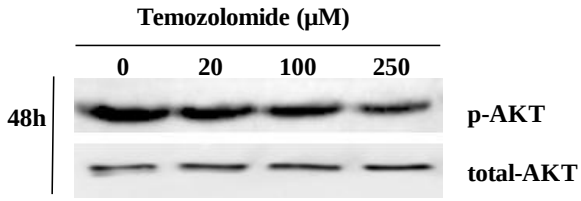


Figure 6 - AKT phosphorylation status. After the incubation of U-118 cells with TMZ, protein extracts were prepared and used for Western blot analysis with anti-p-AKT antibody. Loading control was performed with an antibody for total-AKT. The image is representative of three independent experiments.

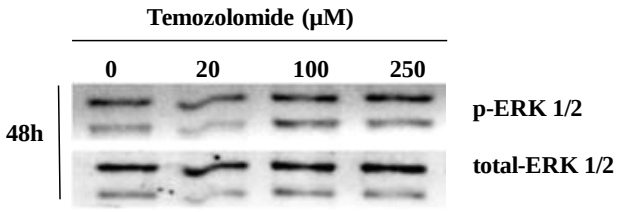


Figure 7 – ERK1/2 phosphorylation status. After the incubation of U-118 cells with TMZ, protein extracts were prepared and used for Western blot analysis with anti-p-ERK1/2 antibody. Loading control was performed with an antibody for total ERK1/2. The image is representative of three independent experiments.

3.7 Induction of apoptosis in U-118 cells

Considering that, TMZ did not altered significantly the active amount of PI3K/AKT and of ERK1/2 MAP kinase and also that these signaling pathways stimulate cellular proliferation, we incubated U-118 cells with wortmannin the inhibitor of PI3K/AKT, and with U-0126 the inhibitor of ERK1/2 MAP in order to determine if the percentage of apoptotic cells increased as compared to that obtained in control cells and in cells incubated with TMZ. In addition, we also incubated cells with rapamycin the inhibitor of mTOR and activator of autophagy. Cell cycle was evaluated 48h after

the addition of the inhibitors and of the autophagy activator by flow cytometer, and the percentage of cells in the sub G0/G1 phase was quantified, figure 8.

The results demonstrated that the percentage of apoptotic cells in the presence of wortmannin was 18.24% ±1.45 which was similar to that observed in the presence of TMZ (16.37%±0.97). However, when wortmannin was used in combination with TMZ, the percentage of apoptotic cells was 43.09% ±1.89, figure 8 .

When cells were incubated with rapamycin the inhibitor of mTOR and activator of autophagy, the percentage of apoptotic cells increased 26.5%±1.01 as compared to that observed in control cells and was 11.13% higher than the percentage of apoptosis detected in the presence of TMZ alone. When cells were simultaneously incubated with rapamycin and TMZ the percentage of apoptotic cells was 37.44%±1.45, figure 8.

Since mTOR is a downstream effector of AKT, we further incubated U-118 cells with wortmannin, rapamycin and TMZ. As shown in figure 8 when cells were incubated with wortmannin plus rapamycin the percentage of apoptotic cells was 49.00%±1.6 and when cells were incubated with wortmannin plus rapamycin and TMZ the percentage of apoptotic cells was 63.01%± 2.1, figure 8.

The contribution of ERK1/2 MAP kinase to glioma cells survival was evaluated in the presence of the U-0126. As shown in figure 8, the percentage of apoptotic cells was 33.3%±1.72 in the presence of U-0126. When U-0126 was combined with TMZ the percentage of apoptotic cells was 51.6%±2.1, which represented an increase of 35.25% as compared to the percentage of apoptotic cells detected in the presence of TMZ alone, figure 8.

U-118 cells were then incubated with rapamycin, U-0126 and TMZ and the cell cycle was evaluated. The percentage of apoptotic cells determined in this condition was

71.0%±2.15 indicating that the survival of glioma cells depends on the activity of mTOR and ERK1/2 MAP kinase, figure 8.

