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Mestrado em Investigação Biomédica

ESTUDO DA CONTRIBUIÇÃO DAS INTEGRINAS NA MOTILIDADE DE CÉLULAS DE GLIOBLASTOMA

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Cover Illustration: F-actin filaments of U-118 MG cells treated with 1µM of Shikonin. Visualized by confocal microscopy (Diana Matias).

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ibili

À minha Tia Cila, Pais e Avós…

…"Pequenas oportunidades podem ser o começo de grandes oportunidades!"

Demóstenes

"Every great advance in science has issued from a new audacity of imagination." ***JOHN DEWEY***

"THE MOST BEAUTIFUL THING WE CAN EXPERIENCE IS THE MYSTERIOUS. IT IS THE SOURCE OF ALL TRUE ART AND ALL SCIENCE." ***ALBERT EINSTEIN***

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ABSTRACT

Background: Glioblastoma (GBM) represente 50% of the tumors that arise within the central nervous system. The main features of GBM are the high proliferative andangiogenic potential, and the capacity of locally invasion. Despite the considerable advances in the knowledge of the mechanisms underlying the genetics, biology and clinical behaviour, GBM pathogenesis is not completely understood and until now, there is not a therapeutic strategy to reduce the invasive and proliferative ability of the glioma cells. The main **objectives** of this work were to evaluate: 1) the integrins expression in the human glioma cell line U-118; 2) the integrins contribution to GBM motility, proliferation and survival using an integrin αv and β3 subunits block, Shikonin; 3) the evaluation of the possible synergistic effect between Shikonin and Temozolomide (TMZ), the *gold standard* in GBM treatment.

Methods and Results: In the human glioma cell line U-118, integrin expression was evaluated by western blot using specific antibodies. Then, taking account the integrin subunit more expressed in U-118 glioma cells, we evaluated the sensibility of cells to Shikonin and the effect on the expression of the integrin αv and β3 subunits. To elucidate the Shikonin effect on cell proliferation and death we used the BrdU/propidium iodide and annexin V assays, respectively, by Flow cytometry. The migration capabilities of U-118 glioma cells were studied by scratch assay. Moreover, to accomplished the results of apoptosis and migration, we observed the nuclear staining by Hoechst 33344 and stained the cells with falloidin to observed the f- actin organization by fluorescence and confocal microscopy, respectively. Regarding the integrin expression, our results showed that U-118 glioma cells expressed mainly the alpha V, beta 3 and alpha 5 subunits. So, we used an integrin blocker, the Shikonin, which induced a cytotoxic effect and also a reduction on αV and β3 integrin expression in U-118 glioma cells treated with this compound. Therefore, we evaluated the effect of Shikonin in growth and survival properties of U-118 glioma cells. The results showed a significant reduction in the migration and proliferation ability of GBM cells incubated with Shikonin and an activation of apoptosis, which was accompanied by a reduction in integrin expression. The simultaneous incubation of cells with Shikonin and Temozolomide induced a synergistic effect between these two drugs which suggests the possibility of Shikonin being used in glioma treatment.

Conclusions: In this work, we demonstrated that the integrins subunits, more specific αV and β3 have a critical role in invasion, migration and proliferation in the glioma cells. The

Shikonin appears to have a stronger modulatory effect in these glioblastoma cells properties. The blockage of integrins expression results in morphological and behavior alteration during the tumor progression. The Shikonin partly blocked the integrin subunits expression in glioma cells. Furthermore, the Shikonin induces apoptosis and decreases proliferation on glioma cells. The synergistic effect obtained between Shikonin and Temozolomide, suggests that combination of compounds with capability to block integrin αV and β3 subunits with conventional therapeutic approaches may represent a future strategy to inhibit tumor growth and the invasion into the tissues that surrounding tumor.

Keywords: Glioblastoma multiforme; integrins; shikonin; temozolomide; motility; survival.

RESUMO

Introdução: O glioblastoma multiforme (GBM) constitui 50% dos tumores que invade o sistema nervoso central. As principiais caracteristicas do GBM são a elevada proliferação, angiogénese e a invasão local. Apesar dos avanços consideravéis relativamente aos mecanismos envolvidos na genética, biologia e no comportamento clínico, a patologia do tumor não está completamente compreendida. No entanto, até agora, não há uma estratégia terapêutica proporcione uma diminuição da invasão e proliferação tumoral nas células de GBM.

Os principais objectivos deste trabalho foram avaliar: 1) a contribuição das integrinas numa linha celular de glioma humano, U-118, 2) a contribuição das integrinas na motilidade, proliferação e sobrevivência do GBM recorrendo a um bloqueador da expressão das subunidades das integrinas, Shikonin; 3) a existência de um efeito sinergístico entre a Shikonin e a temozolamida (TMZ), considerada o "*gold standard*" no tratamento do GBM.

Métodos e Resultados: A expressão das integrinas foi avaliada através de western blot recorrendo a anticorpos específicos para as diferentes subunidade das integrinas. Tendo em conta as subunidades de integrinas mais expressas nas células de glioma U-118 , avaliou-se a sensibilidade que estas células possuem quando incubadas com o bloqueador da expressão das subunidades αV e β3, shikonin. Para além disso, determinou-se o efeito do mesmo na proliferação e apoptose das células através da quantificação do BrdU, iodeto de propidío e anexina V, respectivamente através de citometria de fluxo. Para observar a migração celular usou-se a técnica do *Scracth* nas celúlas U-118. Além disso, para complementar os resultados obtidos da apoptose e migração, observou-se a coloração nuclear com Hoechst 33344 e a faloidina para observar os filamentos de actina através de microscopia de fluorescência e confocal, respectivamente. A proliferação celular e apoptose foram determinadas através de um kit de BrdU/iodeto de propídeo. Para a identificação de apoptose inicial recorremos à marcação com anexina V. Relativamente à expressão das integrinas os nossos resultados demonstraram que a linha celular U-118 expressa maioritariamente as subunidades alfa V, beta 3 e alfa 5. Tendo em consideração este resultado, usou-se a Shikonin, um bloqueador da expressão das subunidades αV e β3, e verificou-se que possui um efeito citotóxico nas células U-118 e que ocorre uma diminuição da expressão das subunidades das integrinas, αV e β3. Portanto, avaliamos o efeito que a shikonin possui no crescimento e sobrevivência das células U-118. Os resultados

demonstraram que a redução da expressão das integrinas é acompanhada pela redução das capacidades proliferativas e migratórias das células U-118, quando incubadas com a Shikonin e consequentemente induz a apoptose celular. Nas células U-118 sujeitas à incubação simultânea de shikonin com temozolomida, observou-se um efeito sinergístico entre os dois fármacos, sugerindo a possibilidade de usar a shikonin como agente quimioterapêutico em combinação com a temozolomida no tratamento do glioma.

Conclusões: Neste trabalho, demostramos que as subunidades das integrinas, especificamente as subunidades αV e β3 possuem um papel importante na invasão, migração e proliferação das células de glioma. A shikonin aparenta ter um grande efeito modulador nas células de glioblastoma. O bloqueio da expressão das integrinas resulta numa alteração morfológica e comportamental durante a progressão tumoral. A shikonin bloqueia parcialmente a expressão das integrinas nas células do glioma. Para além disso, a shikonin induz apoptose e diminui a proliferação das células U-118. Os resultados obtidos do efeito sinergístico entre a shikonin e a temozolomida sugerem que a combinação de bloqueadores das subunidades das integrinas αV e β3 com a terapêutica convencional, poderá constituir uma estratégia futura para a inibição do crescimento e invasão tumoral para os tecidos que rodeiam o tumor.

Palavras-Chave: Glioblastoma; integrinas; shikonin; temozolomida; motilidade; sobrevivência.

FIGURE INDEX

ACRONYMS AND ABBREVIATIONS LIST

ABC	ATP-binding cassette
Akt	Serine- Threonine kinase
Bax	Bcl-2-associated X protein
BBB	Blood- brain barrier
Bcl-2	B-cell lymphoma 2
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin- dependent kinase 6
CD44	Cluster Differentiation 44
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
ECF	Enhanced Chemofluorescence Substrate
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ErbB2	Epidermal Growth factor receptor 2
EORTC	European Organization for research and treatment of cancer
FBS	Fetal Bovine Serum
FDA	Food Drug Administration
Flt-1	Fms- related tyrosine kinase 1
GBM	Glioblastoma
HCl	Hydroxide Chloride
HIF-1α	Hypoxia inducible factor 1, alpha subunit
HRE	Hypoxia responsive element
IDH1	Isocitrate dehydrogenase 1 , soluble
INK4a/ARF	Cyclin-dependent kinase inhibitor 4A
KDR	Kinase insert domain receptor
MAPK	Mitogen- Activated Protein Kinases
MDM2	E3-ubiquitin ligase murine double minute 2
MGMT	O6-methylguanine DNA methyltransferase
MMP	Metalloproteinase
MTT	[3-(4-5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
MYC	v-myc myelocytomatosis viral oncogene homolog
NCAMs	Neural cell adhesion molecules
PDGFR	Platelet derived growth factor receptor
PDGFRA	Platelet-derived growth factor receptor Alpha-type
PlGF	Placental growth factor
PIKK	Phosphoinositide-3-kinase-related protein kinase
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
PIP2	Phosphatidylinositol (4, 5) - biphosphate

PIP3	Phosphatidylinositol (3, 4, 5) - triphosphate
PI3K	Phosphatidylinositol 3-Kinase
PTEN	Phosphatase and tensin homolog
PBS	Phosphate Buffered Saline Solution
P16	Tumor suppressor protein with 16 kilodaltons (Cyclin- dependent kinase inhibitor, 2A)
P53	Protein with 53 kilodaltons
RB	Retinoblastoma protein
RIPA	Radioimmuneprecipitation Assay Lysis Buffer
RTK	Receptor tyrosine kinases
SDS	Sodium Docedyl Sulphate
SHK	Shikonin
TBS-T	Tris-Buffered Solution- 0,1% Tween
TCGA	The cancer genome atlas
TGF-β	Transforming Growth factor beta 1
TMZ	Temozolomide
TP53	Tumor suppressor Protein p53
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
XL184	Cabozantinib
WHO	World health organization classification

CHAPTER 1

THEORETICAL BACKGROUND

Introduction

Glioblastoma (GBM) is considered the most malignant of the astrocytic gliomas and constitutes the most common malignant primary brain tumor (Furnari *et al*., 2007). The first-line for GBM treatment is the maximum surgical resection followed by adjuvant radiation therapy and chemotherapy with the temozolomide (TMZ), considered the *gold standard* for glioma treatment.

However, the median survival of GBM patients is only 12 to 14 months from the time at diagnosis (Darefsky *et al*., 2012). The main reasons for GBM poor prognosis are the late stage of diagnosis combined with chemoresistance to currently available therapies (Blandes *et al*., 2008). Many studies have been performed to understand the cancer biology and, therefore, novel anticancer drugs have been developed, exploiting molecular differences between tumor cells and healthy tissues cells (Nicholas *et al*., 2011). Many targeted therapies have been developed to interfere with cell signaling pathways related with proliferation promoting factors and angiogenesis. However, the clinical results have been largely disappointing, demonstrating lack of efficiency and resistance development in all cancers, in particularly in GBM.

1. Glioblastoma

GBM, the most common and malignant type of brain tumor in adults, is characterized by diffuse infiltration throughout the brain parenchyma, robust angiogenesis, intense resistance to apoptosis, necrosis and genomic instability (Furnari *et al*., 2007). These characteristics confer the high cellular tumor heterogeneity with the presence of reactive astrocytes, microglia, endothelial and cancer stem cells (Vital *et al*., 2010; Lima *et al*., 2012). According to World Health Organization classification (WHO) there are several types of gliomas which are staged in four grades, grade I and II for astrocytoma, grade III for anaplastic astrocytoma and IV for glioblastoma, oligodendrogliomas, ependymomas and mixed gliomas, on the basis of their resemblance to the glial cells (Figure 1) (Louis *et al*., 2007).

As referred, GBM is the most common glioma type and is very biologically aggressive. It has been considerate a grade IV tumor, due to aloud characteristics that have been designed to

this grade, such as the uncontrolled cell proliferation, presence of considerable necrosis and angiogenesis, diffuse infiltration, apoptosis resistance, high genomic instability and high mortality rate (Furnari *et al.,* 2007; Kleihues, 2000).

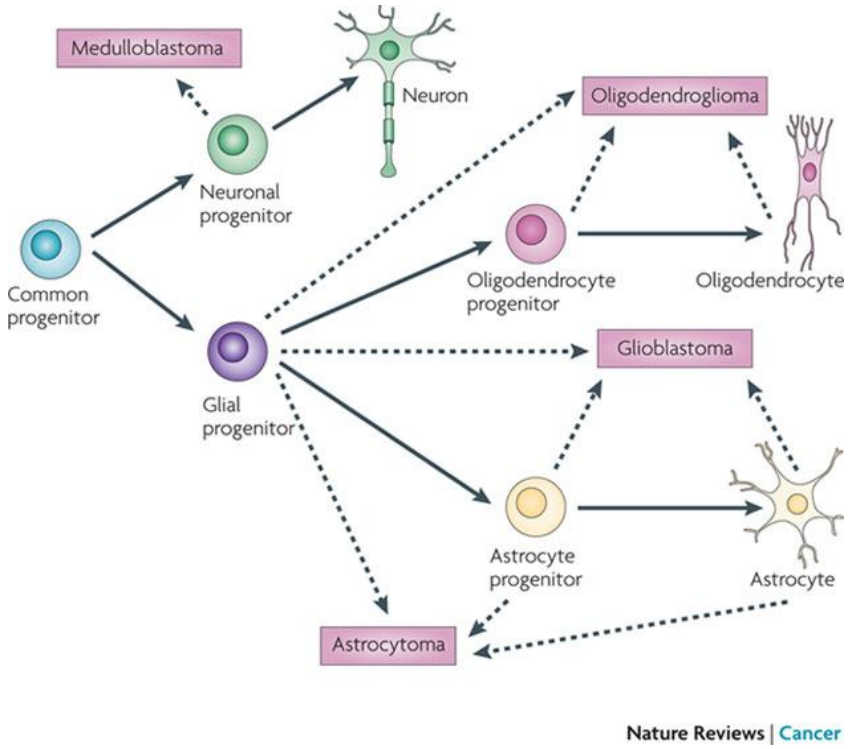
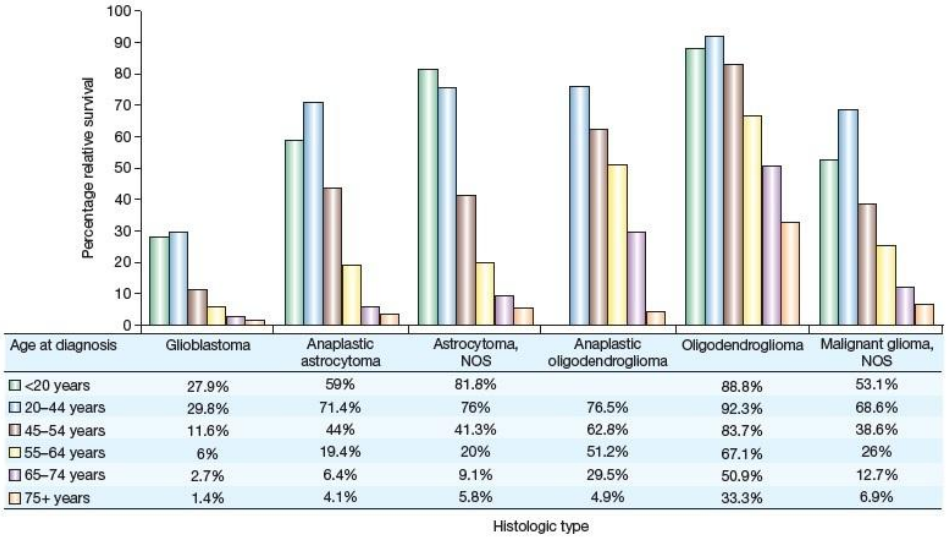


Figure 1 Theory of glioma cells origin. Common progenitors showing self-renewing properties are thought to produce committed neuronal and glial progenitors that eventually differentiate into mature neurons, astrocytes and oligodendrocytes. The astrocytes and oligodendrocytes can originate the different types of glioma, such as astrocytoma, glioblastoma and oligodendroglioma, respectively (adapted from Husen & Hollan, 2010).