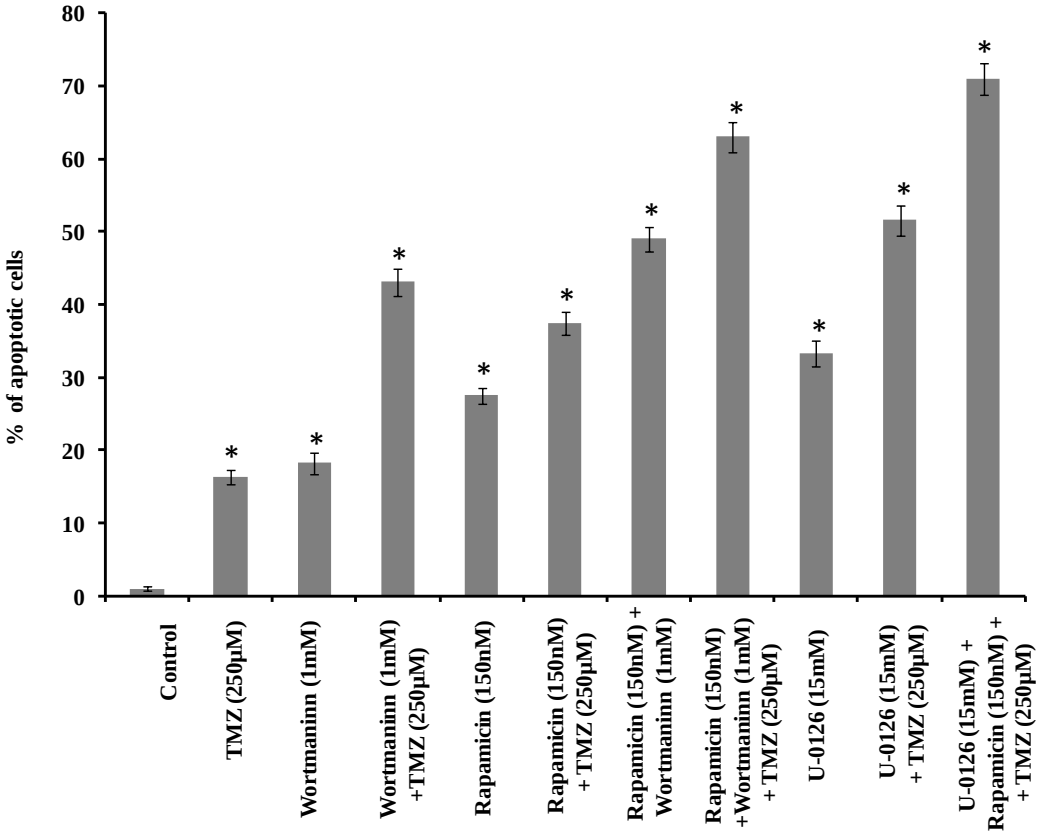


Figure 8 - Cell cycle analysis of U-118 cells. Cells were treated with TMZ 250µM, wortmannin (1mM), rapamycin (150nM) and U-0126 (15mM), for 48h. The results were obtained through the analysis of histograms and are representative of three experiments performed in triplicate. A total of 10.000 events were analyzed for each experiment. *$p<0.01$ as compared to cells incubated with control (250µM).

4. DISCUSSION

The EORTC trial demonstrated an improvement in overall median survival of GBM patients from 12.1 months, in patients submitted to radiotherapy, to 14.6 months in TMZ-treated patients [26, 28, 29]. As a consequence of these results, TMZ is considered the gold standard in GBM treatment [8]. However, the increment in survival is still

reduced and therefore the study of the mechanism of TMZ action and the mechanisms associated to the chemoresistance are fundamental issues.

Regarding the mechanism of TMZ action, the results from previous studies were contradictory. Kanzawa et al. showed that TMZ induced autophagy, but not apoptosis in malignant glioma cells [14]. However, Roos et al. referred that the cell death induced by TMZ was due apoptosis [30]. In addition, Hirose et al. referred that in glioma cells TMZ induced a low level of apoptosis as compared to that observed in lymphoid cells and also that TMZ induced a cell cycle arrest in G2/M [15].

The ability of alkylating agents to induce apoptosis is dependent on the activity of the DNA repair enzymes such as the MGMT[31]. Some GBM tumours are characterized by an increased expression and activity of MGMT that repairs the O^6-meG DNA adducts before the next cell cycle contributing to cell survival and chemoresistance. In others GBM tumours, the expression of MGMT is reduced due to the hypermethylation of the MGMT promoter [16, 18, 20, 30] which contribute to increase the sensitivity of high-grade glioma to alkylating agents [11, 18, 32]. However, recent studies reported that several tumours with low levels of MGMT are also chemoresistant indicating that other mechanisms than the MGMT, contribute to chemoresistance [18, 31]. In fact, it was reported in several tumours, including GBM, that the expression and activity of the survival pathways such as PI3K/AKT, ERK1/2 and autophagy is altered due to the occurrence of gene mutations [25, 28, 33, 34]. However, the contribution of these signaling pathways to the TMZ chemoresistance was not fully investigated.

In this study, we investigated the effect of TMZ in the glioma cell line U-118. This cell line was previously described as having *MGMT* promoter hypermethylated [35, 36]. However, the study of the percentage of apoptotic cells in the presence of TMZ 250μM showed that only 15.36% of cells were apoptotic. These results were in

agreement with those previously reported by Hirose et al and seem to indicate that U-118 present other survival mechanisms that allow cells to circumvent the effect of TMZ. Since the results also showed that TMZ induced a significant reduction in the proliferation ability it was hypothesized that the U-118 cells may induce autophagy in order to survive to the aggressive microenvironment.

The study of autophagy confirmed that in U-118 cells, TMZ induced an increase in the LC3 expression, the protein associated to autophagossomes. Therefore, it is possible that the reduced percentage of apoptotic cells obtained in the presence of TMZ 250µM is associated to autophagy.

The activation of autophagy may also justify the discrepancy between the IC50 concentration (145.2±1.1µM) determined by the MTT assay and the reduced percentage of apoptotic cells in the presence of TMZ 250µM. The MTT assay is based on the mithocondria activity, if TMZ induce an increase in the LC3 expression and autophagy is activated there will be a reduction in cell size and also in the number of mithocondria which consequently will reduce the production of formazan salts and the value of IC50.

Taking in consideration the fact that continuous activation of autophagy can lead to an excessive depletion of cellular organelles and essential proteins and consequently to caspase-independent autophagic cell death [37], U-118 cells were incubated with rapamycin an activator of autophagy. The results showed that in the presence of rapamycin the percentage of apoptotic cells was higher than that obtained with TMZ alone and suggested that autophagy may play a very important role in the treatment of GBM. In order to investigate the existence of a synergistic effect between these two drugs U-118 cells were simultaneously incubated with TMZ and rapamycin. However, the percentage of apoptotic cells obtained in the presence of the two drugs was the sum of the effect of each one, pointing for an additive effect rather than a

synergistic effect. These results seem to indicate that the percentage of apoptotic cells obtained in the presence of TMZ and rapamycin was achieved through different pathways and forces a question that has been subject of great controversy: whether or not apoptosis and autophagy represent two independent processes.

Another important subject raised by the activation of autophagy is whether it may contribute for the reduction of proliferation that was observed in U-118 incubated with TMZ. Several studies reported that autophagy may induce cell cycle arrest and in a later stage may contribute to cellular senescence [38-40] . In fact, the analysis of cell cycle in U-118 cells treated with TMZ revealed that there was a reduction in the percentage of cells in G0/G1 and a slight increase in the percentage of cells in G2/M. Previous studies also reported that autophagy may induce cell cycle arrest in G0/G1 due to the fact that it reduces cellular levels of p53, which is a repressor of autophagy and leads to an accumulation of cells in the G1 phase [41]. On In addition, other studies also reported that treatments used to activate autophagy may also inhibit the Pi3K/AKT pathway and consequently may induce an accumulation of inactive phospho-Cdc2 and phospho-Cdc25C and consequently induce cell cycle arrest in G2/M [42]. In fact, U-118 cells were characterized by constitutive activation of the PI3K/AKT which was slightly reduced when cells were incubated with TMZ. Since, TMZ slightly reduced the amount of the phosphorylated PI3K/AKT, and simultaneously induced the expression of LC3 it is possible that both events contribute to the accumulation of cells in G2/M observed in this study.