1.1. Epidemiology of the disease

Many epidemiological studies have been performed to clarify and identify the genetic, behavioral, environmental and developmental contribution to risk glioma development and to reduce the incidence of the disease. Gliomas are approximately 77% of primary malignant brain tumors. In USA, per year, there are approximately 18.000 of new cases of primary malignant brain and central nervous system (CNS) tumors and approximately 13.000 deaths occur related with this tumors (Smith et al., 2006).

However, survival time for patients with a malignant brain tumor is related to age at diagnosis and histological tumor type (Figure 2). Besides that, the individual sex is very important, so statistical data showed that glioma is more frequent in men than in women (Sun *et al*., 2012).



Age at diagnosis	Glioblastoma	Anaplastic astrocytoma	Astrocytoma, NOS	Anaplastic oligodendroglioma	Oligodendroglioma	Malignant glioma, NOS
<20 years	27.9%	59%	81.8%		88.8%	53.1%
20–44 years	29.8%	71.4%	76%	76.5%	92.3%	68.6%
45–54 years	11.6%	44%	41.3%	62.8%	83.7%	38.6%
55–64 years	6%	19.4%	20%	51.2%	67.1%	26%
65–74 years	2.7%	6.4%	9.1%	29.5%	50.9%	12.7%
75+ years	1.4%	4.1%	5.8%	4.9%	33.3%	6.9%

Histologic type

Figure 2 Patient`s malignant brain tumor survival related with age. Figure represents the Glioma 2-year relative survival probabilities according with age at diagnosis and histologic subtype, based on the follow-up of individuals diagnosed between 1973 and 2002 (adapted from Schwartzbaum *et al*., 2006).

Demographic data showed that Australia, Canada, Denmark, Finland, New Zeeland and the US have higher glioma tumors incidence, and others countries such as Rizal in the Philippines and Mumbai, in India show lower incidence (Dubrow & Darefsky, 2011). The incidence of GBM is fairly low, with 2 to 3 cases per 100.000 people in Europe and North America (Kleihues *et al*. 2000).

The access to health and better medical care is very different between these demographic regions. In fact, countries that have a considerable social and economic condition have access at more health care's and medical treatment than those populations that have low social and economic conditions like the sub-development countries (Schlehofer *et al*., 2005).

The results in the response of GBM to treatment could also depend on the possibilities and medical care offered to the patients. According to the studies performed in 2005 by the European Organization for Research and Treatment of Cancer (EORTC) and National Cancer Institute of Canada (NCIC), GBM patients treated with TMZ during and after chemotherapy have an increased survival rate than those submitted to radiotherapy alone.

So, the administration of TMZ concomitantly with radiotherapy had been considered the GBM standard treatment (Stupp *et al*., 2005). However, the improvement was less prominent among those patients living in areas with a low median household income (Ohgaki & Kleihues, 2007). People living in districts with high median household income were more likely to receive chemo-radiotherapy and undergo a substantial resection. The low median income is generally associated with lower education levels, less social support and less quality of health care. Moreover, given the high cost of TMZ it's likely that some uninsured patients living in low-income districts had impaired access to the chemotherapy (Lawrence, 2011).

During the first decade of the 21st century it has been demonstrated a small, but incremental improvement, in overall survival among GBM patients.

1.2. Glioblastoma Etiology

Some studies have been performed to understand the etiology of glioma. However, it is largely unknown. There are few established risk factors for glioma development such as ionizing radiation, male gender and reproductive and menstrual factors (Brustle *et al*., 1992, Silvera *et al*., 2004). Moreover, other studies appointed that life style as cigarette smoking, diet, alcohol and occupational exposures may be involved in glioma etiology (Zheng *et al*., 2001), but there aren´t yielded consistent findings. Moreover, there are some evidences that hormonal factors might influence glioma risk (Kabat *et al*., 2010).

Asthma and other allergic conditions decrease glioma risk and this protective association has been confirmed by objective evidence from asthma-related germline polymorphisms (Schwartzbaum *et al*., 2006).

The general absence of consistence findings of associations between DNA repair, cell cycle regulation polymorphisms and glioma risk might be attributable to unexamined interactions between these genes and immune regulatory genes or with environmental factors or even both (Schwartzbaum *et al*., 2006).

Some data suggest that energy balance during childhood and/or adolescence may play an important role in the etiology of adult onset glioma (Moore *et al*., 2009). The biological pathways linking energy balance and cancer risk, particularly levels of insulin like

growth factor (IGFs) during childhood, should be more closely investigated as important in glioma etiology. Whether the energy balance and glioma association are due to a protective effect of early-life under- nutrition, a harmful effect of over nutrition, or to other energy balance-related factors, is uncertain. So, to reduce glioma risk it is required weight maintenance and a physically active lifestyle during childhood and adolescence (Moore *et al*., 2009).

1.3. Pathologic features of Glioblastoma

Gliomas are primary brain tumors which can differentiate into different types with own pathological and structural features. The classification of primary brain tumor is made according to neuroepithelial differentiation: astrocytic, oligodendroglial and ependymal (Figure 1). However, the astrocytomas and glioblastoma are the most common, making up 76%, of all glial neoplasm (Rao *et al*., 2003). According with WHO classification, GBM are grade IV tumors, characterized by higher agressivity, invasive ability, increased mitotic activity, pronounced angiogenesis, necrosis and high proliferation, which give a high degree of malignancy. Current models of gliomagenesis coincide with the two clinically recognized forms of GBM, *de novo* and progressive. *De novo* GBMs do not have tumor protein p53 (TP53) alterations and Epidermal Growth Factor Receptor (EGFR) gene amplification, however there are often combined with gene rearrangements that lead to a constitutively active, disable receptor. The progression from a low-grade to a high-grade glioma involves accumulation of genetic alterations, which leads to inactivation of tumor suppressor genes such as TP53, Cyclin-dependent kinase inhibitor, 2A (P16), Retinoblastoma protein (RB), Phosphatase and tensin homolog PTEN or activation of oncogenes such as E3-ubiquitin ligase murine double minute 2 (MDM2), cyclin-dependent kinase 4 (CDK4) and cyclin- dependent kinase 6(CDK6) (Figure 3) (Furnari *et al*., 2007).

Although histological indistinguishable, these IV grade gliomas occur in different age groups and present distinct genetic alterations affecting similar molecular pathways. For example, inactivation of tumor protein 53 (P53) function occurs due to direct mutation in progressive GBMs and P16 INK4aARF mutation/decrease in expression or MDM2 amplification in *de novo* GBMs (Gu *et al.,* 2002). Similarly, activation of the phosphatidylinositol 3-kinases(PI3K) pathway is achieved by several cooperative mechanisms, including EGFR amplification and mutation as well as PTEN mutation, although under-expression of PTEN in the absence of mutation is frequently seen as well (Choe *et al*. 2003).

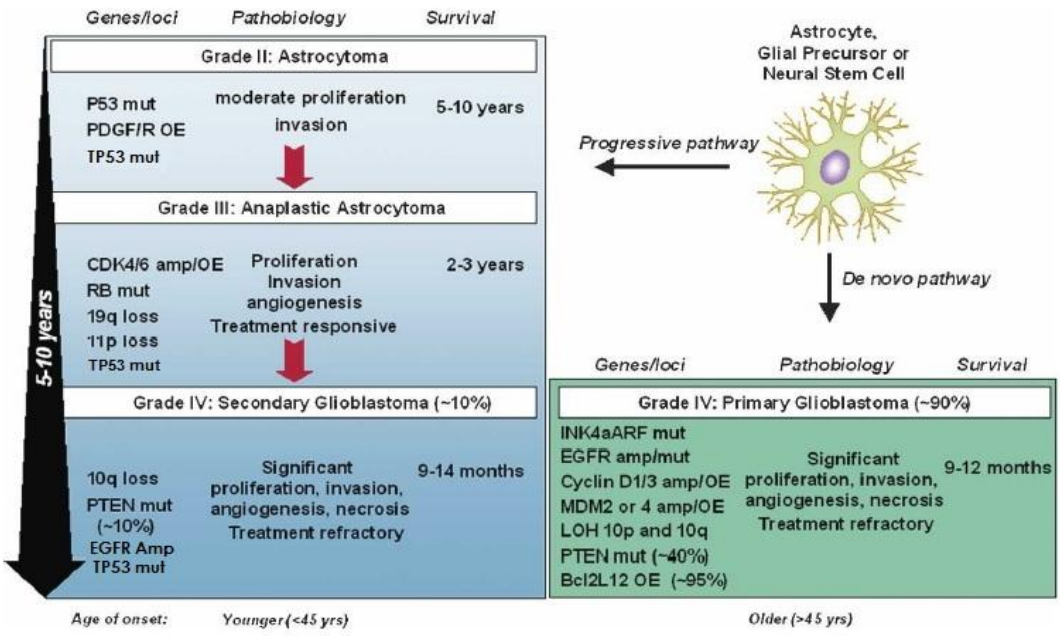


Figure 3 Chromosomal and genetic aberrations involved in the etiopathogeny of glioblastoma. The figure represented the relationships between survival and genetic alterations related with signaling pathways and the molecular lesions that lead to the formation of primary (*de novo*) and secondary (progressive) glioblastomas. Although histologically indistinguishable, these grade IV gliomas occur in different age groups and present distinct genetic alterations affecting similar molecular pathways. Overexpressed (OE); amplified (amp); mutated (mut) (Adapted from Furnari *et al*., 2007).

1.4. Invasion Mechanism involved in GBM

Invasion is a hallmark of malignant gliomas and is the main reason for therapeutic failure and recurrence of the tumor. Thus, there is a great interest to understand the molecular mechanisms governing glioma invasion, in order to develop effective anti-invasive therapies.

The lethality of gliomas is largely related with extensive infiltration into surrounding normal brain tissue (Amberger *et al.,* 1998). So, gliomas appear to be well demarcated glial cells, tipically migrating along the blood vessels and myelinated fiber tracts of the brain (Amberger *et al.,* 1998; Onishi *et al*., 2011). The latter process likely involves myelin-associated ligands while invasion along blood vessels is receptor-mediated (Zagzag *et al*., 2008).

The tumor invasion involves four independent and coordinated processes (Figure 4). The first is the detachment of the tumor mass from the original localization, involving downregulation of neural cell adhesion molecules (NCAMs) and E–cadherin as well as upregulation of cell surface glycoprotein (CD44), which function as an adhesion molecule (Thomas & Speight, 2001). The second process is the interaction with extracellular matrix (ECM), mediated by integrins and tenascin-C (Bellail *et al*., 2004; Alves *et al.,* 2011), leading to the degradation and remodeling of the ECM, including the activity of metalloproteinases (MMPs). These proteases can mediate the breakdown of basal membrane, creating and maintaining a microenvironment that facilitates tumor cell survival (Platen *et al*., 2001). The migration of tumor cells is the last step of invasion, requiring cytoskeletal rearrangements and formation of lamellipodia and filopodia (Figure 4).

Strong correlations have been reported between MMP expression, especially MMP-2 and MMP-9, and glioma cell invasion (Claes *et al*., 2009), and with the poor prognosis in glioma patients (Nakada *et al*., 2007). A promising approach is to inhibit the increase in MMP production by invading tumor cells by targeting the signaling transduction mechanisms that mediate their induction. The identification of intermediates involved in invasion mechanism is very important for the search of potential inhibitors which allows a better tumor resection and decreases the chemoresistance to compounds usually used in the GBM therapy, increasing patient´s survival rate and quality of life.

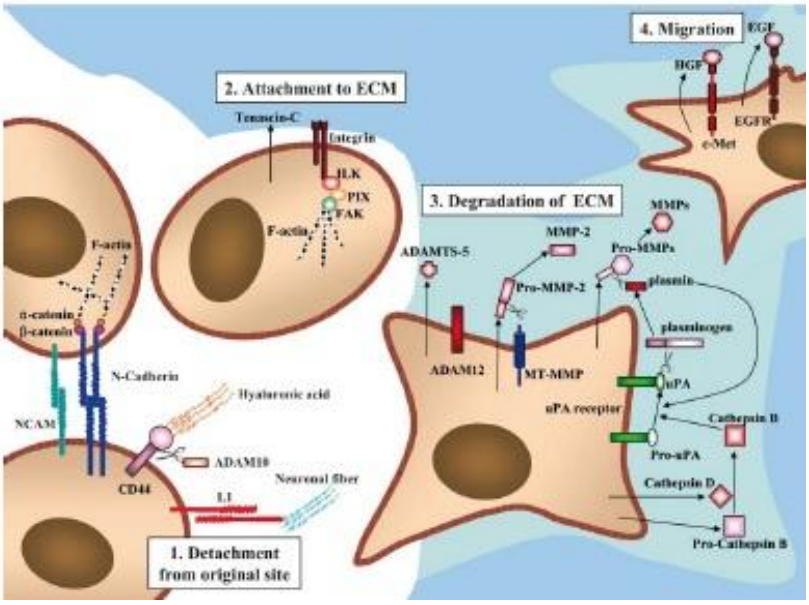


Figure 4 Underlying intracellular and extracellular mechanisms of glioma cell invasion. (I) Detachment from the tumor mass, involving downregulation of neural cell adhesion molecules (NCAMs) and E-cadherin as well as upregulation of CD44, a glycoprotein which functions as an adhesion molecule. (II) Attachment to extracellular matrix (ECM), mediated by integrins and tenascin-C. (III) Degradation and remodelling of the ECM, including the activity of matrix metalloproteinases (MMPs), and (IV) Migration, requiring cytoskeletal rearrangements and formation of lamellipodia and filopodia (Nakada *et al*., 2007).

1.5. Angiogenesis of GBM

Angiogenesis, the formation of new capillaries from pre-existing vessels, is another factor contributing to the pathology of malignant gliomas (Onishi *et al*., 2011). Therefore, inhibition of angiogenesis has been a promising strategy to complement standard treatment with TMZ. Preclinical data indicate that the proliferation and survival of GBM cells depend on angiogenesis. Moreover, the vascular endothelial growth factor (VEGF) and its receptors are key regulators of blood vessel growth and the main target of current anti-angiogenic drugs (Franceschi *et al*., 2011). The inhibition of VEGF function alone is sufficient to stop or slowdown solid tumor growth and also metastasis. Furthermore, blocking VEGF can lead with the reduction of blood vessel density in human tumors (Ferrara *et al*., 2005). However, targeting only one angiogenic pathway (as VEGF) may not be sufficient to block the tumor growth. The most potent activator of angiogenic mechanisms in brain tumors is tissue hypoxia. Some evidences showed that tumor hypoxia can cause angiogenesis and the malignant potential of surviving tumor cells (Hu *et al*., 2012). Specifically, VEGF-A is known to

be upregulated in glioblastoma and is produced by multiple cell types, including tumor, stromal and inflammatory cells (Hanahan & Folkman, 1996).

In May 2009, the US Food and Drug Administration (FDA) organization granted accelerated approval of the single agent bevacizumab for the treatment of patients with GBM that has progressed following prior therapy (Groot & Yung, 2008). Bevacizumab is a humanized monoclonal antibody that targets VEGF, an important mediator of angiogenesis, and is essential for GBM tumorigenesis. Bevacizumab inhibits the binding of VEGF to its receptors (VEGFRs), Fms-related tyrosine kinase 1(Flt-1) and Kinase insert domain receptor (KDR), on the surface of endothelial cells. Neutralization of the biologic activity of VEGF can result in the reduction of tumor vascularization and subsequent reduction in tumor growth (Cohen *et al*., 2009). Anti-angiogenic therapies may arrest tumor growth by mediating the regression of existing tumor vasculature and preventing regrowth over time. As a result, bevacizumab and other anti-angiogenic agents, including cediranib (AZD2171), aflibercept (VEGF Trap), cabozantinib (XL184) and cilengitide (EMD 121974) are being evaluated for the use alone or in combination with chemotherapy and/or radiotherapy in recurrent and newly diagnosed GBM (Beal *et al*., 2011).

1.6. Survival pathways involved in GBM

There are several signaling pathways involved in oncogenic proliferation, cell growth, survival and anti-apoptosis in GBM (Figure 5). This account for the large heterogeneity in cytopathological, transcriptional and genomic levels observed in glioblastoma, but also leads to the development of molecular targeted therapeutic approaches. The GBM treatment allowed the increased of survival rate from 3 to 8 months by surgical resection, and to 6-9 months after the combination with radiotherapy. The median overall survival of 14.6 months is due to the further addition of TMZ concurrently with radiotherapy (Cohen *et al*., 2005). However, there are several difficulties with the removal of tumor due to the localization and diffuse infiltration of tumor cells into normal brain tissue, that can't allow the surgical intervention (Alves *et al*., 2011).

The poor prognosis is mainly responsible to the highly invasive nature of GBM due to a several molecular processes involving deregulation of expression and function of several genes. Moreover, the analysis of primary tumor samples revealed that several signal

transduction pathways are blocked during the glioma progression, such as the alteration of Transforming growth factor beta 1(TGF-beta) and EGFR pathways, that can affect glioma cell migration and invasion by regulation of ECM remodeling which, in turn, involves modulation of MMP expression, ECM components and their integrin receptors. Moreover, invasion can stimulate proliferation and angiogenesis. Also, can increase the resistance to apoptosis providing the progression and maintenance of the cancerous state (Sciumè *et al.,* 2010).

The PI3K/serine-threonine kinase AKT (Akt) and Ras/MAPK pathways are the most important pathways involved in angiogenesis downstream to growth factor receptors, membrane-spanning proteins characterized by intrinsic tyrosine kinase activity (RTKs). Malignant glioma has highly activation of RTKs that can be responsible for the proliferation of tumor cells. The EGFR, platelet-derived growth factor receptor (PDGFR) and Vascular endothelial growth factor receptor (VEGFR) families are some of these examples (Michael & Wong, 2007). Moreover, GBM characteristics, such as the aggressive behavior and the patient's low survival, can be explained by the reduced level of PTEN and the increase in AKT activity. Many cancers, as breast and brain cancer, have PTEN mutations, which can downregulate the PI3K/AKT pathway by the conversion of phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) back to phosphatidylinositol 4, 5- biphosphate (PIP2). In GBM, the metabolic shift is observed, with regulation by genes of oncogenic pathways such as phosphoinositide 3-kinase/protein kinase, v-myc myelocytomatosis viral oncogene homolog (MYC) and hypoxia regulated gene (Hif-1α) (Marie & Shinjo, 2011). New advances in understanding the mechanisms regulating angiogenesis, such as those that promote cell migration and invasion are leading to the development of novel therapeutics for cancer (Figura 5).

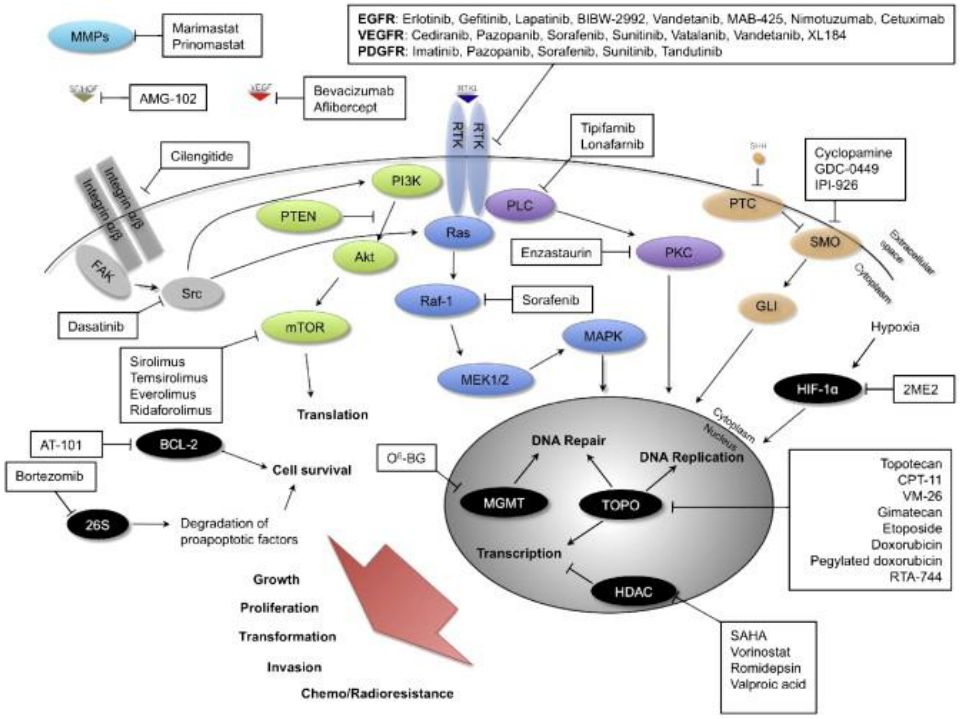


Figure 5 Molecular targeted therapies. The figure shows the signaling pathways and the constituent molecules implicated in glioma transformation, growth, proliferation, invasion and resistance to chemotherapy and radiotherapy. Locations of targeted experimental therapies targeting specific molecules and/or signaling pathways are indicated (Adapted from Arko *et al*., 2010).

1.7. Therapeutic approaches and treatment of glioblastoma multiforme

1.7.1. The Temozolomide- the *gold standard* in GBM treatment

The first step in treating GBM is usually surgery to remove the tumor but, there is a higher risky owing to the tumor location and its diffuse nature. Surgery is followed by radiation and chemotherapy. Even with surgery, the tumor can relapse almost always and the survival time is generally 12 to 15 months. The *gold standard* treatment for glioblastoma is TMZ combined with radiation. This strategy has been shown to increase survival rate from 12.1 to 14.6 months (Cohen *et al*., 2005).

TMZ is an imidazotetrazine derivative and can be metabolized *in vivo* to an active compound that methylates DNA at the O6 position of guanine. This induces the mismatch repair system leading to the DNA damage and induces the tumor cell death (Alvino *et al*., 1999; O'Reilly *et al*., 1993; Lowe *et al*., 1992). Moreover, TMZ can kill cells by inducing endoplasmatic rethiculum (ER) stress and autophagy (Cheng *et al*., 2012). Although TMZ delays tumor progression and improves survival, patients ultimately develop drug resistance and the tumor recurs. One of the mechanisms of resistance is the over-expression of O6-methylguanine DNA methyltransferase (MGMT), a DNA repair enzyme that confers resistance to DNA alkylating agents, like TMZ, by removing the alkyl adducts (Agnihotri *et al*., 2012). Another mechanism of drug resistance is due to multidrug resistance defined as simultaneous resistance of tumor cells to a broad spectrum of structurally and functionally diverse drugs.

TMZ does not have any anti-angiogenic activity, which had also been confirmed by Kim *et al.,* (2006). Moreover, TMZ does not have any effect on cancer stem cells. The TMZ capability to cross the blood brain barrier is derived of lipophilic proprieties and orally delivering (Kim *et al*., 2006). Preliminary clinical studies conducted by the Cancer Research Campaign showed that temozolomide has meaningful efficacy and an acceptable safety profile in the treatment of patients with malignant glioma (Friedman *et al*., 2000). But due to increasing TMZ-resistance to gliomas, there is an urgent need to develop more effective therapies for treating GBM.

1.7.2. Anti-Angiogenic drugs

Gliomas are highly vascular tumors that depend on angiogenesis for growth and progression. In last year's anti-angiogenic therapy has been used in GBM patients treatment showing interesting results. Since the VEGF pathway is the major pro-angiogenic pathway in gliomas, VEGF and its receptor VEGFR, have been the main target for anti-angiogenic therapies in GBM. The humanized monoclonal antibody against VEGF, bevacizumab, has been used in phase II trials, showing good response rates of 50-66% for recurrent and anaplastic glioma (Vredenburgh *et al*., 2007). Other drugs that target VEFG or its receptor, like cediranib, vatalanib, sorafenib, sunitinib are also been investigated (Figure 6) for their role in malignant glioma therapy (Norden *et al*., 2008).

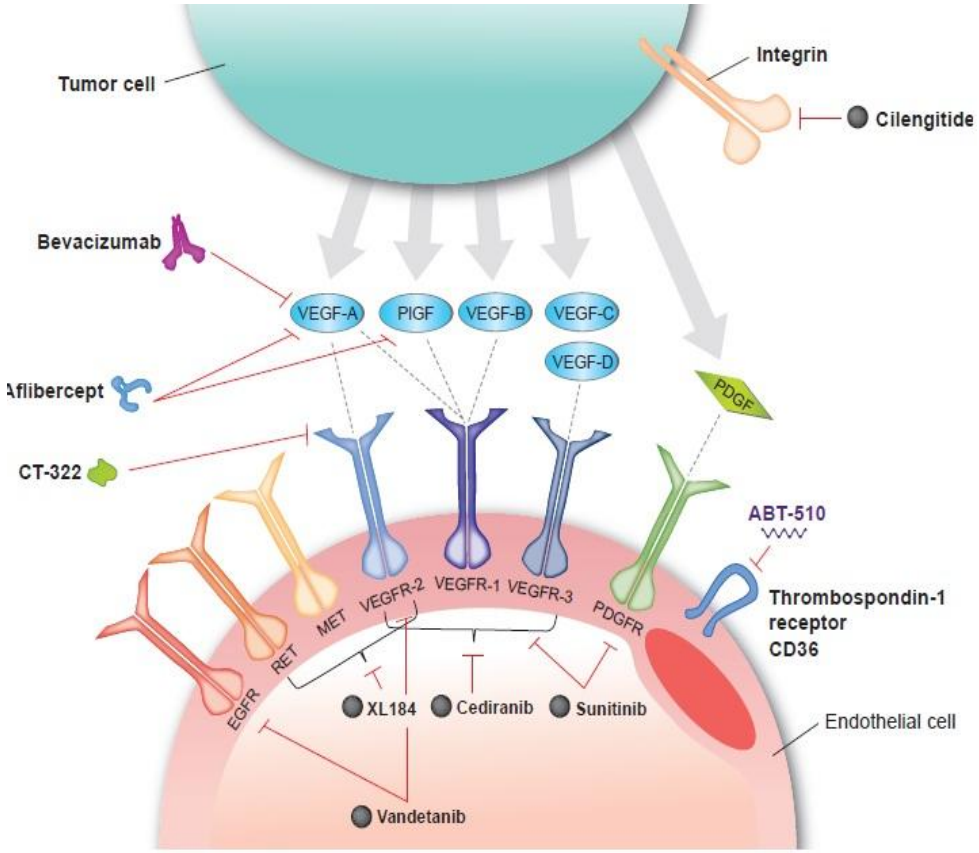


Figure 6 Molecular targets of anti-angiogenic agents in glioblastoma. Cilengitide is a cyclic peptide that binds and inhibits the activities of the alpha(v)beta(3) and alpha(v)beta(5) integrins. Bevacizumab is a humanized monoclonal immunoglobulin G1 antibody that binds and inhibits VEGF-A. Aflibercept is a fusion protein that binds all isoforms of VEGF-A, as well as PlGF. Cediranib, sunitinib, vandetanib, XL184, and CT-322 are multireceptor tyrosine kinase inhibitors. ABT-510 is a nonapeptide that targets the thrombospondin-1 receptor CD36 (Adapted from Beal *et al.,* 2011).

Sorafenib is a multikinase inhibitor of RAF, VEGF, PDGFR-b and c-KIT, and can suppresses angiogenesis via inhibition of VEGFR and PDFGR activities in endothelial cells (Adnane *et al*., 2006). Phase II trials with sorafenib in patients with malignant gliomas are ongoing to reduce the angiogenesis (Wong *et al*., 2009).

In parallel, cilengitide, an intravenous inhibitor of αvβ3 and αvβ5 integrins, demonstrated efficacy against an animal malignant glioma model and also inhibits blood vessel formation in vivo (MacDonald *et al*., 2001; Fu *et al*., 2007).

1.7.3. Anti-invasion treatment in GBM

The GBM is an extremely invasive tumor characterized by genetic heterogeneity affecting signaling pathways involved in invasion and migration. Due to these characteristics, many studies have been performed to reduce and inhibit these pathways or molecules. Recent studies have identified new molecules that are applied in the clinic showing significant results (figure 7). The most of them are new therapies that target molecular mediators of glioma migration and invasion. The common treatment, after detection, has several components including: 1) Surgical resection of tumor from the primary site; 2) Lining of the primary site with carmustine wafers designed to kill cells in the close proximity; 3) the chemotherapy with oral alkylating gent, temozolomide and 4) Radiotherapy directed to the brain. As previously mentioned, in the case of recurrence, this treatment is coupled with anti-angiogenic therapy to prolong patient life (Beal *et al*., 2011). The limitative response of GBM patients to the treatment may not be a suitable measure of efficacy of the drug (Franceshi & Brandes, 2011). The effectiveness of traditional chemotherapies such as procarbazine, lomustine and vincristine are limited by their side effects and their inability to cross the blood-brain barrier (BBB) in sufficient concentration (Goerne *et al*., 2008; Stupp *et al*., 2007).