Since TMZ only induced a slight reduction in the phosphorylation status of the U-118 cells it is possible that the remained phosphorylated PI3K/AKT contribute to glioma cells survival. To investigate this hypothesis cells were incubated with wortmannin a specific inhibitor of PI3K/AKT and the percentage of apoptotic cells

was measured. In the presence of wortmannin the percentage of apoptotic cells increased 17.24% as compared to control cells, confirming that PI3K/AKT should also be considered a therapeutic target in the GBM treatment. The incubation of U-118 cells with wortmannin plus TMZ showed that the association of these two drugs has a synergistic effect since the percentage of apoptotic cells obtained with the drug combination was higher than the sum of the percentage of each one. As previously showed by other studies, activated PI3K/AKT is involved in the regulation of proteins involved in cell cycle, proliferation, apoptosis and invasion, therefore once deregulated AKT may contribute to tumorigenesis [9, 21, 43]. In fact, activated AKT induces the stabilization of cyclin D and the promotion of the nuclear entry of MDM2, leading to the degradation of p53 and consequently to the deregulation of cell growth [15, 43]. On the other hand, activated AKT induces the phosphorylation and inactivation of pro-apoptotic signaling proteins, such as BAD and caspase-9 exerting anti-apoptotic activity and contributing to chemoresistance [44]. Taking in consideration these studies, U-188 cells were incubated with wortmannin and the percentage of apoptotic cells evaluated. The results obtained in the presence of wortmannin and TMZ confirmed that PI3K/AKT contributed to the survival and to the TMZ-chemoresistance of U-118 cells and emphasized the need to develop a combined treatment for GBM that activates autophagy and reduces the activity of the PI3K/AKT.

In addition to autophagy and to the activity of PI3K/AKT it is also important to study the contribution of ERK1/2 MAP kinase which controls cell proliferation, survival, migration and differentiation in several cell types including astrocytes [45, 46]. In tumour cells the role of ERK is contradictory. Some studies reported that, in tumour cells, including GBM cells, ERK may induce cell death by apoptosis and by autophagy [47-49]. But in another studies it was reported that activated ERK contributed to G1- to S-

phase progression and protected cells from apoptotic signaling [24, 46] contributing to chemoresistance [50]. In the U-118 cells ERK1/2 was constitutively active and the incubation of cells with TMZ did not changed the activation status of this signaling pathway. However, when U-118 cells were incubated with U-0126 the percentage of apoptotic cells was 33.3% indicating that this signaling pathway contributed in a very significative way to the survival of U-118 cells. When cells were treated with U-0126 plus TMZ the percentage of apoptotic cells was 51.6%, approximately the sum of the percentage of each one indicating the absence of a synergistic effect between these two drugs. This same observation was observed when U-0126 was combined with the inhibitors of PI3K/AKT or of autophagy. These results showed once more how complex is the understanding of chemoresistance and how difficult is to circunvent it. It is possible to hypothesize that glioma cells could have the ability to switch from one signaling pathway to another in order to bypass the difficulties imposed by drugs. In fact, one of the signaling pathway that was not evaluated in this study but could also contribute to glioma cell survival is the protein kinase C. Future studies will be necessary to clarify the role of these signaling pathways to the survival and proliferation of glioma cells.

5.CONCLUSIONS

Altogether, our results indicated that TMZ by itself is not very effective in inducing cell death even in the U-118 cell line which has the *MGMT* promoter hypermethylated. In addition, our results also emphasize that TMZ-chemoresistance of GBM cells is a complex question that depends on the activity of several signaling pathways such as autophagy, PI3K/AKT and ERK1/2. Furthermore, we showed that

each signaling pathway could be considered a therapeutic target since the inhibition of each one significantly reduced the survival of the U-118 cells. Our results also indicated that there is a synergistic effect between wortmannin and TMZ which could be a very important therapeutic advantage.

Therefore, in order to achieve a more efficient therapy of GBM it is important to combine traditional chemotherapy with molecular-based therapy, in particular the therapy associated with signaling pathways. Further studies are needed to clarify the role of each signaling pathway and the molecular interconnections of those signaling pathways.

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