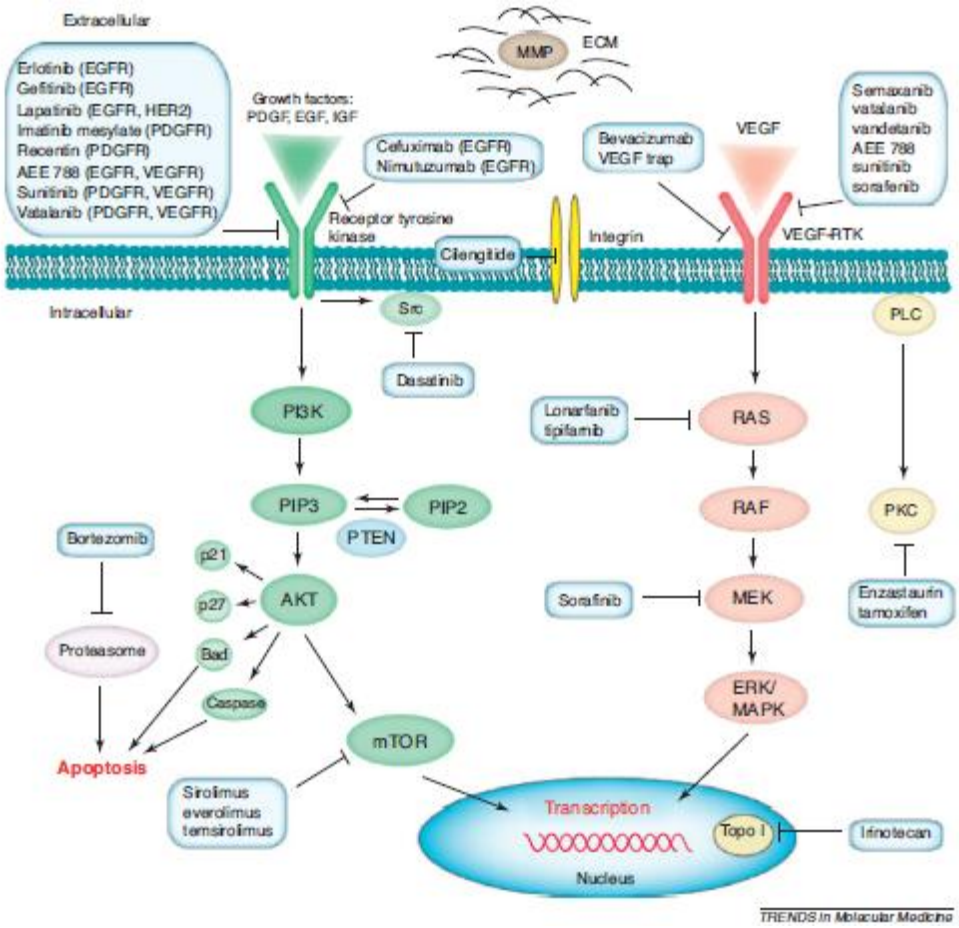


Figure 7 Mediators of glioblastoma treatment resistance. The figure represents a schematic overview of current molecular targeted therapies of GBM, depicted the various treatment resistance mechanisms and pathways differentially expressed or regulated in glioma. Aberrant oncogenic *RTK* pathways are frequent therapeutic targets in GBM. The PI3K-AKT (green) and *RAS* (pink) oncogenic pathways are often targeted intracellularly with small molecule inhibitors. EGF, VEGF and PDGF, as well as their receptors, can be blocked by small molecules and monoclonal antibodies. Items in blue boxes include examples of drugs that target the respective pathways (Adapted from Bai *et al*., 2011).

Another novel therapy is the photodynamic therapy which relies on a photosensitizer selectively taken up by tumor cells where upon light activation produces selective tumor destruction. The efficacy of photodynamic therapy is similar to chemotherapy with a median survival rate of 14 months in GBM patients, although some long-term survivors have been reported (Michael & Wong, 2007).

The Cancer Genome Atlas (TCGA) analysis showed that in GBM there are aberrations occurring more commonly in genes whose proteins products can regulate cell growth signaling pathways, such as TP53, NF1, Alpha-type platelet-derived growth factor receptor (PDGFRA), isocitrate dehydrogenase 1 (NADP+), soluble (IDH1), EGFR, PTEN,

phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1), NF1 and Human Epidermal Growth Factor Receptor 2 (ERBB2) (The Cancer Genome Atlas Research Network, 2008).

The receptor tyrosine kinase (RTK)/phosphatidylinositol 3-kinase PI3K)/Akt/mammalian target of rapamycin (mTOR) cascade is an important pathway which is frequently downregulated (Fan & Weiss, 2010). According to Parsons, 86% of clinical samples analyzed by the TCGA with both copy number and sequencing data had a genetic alteration in this pathway (TCGA research network, 2008). All tumors have to be sequenced and the genetic aberrations documented before selecting the targeted therapy.

1.8. Extracellular Matrix and glioma cells interactions

GBM is the most aggressive subtype of glioma, whose characteristics like diffuse infiltration into the normal brain parenchyma and the interaction with extracellular matrix (ECM) components in the brain are particularly. GBM cells are locally invasive and involve the basement membrane of blood vessels and myelinated nerve fibers of white matter tracts (Onishi *et al.,* 2011). The new ECM molecules and receptor adhesion molecules are expressed by glioma promoting the glioma cell-ECM interaction and signaling. The most common molecules that allow glioma cells to adhere to ECM are the integrins. However, ECM secrets molecules, such as tenascin-C, which is known to be associated with cell motility and angiogenesis which are essential for the tumor development (Alves *et al*., 2011).

1.8.1. Integrins

Integrins play an important role in cancer initiation and progression. Integrins are involved in adhesion, migration and survival processes of tumor cells and establish the interaction between microenvironment and tumor cells, including angiogenic endothelial cells (Figure 8).

The interaction between integrins and ECM ligands such as collagens, laminins, vitronectins and fribronectins can activate downstream signalling pathways through PDGFR, EGFR and VEGFR (Baker *et al*., 2009). Integrins have been considered as a promising therapeutic target in cancer and a key for the cellular processes of tumor growth. Antagonists of these integrins can suppress cell migration and invasion of primary and transformed cells and also induce apoptosis of primary cells. Integrin antagonists also block tumor angiogenesis and metastasis. Currently, humanized antibody antagonists and peptide inhibitors of the integrins α5β1/ αVβ3 are under evaluation as angiogenesis-inhibiting therapeutics in clinical trials (Bonfoco *et al*., 2001).

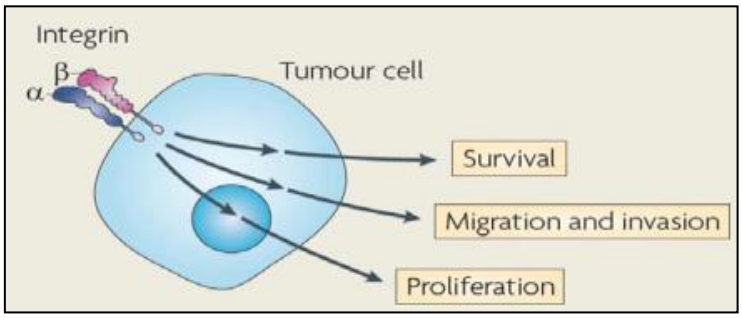


Figure 8 Integrins role in tumor cells. Integrins are involved in several survival pathways, migration, invasion and proliferation mechanisms in tumor cells *(Adapted from Desgrosellier & Cheresh, 2010)*

1.8.1.1. Integrin structures, ligand binding and activation

The integrin cell surface receptor is a heterodimer composed of 2 subunits, α and β. The α and β subunits interact and bind to their ligands in the extracellular matrix via their extracellular domains, which contain 'head' regions which can be folded in several states of activity (Figure 9). The integrins are a family of transmembrane adhesion receptors comprising of 19α and 8β subunits that interact nonconvalently to form up to 25 different heterodimeric receptors (Figure 10). The combination of different integrin subunits on the cell surface allows cells to recognize and respond to a variety of different ECM proteins including fibronectin, laminin, collagen and vitronectin (Chatterjee *et al*., 2000). Because integrins are the primary receptors for cellular adhesion to ECM molecules, they can act as crucial transducers of bidirectional cell signaling, regulating cell survival, differentiation, proliferation, migration, cytoskeletal organization and tissue remodeling, in many processes including fertilization, implantation and embryonic development, immune response, bone re-absortion and platelet aggregation. Integrin has been greatly implicated in tumor

development. There are extracellular factors that allow the integrin activation as the ligand binding, the divalent cation concentration, the chemokine signaling and the mechanical stress (Millard *et al*., 2011).

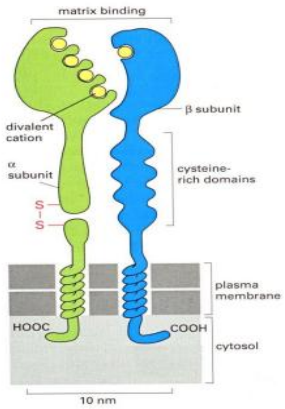


Figure 9 Integrin structure. Integrins are heterodimeric cell surface receptors composed of noncovalently-linked α and β subunits. Both subunits have large extracellular domains, which bind extracellular ligands. The small transmembrane domains are relatively short cytoplasmic domains, which bind intracellular anchor proteins. The α subunit is characterized by a large extracellular domain containing four divalent-cation binding sites. In some integrins, this domain is connected to the transmembrane domain via a disulfide bond. The extracellular domain of the β subunit contains a single divalent cation binding site,as well as a repeating cystein-rich region, where intrachain disulfide bonding occurs (Adapted from Alberts *et al*., 2002).

The absence of cell integrin-ligand interaction inhibits cell migration and induces apoptosis (Mattern *et al*., 2005). In normal cells, some integrins regulate survival, when connected, and induced apoptosis, when disconnected (Jin & Varner, 2004). Integrins can regulate cell migration by making contact with the substratum and by promoting signal transduction cascades that support migration. The heterodimer α6β1 play a pivotal role in maintaining adhesion to the ventricular zone and contributes for tumor cell proliferation, survival, self-renewal and *in vivo* growth (Lathia *et al*., 2010). At the same time, the integrins αVβ3 and αVβ5, among others, are highly expressed not only on the tumor vasculature and angiogenic endothelial cells, but also on tumor cells, including gliomas (Gladson *et al*., 1991). Moreover, GBM express integrin α3β1

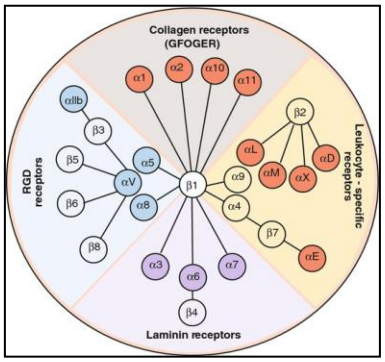


Figure 10 Representation of the integrin family. In higher vertebrates, the integrin family has 25 prototypical members (Adapted from Barczyk *et al*., 2010)

as a major receptor for the extracellular matrix both *in vivo* and *in vitro (*Fukushima *et al*., 2008).

1.8.1.2. Integrin Antagonists: applications in cancer

Since integrins are extremely important during glioma growth, invasion and angiogenesis, integrin inhibitors might be ideal therapeutic tools with synergistic activities in conjunction with already established therapeutic modalities, i.e., radiotherapy and cytotoxic chemotherapy. Furthermore, integrins are promising targets in glioblastoma therapy, specially αVβ3 and αVβ5 which are overexpressed on both glioma cells and tumor vasculature (Reardon *et al*., 2011; Gladson *et al*., 1991). Cilengitide is a cyclic peptide inhibitor of αvβ3/ αvβ5 integrins, used in cancers, such as glioblastoma (Lomonaco *et al.,* 2011; Maurer *et al*., 2008; Burke *et al*., 2002; Eskens *et al*., 2003). Thus, the αV-antagonist, cilengitide, suppresses brain tumor growth through induction of apoptosis in both brain capillary and brain tumor cells by preventing their interaction with the matrix proteins, vitronectin and tenascin. The dual action of this peptide explains its potent growth suppression of orthotopically transplanted brain tumors (Taga *et al*., 2002).

Abdollahi *et al*., showed that the RGD peptidomimetic integrin antagonist (S247) enhanced radiation-induced antiangiogenic effects in human endothelial cells *in vitro* and significantly augmented the local antitumor effect of fractionated radiation in tumor models *in vivo* without apparent increase in toxicity (Abdollahi *et al*., 2005).

Shikonin, an active chemical component isolated from Zicao purple gromwell (the dried root of *Lithospermum erythrorhizon* a chinese herbal medicine) (Figure 11), has numerous pharmacological properties, including anti-inflammatory and anti-tumor.

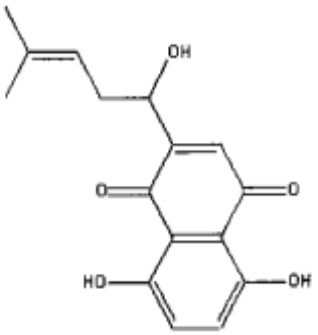


Figure 11 Chemical Structure of Shikonin ($C_{16}H_{16}O_5$: molecular weight, 288). Shikonin is a major active chemical component isolated from zicao (Adapted from Tabata *et al*., 1974)

Furthermore, shikonin can have the ability to promote wound healing activity (Chen *et al*., 2003). Some studies showed that Shikonin can block chemokine receptor functions by interfering with downstream signals, which may suppress inflammation and HIV-1

replication (Andújar *et al*., 2010; Chen *et al*., 2002). Moreover, Guo and colleagues *et al*. (1991) investigated the effects of Shikonin on lung cancer and observed that the Shikonin mixture could inhibit the growth and improve the immune function of the body. Besides that, Shikonin have been studied in osteosarcoma, and results showed by Chang *et al*. (2010) demonstrated that Shikonin increased ROS generation and ERK activation and reduced BCL2, which consequently caused the cells to undergo apoptosis. Other studies should be made to understand the Shikonin properties and the role in cancer cells.

Aims

Glioma cells are unique in their striking property to migrate and invade normal brain, often along preformed structures such as white matter tracts and subependymal space. These features are not shared with other cancer cells that derive from other organs and are often metastatic to the brain. Several studies have confirmed that integrins over-expressed on glioma cells enhanced the interactions with the extracellular matrix for the migration and invasion of glioma cells (Frielander *et al*., 1996; Uhn *et al*., 1999; Brizzi *et al.* 2012).

As so, in this study, we had intended to elucidate the motility of glioma cells, giving a particular attention to the role played by integrins during tumor invasion. To attaint this objective, we had used the integrin blocker, Shikonin, alone or in combination with the gold standard for glioblastoma treatment, temozolomide.

In summary, the main objectives of this work were: i) to evaluate the integrin basal expression in a U-118 glioma cell line; ii) to verify if the blocking of integrins expression in U-118 cell line can modify proliferation, cell cycle and cytoskeleton; iii) to elucidate if shikonin in combination with temozolomide can have synergistic effect.

CHAPTER 2

MATERIAL AND METHODS

2. MATERIAL AND METHODS

2.1. Material

In all experimental solutions, plastic material and glass used were sterilized by autoclaving at 121 ° C or by filtration a pore size of 0.22 m in the case of solutions.

Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10 % fetal calf serum was purchased from Invitrogen (Paisley, UK).The protease and phosphatase inhibitor cocktails were obtained from Roche (Mannheim, Germany). The anti-actin antibody was purchased from Millipore (Bedford, MA). The alkaline phosphatase-linked secondary antibodies and the enhanced chemifluorescence (ECF) reagent were obtained from GE Healthcare (Chalfont St. Giles, UK). The polyvinylidene difluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA). All other reagents were from Sigma Chemical Co., (St. Louis, MO) or from Merck (Darmstadt, Germany). The integrin antibody Sample kit was purchased by Cell signaling (Spain). The In situ cell proliferation kit was obtained by Roche (Spain), shikonin by Merck (Germany) and temozolomide from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 568 phalloidin, propidium iodide (PI), Hoechst 33342, Annexin V from and DAPI were acquired from Invitrogen (Paisley, UK). Vectashield was acquired from Vector Laboratories, Peterborough, England. All other reagents were acquired from Sigma-Aldrich Corporation (St. Louis, MO, USA).

2.2. Cell Culture

The cell culture is used to applying the experimental manipulation of cells and tissues *in vitro*. This technique is based on creating a system of cellular proliferation, with characteristics as close as possible to their *in vivo* environment. Is necessary a culture medium with appropriate concentrations of nutrients, whether amino acids, inorganic salts, vitamins, hormones and / growth factors, and favorable environmental conditions, and constant parameters, including pH, temperature, CO2-enriched atmosphere, humidity and asepsis. However, the cell culture also has disadvantages, including alteration of cell phenotype and genotype after a long period of growth (Freshney, 2000).

The human glioblastoma cell line, U-118 cells (cells isolated from a glioblastoma of a 50 years Caucasian male), was grown in Dulbecco's modified Eagle's medium (DMEM)

(Sigma-Aldrich) supplemented with 10 % fetal calf serum (FCS) (Gibco, Invi-trogen, UK), 100 units/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). The cells were maintained at 37 °C, 5 % CO2, and 95 % air in a humidified incubator and routinely passaged at time confluence (±80 %). All experiments were performed on cells seeded out 48 hours in advance, allowing the cells to attach to the surface of the tissue culture plate and to reach a cell density appropriate for the particular experiment.

2.3. Assessment of integrin expression in U-118 cells by western blot

To determine the integrin expression on U-118 cell line, the cells were treated with different concentrations of shikonin (8,649 mM) alone or in combination with temozolomide (0,133mM) during 48h. After that, cells were collected and lysis buffer (RIPA buffer: 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and 1 mM DTT, freshly supplemented with protease and phosphatase inhibitor cocktails was added at the end of each incubation period. The nuclei and the insoluble cell debris were removed by centrifugation at 4°C, at 12.000 g for 10 min. The postnuclear extracts were collected and used as total cell lysates. Protein concentration was determined using the bicinchoninic acid method and the cell lysates were denatured at 95°C, for 5 min, in sample buffer (0.125 mM Tris pH 6.8; 2% w/v SDS; 100 mM DTT; 10% glycerol and bromophenol blue) for its use in western blot analysis. Thirty µg of total protein was added to each lane of a 12% SDS-PAGE and transferred to a PVDF membrane. The immunodetection of integrin expression was performed incubating the membrane overnight in the presence of a rabbit anti-integrin α(4, 5 and V) and rabbit anti-integrin β (1, 3 and 5) (1:1000) and the subunits were visualized by the ECF method. Immunoblotting, using anti-actin were used to confirm comparable loading of integrin protein in each lane. In order to reprobe membranes with additional primary antibodies, namely those used against actin, used as experimental loading control, ECF was removed by washing the membranes with TBS-T for 30 min. After this washing step, the membranes were washed for 5 min with 0.2 M NaOH and washed again, abundantly with water. Membranes were again blocked for 1 h, with 5% non-fat milk in TBS-T, and incubated with the anti-actin antibody (1:20.000).

Secondary antibodies were incubated for 1 h at room temperature. Each experiment was repeated three times.

2.4. Evaluation the effect of Shikonin and temozolomide by MTT assay

The cell viability of the human GBM cell line U118-MG was performed by MTT assay. For the experiments cells were incubated during forty-eight hours at different concentration in alone/association of Shikonin (8.649 mM) and temozolomide (0.133 mM). One hour before the cells completes the forty eight hours of incubation, we taken 1,1ml of medium and MTT (5mg/ml) was addicted to the cells and stained them along forty five minutes. After that, the all medium are removed and the cells were centrifugated at 2500rpm during ten minutes. The supernatant are removed and the pellets dissolved with isopranol acid (0,04N) and measure the absorbance in ELISA plate at 570nm and reference 620nm. The percentage of viable cells in relation to the controls (untreated cells) was calculated using the following formula (1):

$$(1) \qquad \%\ \text{cellular viability} = \frac{\text{Absorbance (sample)}}{\text{Absorbance (Control)}} \times 100$$

A dose response-curve was plotted on a semi-log scale with a percentage of viable cells *versus* concentration of SHK alone or combinated with TMZ and fitted to a sigmoidal function, according to the equation (2). The concentration of drug necessary to inhibited cell proliferation by 50% (IC_{50}) was determined.

$$(2) \qquad y = A1 + \frac{A1-A2}{1+10^{(Logxo-x)p}}$$

Where A1 and A2 are the bottom and top asymptotes, respectively, x0 is the IC_{50} and p the slope. The curve fitting was performed using OriginPro8 software (OriginaLab Corporation).

2.5. Analysis of Proliferation, Cell cycle and Cell death by Flow Cytometry

The U-118 cells were incubated for 48h, in the presence and/or absence of integrin blocker shikonin (8.6mM) alone and in combination with temozolomide (0.133mM). After 48h, the cells were incubated with BrdU labelling solution (10mM) for 30-60 min at 37ºC in a humidified atmosphere (5% CO_2). The cells were fixed with fixative solution (ethanol-fixative with glycyne 7:3) and denatured with HCL (4M). Between all steps, the cells were washed with PBS and centrifugated at 500xg. The samples were analysed by fow cytometry, for detection of incorporation of BrdU with the conjugated anti-BrdU monoclonal antibody, $F(ab')_2$ fragments (anti-BrdU FLUOS). Thereafter, the cells were incubated with cocktail of Iodete Propidium and RNAse (0,5mg/mL). Moreover, we evaluated the cell death using an annexin V and PI staining protocol. The cells were incubated with bidding buffer solution (10mM HEPES, 140mM NaCl, 2.5mM CaCl2, pH 7.4) and washed two times with PBS 1x. Then, 10µL the mix of annexin V and PI was added to the cells and analyzed in Flow cytometry.

2.6. Analysis of apoptosis by fluorescence microscopy

U-118 cells were plated in round coverslips into the twenty-four-well plates and then incubated with Shikonin alone or with combination of temozolomide for 48 h. After the incubation period, cells were washed with PBS three times. Then, the cells were fixed for 10 min with methanol/acetone (1:1) and incubated for 5 min with Hoechst 33342 (5µg/ml). Cells were then washed three times with PBS, coverslips were mounted onto slides using Vectashield Mounting Medium and visualized in a fluorescence microscopy.

2.7. Evaluation of the U-118 cells migration capacity

Cell migration was studied according the method described by Liang *et al*. (Liang *et al*., 2007). Briefly, U-118 cells were plated on twelve-well plates and allowed to adhere to the

collagen matrix. When cells were confluent, the cell monolayer was scraped in a straight line, with a p200 pipet tip, debris were 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 as the zero time. Next, shikonin was added to each well and cells were photographed during the next 4h after the additions. Each experiment was repeated three times.

2.8. Characterization of actin filaments in GBM cells

U-118 cells were plated in round coverslips into the twenty-four- well plates and then incubated with shikonin for 48 h. For detection of actin filament, cells were fixed with 4% paraformaldehyde/PBS for 20 min, submitted to permeabilization with 0.1% Triton X-100/PBS for 3 min and then incubated for 45 min with the Alexa Fluor 568 phalloidin staining solution (5 U/ml) in PBS containing 1% BSA for 20 min. Nucleus were stained with DAPI for 2 min. The coverslips were mounted onto the slides using Vectashield Mounting Medium and visualized in a confocal microscope (Zeiss LSM, 510 Meta) using a filter set with an excitation filter of 568 nm and a barrier filter of 585 nm.

2.9. Statistical analysis

All values are expressed as mean ± SEM. After proving the assumption of normality and homogeneity of variance across groups, 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 was set at $p < 0.05$, $p < 0.01$, $p< 0.001$ and $p<0.0001$.

Statistical analysis was performed using the software Originalab.

CHAPTER 3

RESULTS

3. RESULTS

3.1 Characterization of integrin subunits expression in the U-118 glioma cell line

Glioblastoma display enhanced expression of several integrins along with their ECM ligands such as tenascin and vibronectin (αvβ3 and αvβ5), fibronectin (α5β1), collagen (α2β1) and laminin (α3β1, α6β4 and α6β1) (Bellail *et al.,* 2004). To analyze the basal expression of integrins in U-118 glioma cells, we used specific antibodies to detect the integrins subunits (αV, α4, α5, β3, β5 and β1) by western blotting. We found that U-118 cells expressed the αV, α4, α5, β3, β5 and β1 integrin subunits (Figure 12). However, as shown in Figure 12, in the U-118 glioma cell line, the α5, β3, αV are the integrin subunits more expressed in this cell line.

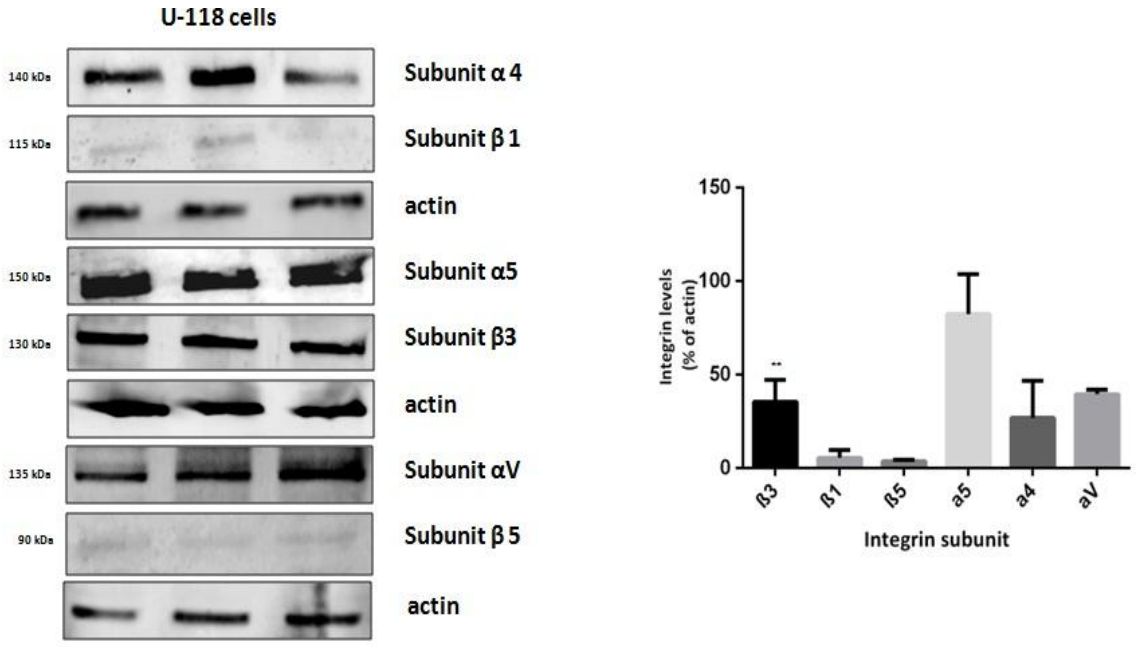


Figure 12 Evaluation of integrin levels by western blot analysis in U-118 glioma cells. After the incubation period of 48 hours, the cell lysates were prepared and used for Western blot analysis to determine the basal expression levels of each subunit of integrin with specific antibodies (anti-α4,α5, αV and anti-β1, β3, β5) according with the described in materials and methods. All values are representative of three independent experiments. * p< 0,5

3.2 Susceptibility of the U-118 glioma cell line to treatment with shikonin

3.2.1 Effect of shikonin in glioma cells

In order to evaluate the contribution of integrin expression to proliferation, migration and apoptosis of the U-118 cells, it was decided to incubate cells with shikonin (SHK). SHK and its derivates have been investigated as potential anti-cancer drugs. Some evidences showed that shikonin and its analogues could induce necroptosis in breast cancer cells (Han *et al*., 2012). Also, shikonin is a unique small molecule that can act on multiple cellular targets and trigger multiple death pathways, thus making it a promising candidate for cancer treatment, particularly in multi-drug resistance cancer cells (Han *et al*., 2007; Ahn *et al.,* 2000 and Kim *et al*., 2001). To determine the effect of shikonin on the viability of U-118 glioma cells, the cells were incubated with 0, 0.2, 0.5, 0.75, 1, 1.5, 2, 5, 10, 15 and 20 µM of the compound for 48 hours.

The data points of individual dose-response curves were fitted with a sigmoid function for the calculation of the IC_{50} values that correspond to the drug concentration that inhibits cells viability in 50%, as represented in Figure 13. This parameter was considered as a measure of cell sensitivity to Shikonin. The obtained dose-response curve of Shikonin and corresponding IC_{50} of Shikonin are presented in Figure 13. As we can observe in this Figure, the treatment with shikonin induced a decrease in glioma cells viability in a dose-dependent manner. Our results showed that the IC50 of shikonin was 0.27±1.87 µM ($p<0.001$) for U-118 cells (Figure 13).

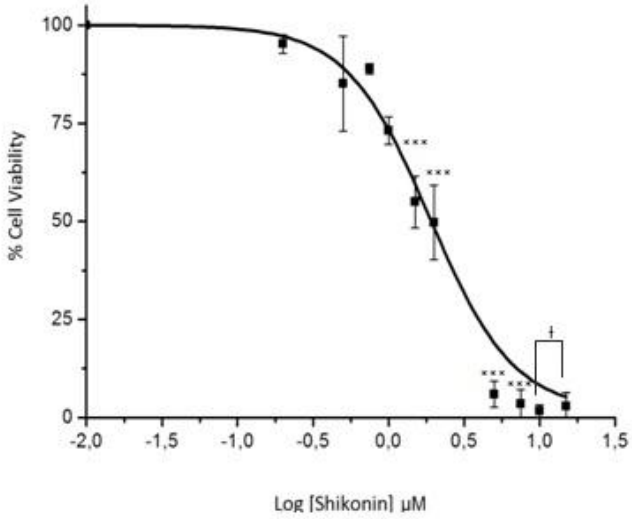


Figure 13 Evaluation of U-118 glioma cells viability after Shikonin treatment by the MTT assay. U-118 cells survival rate was obtained by the MTT assay after 48 hours of treatment of the cells with different Shikonin concentrations (0 to 20µg/mL). All values are the mean ± SD of three independent experiment each of them performed in triplicate ***p<0.001 ǂ p<0.0001.

3.2.2 Effect of shikonin in integrin subunits expression levels

In order to verify the effect of shikonin in integrin expression in our glioma cell line, U-118 cells were incubated with 0.75, 1, 1.5 and 2µM for 48h and then the expression of β3 and αV was evaluated by western blot. Hisa *et al*., (1998) showed the shikonin effect in migration and proliferation to prevent the network formation by endothelial cells via blocking integrin alpha v and beta 3 expression. In particular, the integrin αVβ3, which binds to ECM ligands such as fibronectin, vitronectin and tenascin C, plays an important role in glioma invasion (Bellail *et al*., 2004; Alves *et al*., 2011). Moreover, increased expression of integrin αVβ3 leads to the increase of the motility of human glioma cells with a concomitant decrease in apoptosis sensitivity (Platten *et al*., 2000).

Taking into account that the integrin subunit more expressed by U-188 cells showed in first section, we proposed to characterize the expression of integrins in the presence of the integrin expression blocker, the shikonin (Figure 14). Our results showed that, in the presence of SHK, there was a reduction in both subunits in a dose-dependent manner. However, the reduction was more evident in the αV subunit. These results suggest that shikonin could be used for blocking the integrin αV and β3 expression in U-118 glioma cells.

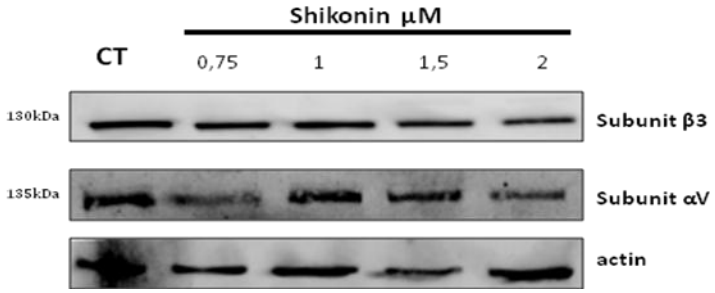


Figure 14 Shikonin reduces the αV and β3 integrin subunit levels in U-118 glioma cells. After the incubation period of 48 hours, the cell lysates were prepared and used for Western blot analysis, in order to investigate the expression levels of αV and β3 integrin subunits using specific antibodies (anti-αV and anti-β3) after shikonin treatment.

3.2.3 Shikonin inhibits proliferation in glioma cells

The contribution of integrins in U-118 cells proliferation was evaluated in the presence of 0.75, 1 and 1.5 µM of shikonin. The proliferation rate of U-118 cells was assessed using the method of BrdU, which consists in measuring the amount of the incorporated BrdU in DNA during the S phase of cell cycle. The incorporated BrdU was detected by a monoclonal antibody against BrdU, conjugated with a fluorochrome. Shikonin was added at concentrations bellow IC_{50} (0.75, 1 and 1.5 µg/mL) over a period of 48 hours and added 1:10 volume of BrdU(10mM). The results showed that 48 hours after the addition of 1 µM of shikonin the incorporated BrdU decreased about 20 % ($p < 0.001$) as compared to the incorporated BrdU in control cells considered as 100% proliferation rate, as we shown in Figure 15. However, at lower concentrations, namely 0.75 µM of SHK, almost no impairment of proliferation on glioma cells was observed compared with the control ($p<0,001$). These results suggest that Shikonin can decrease the proliferation of U-118 glioma cells in a dose-dependent manner.

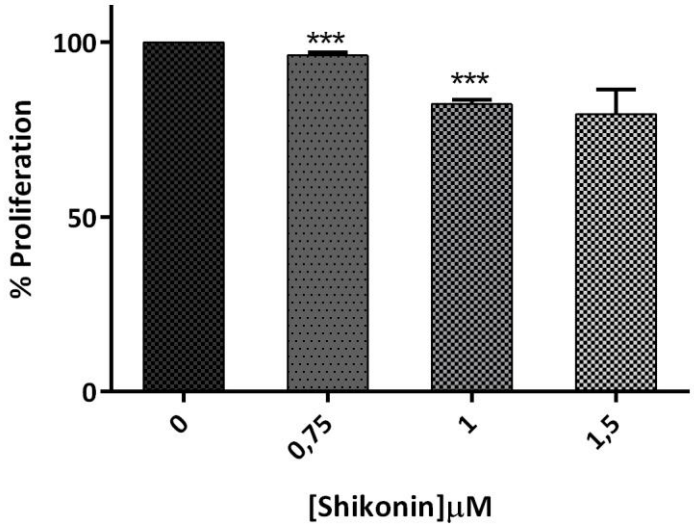


Figure 15 Shikonin reduces proliferation of U-118 glioma cells. U-118 cells were incubated for 48 hours in the presence of different concentrations of Shikonin. All values are mean ± SD of three independent experiments each of them performed in duplicate. ***p<0.001

3.2.4 Cell cycle U-118 cells analysis after Shikonin treatment

The cell cycle analysis was obtained by flow cytometry using a mix of PI/RNAse staining, allowing the calculation of the percentage of cell in a specific cell cycle phase. Since the G0/G1 fraction of cells have an N amount of DNA, the G2/M cells have 2 N while the fraction of cells that have an intermediate DNA amount of DNA are considered to be in S phase. Cells that rest on the left side of the G0/G1 peak (pré-G0/G1) are considered apoptotic cells related with DNA fragmentation (Figure 16A).

So, we evaluated the effect of SHK in the percentage of cells in different phases of cell cycle using the ModFit software (Figure 16A). Our results showed the fluorescence peak on the left of G1 peak of control cells corresponded to the cells in G0/G1 phase, since these cells are the most abundant in normal unsynchronized cell population. The G2/M peak is usually significantly smaller comparing with G0/G1 phase and is not always well defined. Therefore, the presence of the G2/M peal in the histogram has to be calculated by doubling the value obtained for the G0/G1 peak. Furthermore, our results showed that the U-118 glioma cells treated with 0.75, 1 and 1.5μg/mL of shikonin during 48h demonstrated a significant alteration in the cell cycle (Figure 16B). In fact, in cells treated with 0.75 and 1μM

of shikonin, we observe a decrease in the percentage of cells in the S-phase and and an increase in the G2 phase (Figure 16B), however these results are not significant.

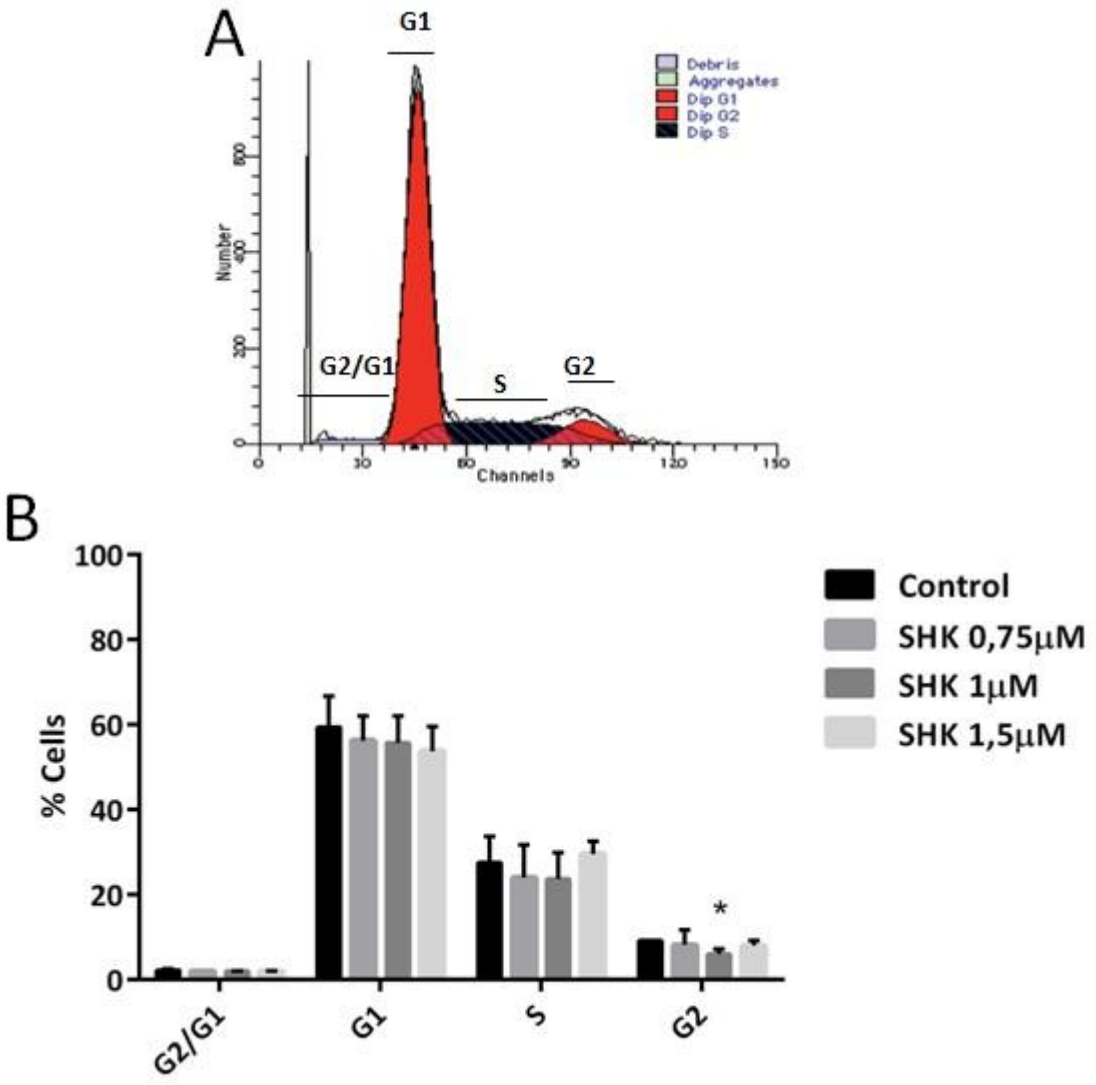


Figure 16 Cell cycle analysis of U-118 treated with Shikonin for 48h. The histogram is representative of the cell cycle phases of cells in absence of treatment with SHK (A); A total of 10.000 events were analyzed for each experiment. For cell cycle analysis, the U-118 cells were incubated with different concentrations of SHK (0.75µM, 1 µM and 1.5 µM) (B). After the incubation time, the cells were stained with PI and analyzed by flow cytometry. The percentage of cells in each cell cycle phase was determined by using the ModFit LT™ software. All values are mean ± SD of three independent experiments each of them performed in triplicate. *P<0.05.

3.2.5 Determination of apoptosis in U-118 cells incubated with Shikonin

Taking in consideration that shikonin reduced the proliferation rate of U-118 cells we decided to evaluate if shikonin also altered the apoptotic rate of U-118 cells. For that, U-118

glioma cells were treated with different concentrations of shikonin during 48h and apoptosis was studied by flow cytometry using Propidium iodide (PI) and Annexin V. PI and Annexin V were used to evaluate the viable cells, apoptotic or necrotic through differences in plasma membrane integrity and permeability (Rieger *et al*., 2011).

For that, the U-118 cells were cultured on six-well plates, treated with different concentrations of shikonin (0.75 at 1.5 µM) during 48hours. Then, cells were removed from the wells and 5µL of annexin V and 5µL of PI were added. The percentage of cells in necrosis was measured by analyzing the amount of PI bound to DNA of plasma membrane permeabilized cells and the percentage of cells in apoptosis by analyzing Annexin V binding to exteriorized phosphatidilserine in intact membrane cells. Our results displayed that Shikonin induced a decrease in viable cells and an increase in apoptosis, namely in late apoptosis in U-118 glioma cells (Figure 17). As we can observe in Figure 17, U-118 cells treated with SHK 0.75µM shown an increase in percentage of cells in apoptosis (2%) compared with the control cells ($p<0.0001$), which increase to 5% in cells treated with SHK 1 and 1.5 µM ($p< 0.0001$ and $p<0.01$, respectively). Moreover, we observed a decrease of viable cells in a dose-response manner compared with control a cell (30%), which was accompanied with an increased of late apoptosis (23%). In cells treated with 0.75µM of SHK we observed an increase of 5% of cells in necrosis state compared with control ($p<0.001$). However, in cells treated with 1 and 1.5µM of SHK there was a decrease in necrotic cells of 3% compared with cell treat at low concentration of SHK (n.s $p>0.05$).

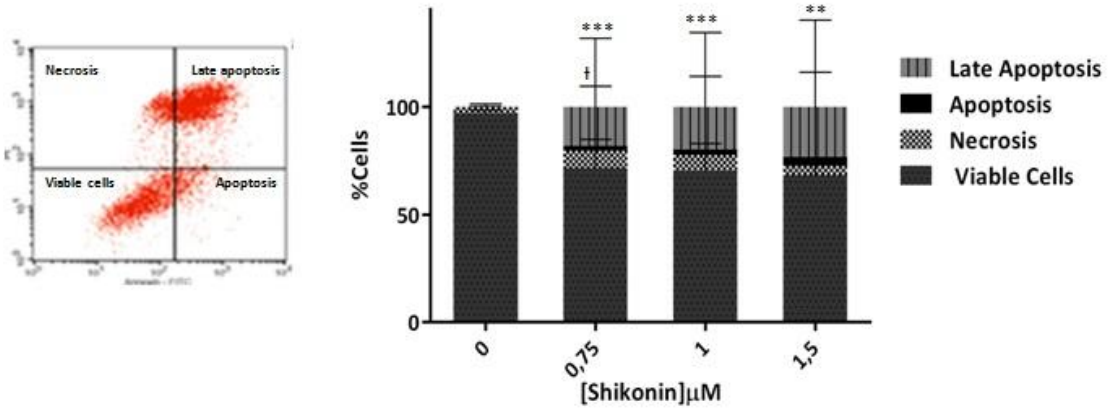


Figure 17 Effect of Shikonin in glioma cells survival and death. Apoptosis of U118 cells was detected by Flow cytometry using Annexin V and necrotic cells were stained using PI. The U-118 glioma cells were treated with different concentrations of Shikonin for 48 h.. Data represent the means and standard deviation of 3 independent experiments. ** $p<0.01$, *** $p<0.001$, ł $p<0.0001$

3.2.6 Nucleus visualization with Hoechst dye

In order to confirm the results obtained in cell cycle analysis and annexin V assay we evaluated if SHK induced morphological alterations compatible with apoptosis characteristics, such as chromatin condensation and pyknotic nuclei. For that, U-118 cells were stained with Hoechst 33342 dye (1µg/mL). The U-118 cells were cultured in 12 multi-well plates and incubated with different concentrations of Shikonin for 48 hours. Then, the cells were stained with Hoechst 33342 and nuclei were visualized on a fluorescence microscope.

The analysis showed that control cells exhibited a great number of nucleioli in the nuclei indicating that these cells have a high transcription activity. When cells were incubated for 48 hours with different concentrations of SHK (0.75, 1 and 1.5µM), the results in Figure 18B, C and D, respectively, showed an increase in the fluorescence intensity of the Hoechst which seems to indicate that in the presence of SHK there is a condensation chromatin (Figure 18).

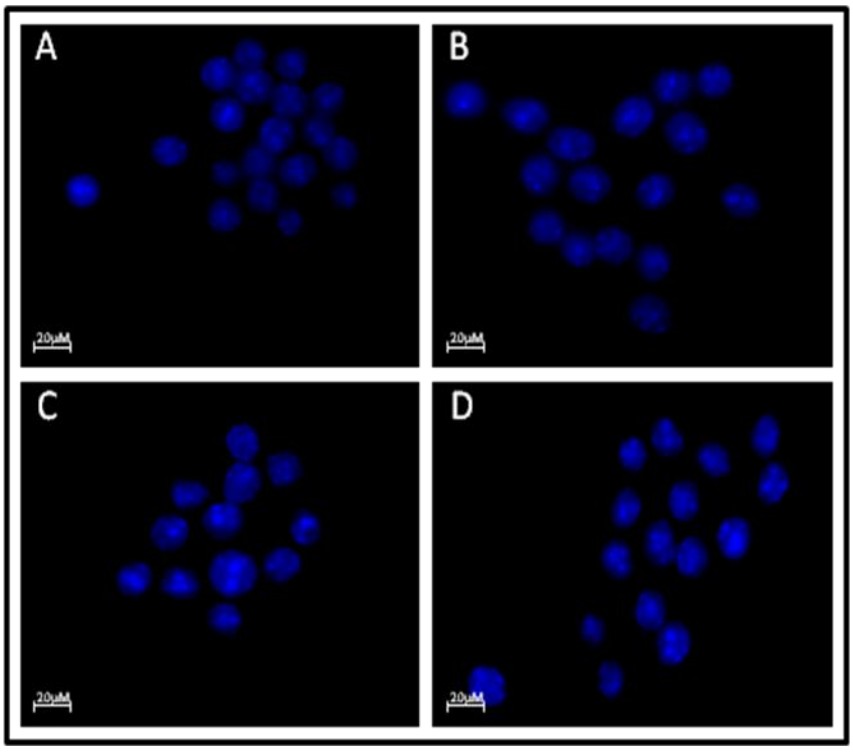


Figure 18 Shikonin promotes the cell death of U-118 glioma cells. The U-118 glioma cells were incubated in absence (control cells) (A) and in presence of different concentrations of Shikonin, with 0.75µM (B); 1µM (C) and with 1.5µM (D), for a period of 48h. After fixation, the cell death was evaluated by fluorescence microscopy with Hoechst 33342 staining for nuclear morphology analysis. The images are representative of three independent experiments.

3.2.7 Contribution of the Shikonin in glioma cell migration

Some studies revealed that glioma invasion into the healthy brain tissue required the interaction with extracellular matrix molecules and with surrounding cells (Bellail *et al*., 2004). Integrins plays an important role in glioma cells proliferation and invasion. Moreover, the interactions between integrins and ECM ligands leads to intracellular signaling events affecting cells processes such as migration, proliferation and survival (Farber *et al*., 2008). We evaluate whether shikonin could regulate the motility of glioma cells on a collagen matrix. For that, we had used the scratch assay according to the method described by Liang *et al*., (2007). The cells were incubated with shikonin at different concentrations (0.75, 1, 1.5µM) during 4 hours. The results showed that upon the removal, by scratching, of a part of the cell monolayer, some of the remained cells migrated, even without stimuli, into the scratch. Two hours after the addition of shikonin at different concentrations, it was not observed a significant difference in the number of cells that moved into the scratch in cells treated with 0.75µM SHK, as compared to the control experiment (Figure 19). In fact, the incubation of U-118 cells with SHK 0.75 µM did not alter the migration ability of U-118 cells. However, when cells were treated with 1µM and 1.5µM there was a decrease of the migrated cells into the scratch as compared to the control (Figure 19) .Reduction of the migration ability was more evident four hours after the addition of SHK.

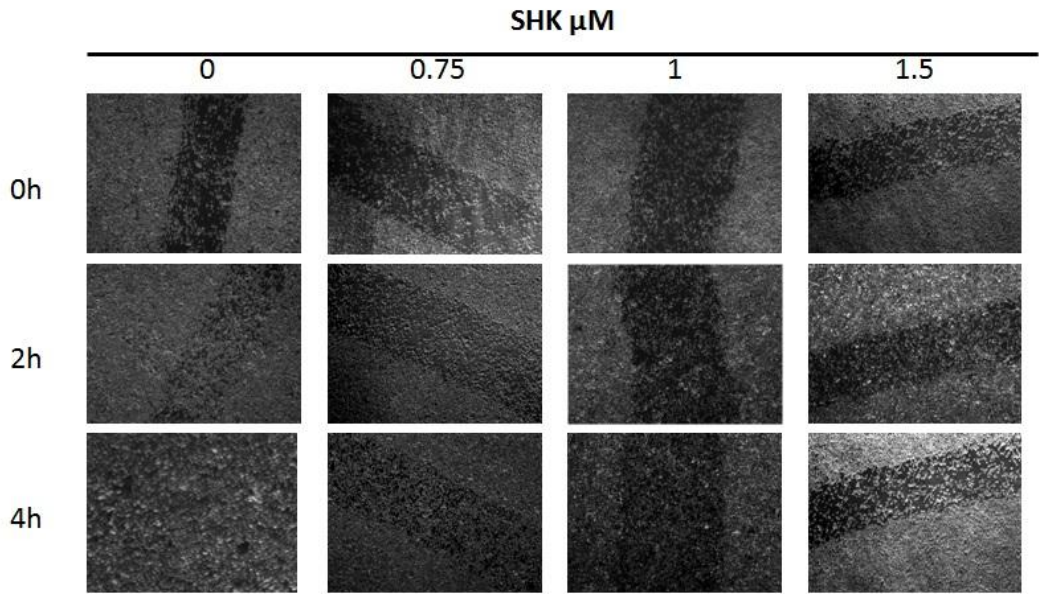


Figure 19 Shikonin can reduce the migration of U-118 glioma cells. To study U-118 cells migration through collagen matrix, cells were incubated during 4hours in the presence of different concentrations of Shikonin (0.75, 1 and 1.5µM).

3.2.8 Shikonin regulates microfilament dynamics in glioma cells

To accomplish the results obtained with migration studies, we evaluated if Shikonin could modify the actin cytoskeleton in U-118 glioma cells. The results showed that F-actin assembly in the shikonin cells were significantly disturbed compared to control cells (Figure 20). Control cells (Figure 20A) presented a well organized actin cytoskeleton. When cells were incubated with SHK, it´s possible to observe a disorganization of actin filaments (Figure 20B, C and D) in a dose-dependent manner, reflecting less lamellipodia and filopodia formation. The disorganization of F-actin filaments were observed for all SHK concentrations (Figures 20B, C and D).

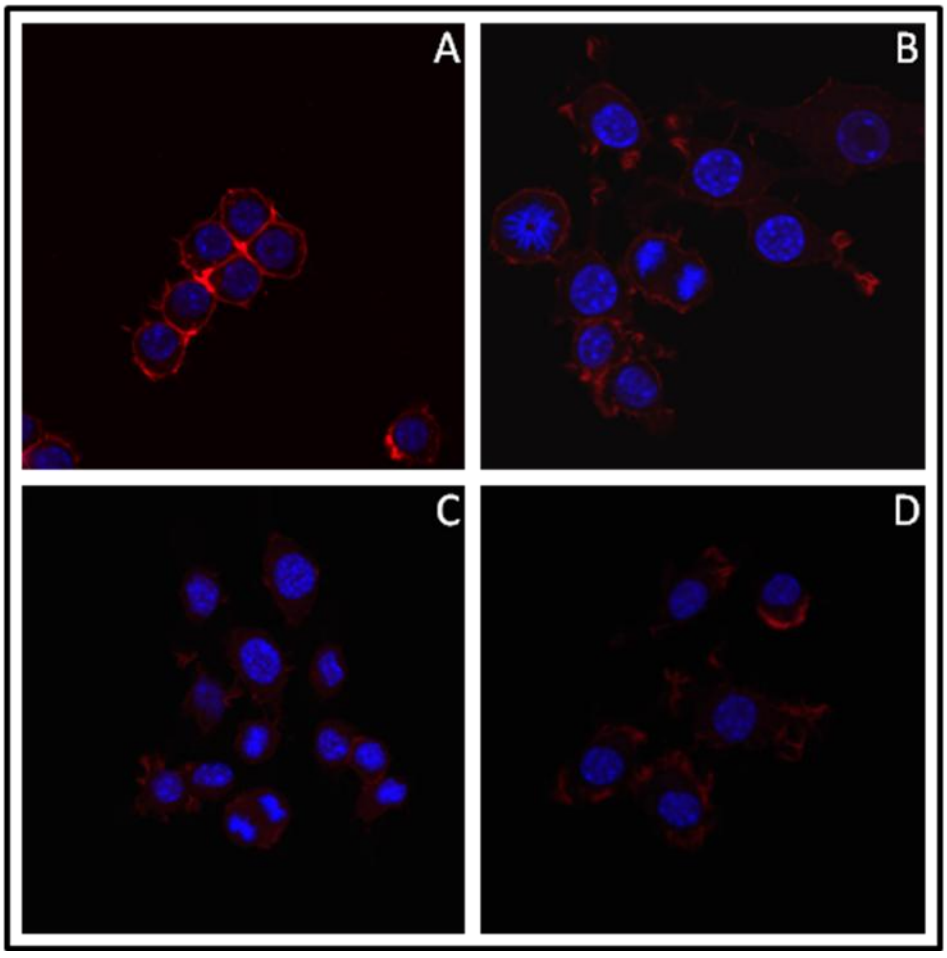


Figure 20 F-actin filaments organization in glioma cells treated with shikonin. U-118 cells were incubated for 48 hours in the presence of different concentrations of shikonin. Cells were then fixated and f-actin was stained with Alexa Fluor 568 phalloidin. Nuclei were counterstained with DAPI. Red represents f-actin staining and blue represents cell nuclei counterstaining. (A) Control cells; (B) Cells incubated with shikonin 0,75 µM; (C) Cells incubated with shikonin 1 µM;(D) Cells incubated with shikonin 1,5 µM. The images are representative of three independent experiments obtained by confocal microscopy with a magnification of x100.

3.3 The synergistic therapeutic effect of Shikonin and Temozolomide in U-118 glioma cells

Nowadays, the current standard of glioma patient's treatment consists of cytoreductive surgery, followed by concomitant radiation and chemotherapy with the oral DNA-alkylating agent, temozolomide (TMZ) (Barvaux *et al*., 2004; Hegi *et al*., 2005; Caporali *et al*., 2004). Temozolomide is considered the gold standard of GBM treatment after the randomized study performed by Stupp (Stupp *et al*., 2006). Preclinical studies and clinical trials are being investigated for the possibility to increase the anti-cancer potency of TMZ by combining it with other pharmacological agents (Goellner *et al*., 2011; Agnihotri *et al*., 2012).

3.3.1 Shikonin enhances the cytotoxic effect of temozolomide in U-118 glioma cells

Taking in consideration that SHK may reduce the proliferation and migration ability of U-118 cells and may also increase the apoptotic rate of these cells, we evaluated if a combined therapy, SHK plus TMZ, had a synergistic effect. The cells were incubated with different concentrations of TMZ (50µM, 100µM, 150µM, 200µM and 250µM) for 48 hours and cell viability was evaluated using the MTT method as previously described (Figure 21). As we can observed in Figure 21, the IC50 of TMZ was verified at 160µM.

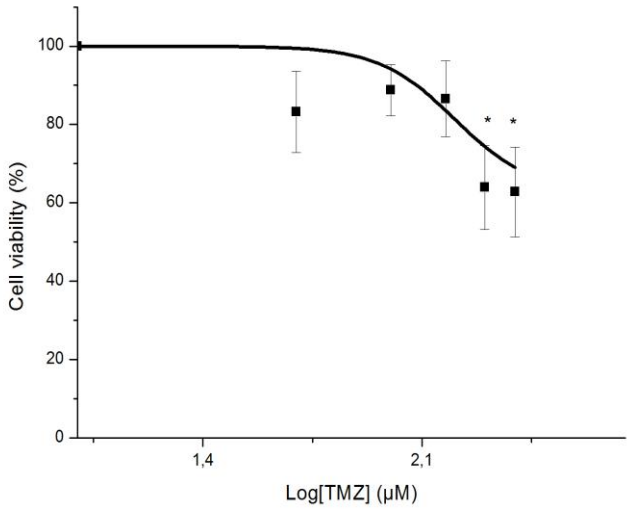


Figure 21 Effect of temozolomide in U-118 glioma cells after 48h by the MTT assay. U-118 survival rate after the treatment with temozolomide (0, 50, 100, 150, 200, 250 µM) for 48 hours was determinate by the MTT assay. All values are mean ±SD of three independent each of them performed in triplicate. *p<0.01

To verify if SHK could enhanced the TMZ effect in U-118 glioma cells, we incubated the U-118 cells during 48 hours with combination of different concentrations of SHK (0, 0.2, 0.5, 0.75, 1, 1.5 µM) and TMZ (0, 150µM, 200µM and 250µM). Our results showed, the IC50 of the combination of TMZ (150,200 and 250µM) with SHK (0.2 at 1.5µM) compared with the IC50 of SHK in monotherapy, determinated in section 3.2.1 (Figure 13), is decreased from 1.87µM to 0.176µM ($p<0.05$), respectively (Figure 22A). Also, if we compared the effect of TMZ in monotherapy (Figure 21) with TMZ in conjugation with SHK, we saw a decrease of IC50 from 160.18 µM to 51.014µM ($p<0.05$) of TMZ (Figure 22B). These results suggest, there is a synergistic effect between both drugs.

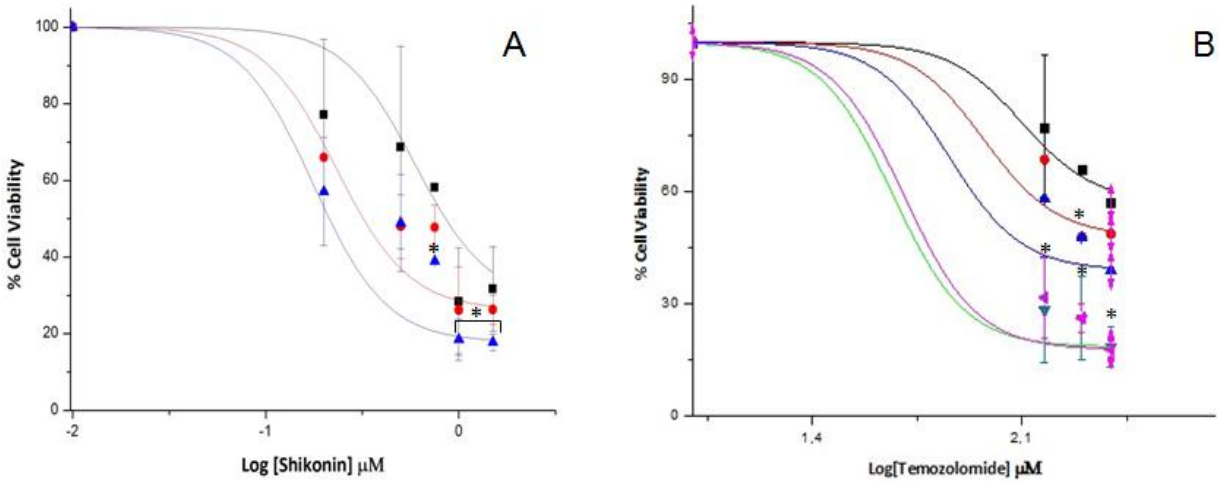


Figure 22 Synergistic effect of shikonin and temozolomide combination in U-118 glioma cells. The U-118 glioma cells were treated with different concentrations of Shikonin (0, 0.2µM, 0.5 µM, 0.75 µM, 1 µM and 1.5 µM) combinated with different concentrations of Temozolomide (0, 150µM, 200µM and 250µM) during 48 hours. The U-118 survival rate was evaluated by the MTT assay. A) The black line correspond to the effect of Shikonin combinated with 150 µM of Temozolomide. The red Line is the effect of combination between Shikonin with 200 µM of Temozolomide and the blue line correspond to the effect of combination between Shikonin with 250µM of Temozolomide. B) The black line correspond the effect of Shikonin at 0.2µM with different concentrations of Temozolomide. The red line is the effect of combination between Shikonin at 0.5 µM with Temozolomide. The blue line is the effect of temozolomide with Shikonin 0.75 µM. The green line represented the effect of the combination between Shikonin 1 µM and temozolomide. The purple line is the effect of temozolomide combinated with Shikonin 1.5 µM. All values are mean ± SD of three independent experiment each of them performed in triplicate.*$p<0.05$

Taking into account the results obtained by MTT assay (Figures 21 and 22), we wanted to understand if the synergistic effect observed could also affect the integrins expression levels compared with the effect of Shikonin in monotherapy (Figure 13). For that,

we incubated the U118 glioma cells with three concentrations of shikonin bellow the IC_{50} concentration (0, 0.75, 1 and 1.5µM) in combination with three concentrations of temozolomide bellow the IC_{50} concentration (0, 150, 200 and 250µM), during 48 hours. Western blot analysis showed a significant a decrease in the integrin subunit expression, dose-induced (Figure 23).

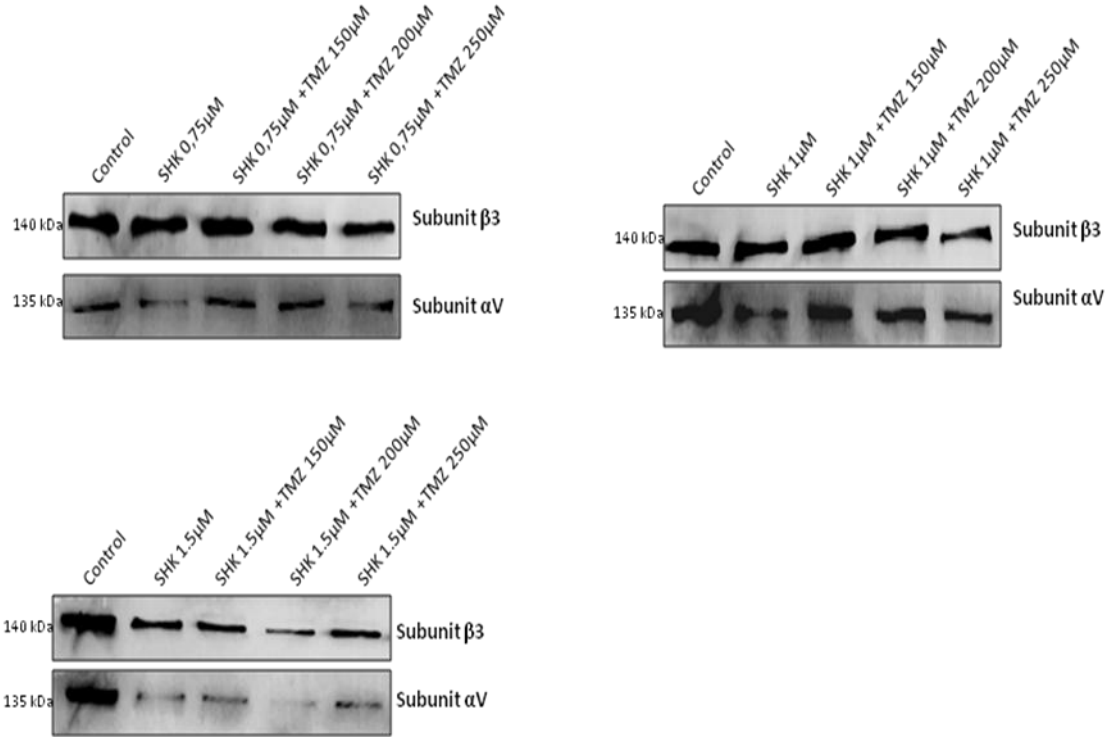


Figure 23 Combination of Shikonin with temozolomide has a synergistic effect in integrin expression in U-118 glioma cells. The U-118 glioma cells were treated with concentrations of SHK with TMZ bellow of IC_{50} concentrations and the expression was evaluate by western blot analysis.

The effect of combination with SHK (0, 0.75, 1, 1.5 µM) and TMZ (0,150, 200 and 250µM) in the proliferation rate of U-118 cells was evaluated by Flow cytometry using the BrdU method. This method consists in measuring the amount of the incorporated BrdU in DNA during S phase of the cell cycle. The incorporated BrdU was detected by a monoclonal antibody against BrdU, conjugated with a fluorochrome. SHK was added at bellow concentrations of IC_{50} (1.87µM) in combination with TMZ (150, 200 and 250µM) over a period of 48 hours and BrdU was added to the cells. The results showed that 48 hours after the addition of 1.5 µM of SHK with 150 µM TMZ concentrations the incorporation of BrdU

decrease around 65 % ($p < 0.05$) when compared with the BrdU incorporation in control cells, considered as 100% proliferation rate (Figure 24). As shown in Figure 24, we observed a dose-dependent reduction of glioma cell counts. These results suggest that the combination between SHK and TMZ decrease U-118 glioma cells proliferation, demonstrating a synergistic effect between the SHK and TMZ compared with the results obtained in monotherapy. In this condition cells treated with SHK alone showed a reduction in proliferation around 20% ($p<0.001$) (Figure 24).

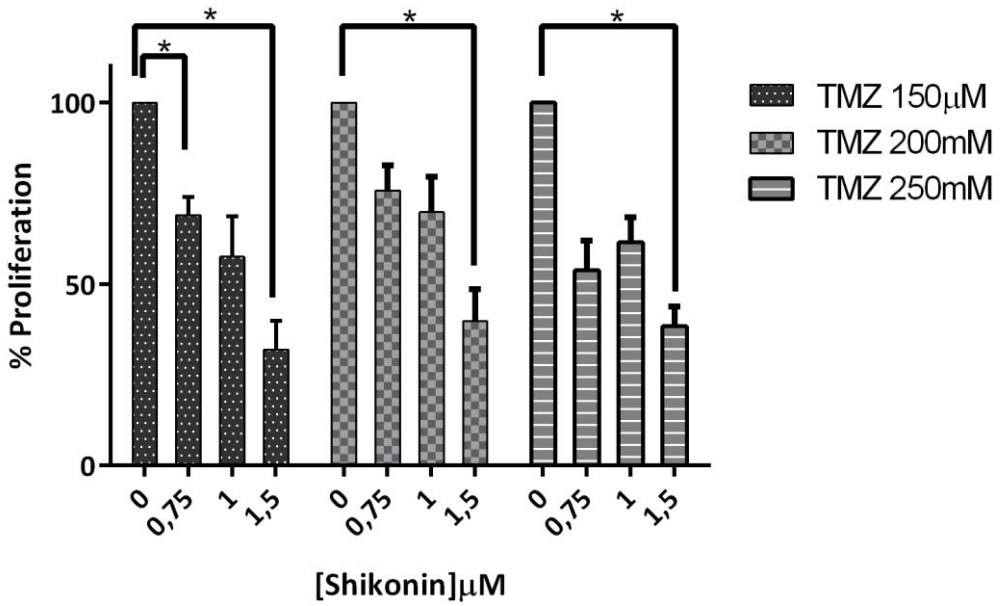


Figure 24 Synergetic effect of shikonin on U-118 MG cells proliferation. U-118 cells were incubated for 48 hours in the presence of different concentrations of SHK (0, 0.75, 1 and 1.5μM) in combination with TMZ (0, 150, 200 and 250μM). The results showed that the combination between SHK and TMZ can decrease glioma cells proliferation. The cells treated with SHK at 1.5 μM and TMZ 150μM compared with non treated cells had a decrease of 65% in proliferation ($p<0.05$). The same was observed in cells treated with 1.5μM SHK and TMZ 250μM ($p<0.05$). The combination between TMZ and SHK decrease U-118 cells proliferation in a dose-response manner. All values represented the mean ± SD of three independent experiments each of them performed in duplicate. *$p<0.05$

CHAPTER 4

DISCUSSION

Discussion

Many studies have been performed to understand the glioblastoma multiforme biology and, therefore, novel anticancer drugs have been developed, exploiting molecular differences between tumor cells and cells of healthy tissues (Nicholas *et al.,* 2011). The identification of the molecular and genetic mechanisms underlying treatment resistance constitutes the main objective for the development effective therapies against GBMs. An important research area includes the understanding of the integrin contribution to tumorigenesis and also its role in the modulation of the chemotherapeutic response of cancer cells. The integrin signaling regulates diverse function in tumor cell, including migration, invasion, proliferation and survival (Desgrosellier & Cheresh, 2010). There is evidence that inhibiting integrins might enhance the efficiency of therapy, however little is known if the integrins expression could contribute for the GBM resistance. Some studies showed that down-regulation of integrins, such as β1, α6 and β4, contributes for tumorigenicity in other cancer cells (Kim *et al*., 2011). Moreover, the glioma cells expressed high levels of integrins αVβ3 and αVβ5 (Tabatai *et al*., 2010). Taking into account the role of integrins in promoting glioma growth, invasion and angiogenesis (Wild-bode *et al*., 2001), integrin inhibitors could be used as therapeutic tools. Although integrins could contribute for the GBM malignancy, is not yet explored the possibility of the synergistic effect between the integrin expression blocker with therapeutic treatment already established such as TMZ, for the reduction of the aggressive behavior and the chemoresistance characteristic of these tumors.

In this study we proposed to understand the contribution of integrins to proliferation, survival and motility of U-118 glioma cells. For this propose, we evaluated the basal expression of integrin subunits in GBM cell line U-118 by western blot. Then, we studied the contribution of integrins to proliferation, apoptotic rate and motility of U-118 glioma cells. In addition, we determined if the combination of shikonin with temozolomide, the *gold standard* of glioma treatment, may contribute to decrease glioma cells proliferation and motility and to increase the apoptotic rate.

Our findings demonstrate that the U-118 glioma cells expressed mainly the integrins subunits α5, αV and β3 (Figure 12). These results are in accordance with those demonstrated by Mattern *et al*. (2005) and Gladson *et al*., (1991). These authors also

showed that the subunits organized into heterodimers and also that one of the most expressed heterodimers was αVβ3 in GBM cells. Taking in consideration our results and also the results from Mattern *et al*., (2005), we used shikonin, a drug that blocks αV and β3 subunits expression, (Hisa *et al*., 1998) and evaluated its effect on integrins expression. Glioma cells were incubated with different concentrations of shikonin and the viability, proliferation, survival and motility were analyzed. The viability that U-118 cells have to shikonin was evaluated by MTT assay. The results obtained from dose-response curves to shikonin showed that U-118 glioma cells were sensitive to Shikonin treatment in a dose-dependent manner, as shown in Figure 13. The viability study revealed that an IC50 of U-118 glioma cell in response to the incubation with shikonin was 0.27±1.87μM. Previous studies performed by Chang *et al*., (2010), whose showed that shikonin exhibited dose-dependent inhibitory effects on the viability of ostesarcoma cell. However the IC50 determined by Chang *et al*., (2010) was 2.01μM which indicates that glioma cells are more sensible to shikonin than osteosarcoma cells. To determine the effect of shikonin in the expression of integrins U-118 cells were incubated with shikonin and the level of integrins expression were determined and compared with the integrin basal expression (Figure 12 and 14). Our results showed that in the presence of shikonin below IC50 concentrations (0.27±1.87μM), there was a reduction in the expression of both subunits in a dose-dependent manner. However, the decrease was only statistically significant for the β3 expression subunit ($p<0.01$).

To determine the contribution of integrin expression to proliferation of glioma cells we evaluated the incorporation of BrdU in S phase of cell cycle. Our results showed that in the presence of 0.75 and 1μM shikonin there was a significant reduction of U-118 cells proliferation that induced a decrease as described in Figure 15 ($p<0.001$). The analysis of cell cycle showed that there was a cell cycle arrest in G0/G1 phase which was accompanied by a significant reduction of cells in G2/M phase, ($p<0.05$). In fact, these results are in agreement with those previously published by Chang and colleagues, which in osteosarcoma cells treated with shikonin, also reported cell cycle arrest in the G0/G1 (Chang *et al.,* 2010). Moreover, our results showed that the inhibition of integrin expression by shikonin may contribute to reduce the aggressive behavior of glioma cells.

In order to evaluate if the cell cycle arrest was associated with an increase in the apoptotic rate we determined the percentage of cells in necrosis stage, measuring the

amount of PI bound to each cell's DNA, and the percentage of cells in apoptosis by flow cytometry using the Annexin V and PI double staining. Our results showed that shikonin can induced early and late apoptosis on glioma cells (Figure 16), which is in agreement with Chen *et al.,* (2002) that also showed an increase in apoptotic rate in human glioma cells. In future studies, it will be necessary to understand which intrinsic and/or extrinsic apoptosis pathways may be involved in U-118 cells under the shikonin effect. Hsu *et al*., (2004) showed that shikonin led to cell apoptosis through up-regulation of p27, p53, Bax and down-regulation of Bcl-2 and Bcl-xL in human colorectal carcinoma but in glioma cells the mechanism is not completely understood.

To confirm the results obtained with annexin and PI we stained the nuclei with Hoeschst 33342. The analysis of our results showed that the nuclei of U-118 cells treated with shikonin showed an increased chromatin condensation accompanied by pyknosis, which are features associated with of apoptosis. Taking these results altogether we may suggest that the reduction of integrin expression is associated with an increase in apoptosis which reinforce the contribution of integrin signaling pathways to the malignant potential of U-118 glioma cells.

Glioma cells features are also characterized by an increase ability to migrate into surrounding tissues, for this reason and also because integrins interact with cell-ECM we decided to contribute the motility of glioma cells. For that, we observed the migration ability of the glioma cells using the scratch assay in the presence of shikonin. Our results showed that when U-118 cells were incubated with shikonin there was a reduction in motility. To determine if this reduction was accompanied by disorganization in F-actin filaments U-118 cells were incubated with different concentrations of shikonin and F-actin was stained with phaloidin. The results showed that in control cells F-actin filaments were organized. However, in cells treated with shikonin we observed a disorganization of F-actin. These results are in accordance with the results obtained by scratch assay, showing that shikonin reduces the migration capabilities of U-118 glioma cells. These results are also in agreement with those reporting that integrins mediate cell-ECM interactions necessary for cell motility and survival (Stromblad & Cheresh, 1996).

Altogether, our results suggest that integrin signaling pathway contribute to the aggressive behavior of the U-118 glioma cells since the inhibition of the alpha V and beta 3 subunits reduce the proliferation and the motility ability and increased the apoptotic rate.

Therefore, it seems possible that integrins could be considered a potential therapeutic target in glioma treatment.

The treatment of GBM is based on surgery and on the use of temozolomide, which is considered the *gold standard* for glioma treatment. One of the main problems in GBM treatment is the development of chemoresistance and the toxicity associated to the use of temozolomide which is an alkylating drug. Therefore, we hypothesized that if the inhibition of integrins expression reduces the aggressiveness of U-118 cells then it is possible that if we incubate U-118 cells with shikonin plus temozolomide, the temozolomide dose needed to reduce proliferation could be reduced. To test this hypothesis U-118 cells were treated with different concentrations of shikonin in combination with different concentrations of temozolomide bellow to the IC_{50} concentration, as we shown in Figure 22. Our results showed that there was a synergistic effect between the Shikonin and temozolomide. The IC50 of the combination of temozolomide (150, 200 and 250µM) with shikonin (0.2 at 1.5µM) compared with the IC50 of shikonin in monotherapy, determinated in section 3.2.1 (Figure 13), is decreased from 1.87µM to 0.176µM ($p<0.05$), respectively (Figure 22A). Also, if we compared the effect of temozolomide in monotherapy (Figure 21) with temozolomide in conjugation with shikonin, we saw a decrease of IC50 from 160.18 µM to 51.014µM ($p<0.05$) (Figure 22B).

In order to determine if temozolomide interfere with integrin expression we evaluated by western blot the expression of integrins in U-118 cells treated with shikonin and temozolomide. For that, the cells were treated with the combination of shikonin with temozolomide in the concentrations bellow the IC_{50} (± 160.18 µM). The results showed a decrease in the integrin expression, dose-induced (Figure 23), compared with the results obtained with shikonin in monotherapy (Figure 14), however the decrease is not statistically significant.

Taking in consideration these results we evaluated the effect of the combination shikonin plus temozolomide to the proliferation ability of U-118 cells. Our results showed that in the presence of 0.75µM shikonin plus 150µM temozolomide (±69% cell proliferation) there was a significant reduction of 20% in proliferation ($p< 0.05$) as compared to that obtained in the presence of 0.75µM shikonin (± 90%) alone. In addition when cells were incubated with 1.5µM shikonin plus 150µM temozolomide we observed a significant decrease to half in cell proliferation as compared with the results acquired in the presence of

1.5µM shikonin alone (from 60% in monotherapy to 30% shikonin and temozolomide combination) ($p<0.05$).

In summary, our results indicate that shikonin is a promising chemotherapeutic agent for treating glioma. Also suggest that the combination between shikonin and temozolomide has a synergistic effect in U-118 glioma cells, which could reduce secondary toxicity and ameliorate patient quality of life.

CHAPTER 5

Conclusion and Future Perspectives

Conclusion and Future Perspectives

In this work, we demonstrated that the integrins, more specific the α3βV subunit, have a critical role in invasion, migration and proliferation in the glioma cells. The shikonin appears to have a stronger modulatory effect in glioblastoma cells. The blockage of integrins expression results in morphological and behavior alteration during the tumor progression. The shikonin partially blocked integrin subunits expression in glioma cells. Furthermore, the shikonin induces apoptosis and decrease glioma cells proliferation. The synergistic effect obtained between shikonin and temozolomide, suggests that the combination of integrin αVβ3 antagonists and other conventional therapeutic approaches may represent a future strategy to inhibit tumor growth and the invasion into the tissues that surround the tumor, allowing a better tumor resection and decreasing the chemoresistance to the compounds used in GBM therapy, thus increasing the survival rate and quality of life in patients.

Taking into account the results obtained, it is clear that more studies must be performed to better understand the modulation of the glioma cells behavior during the tumor progression by integrin inhibition. Therefore, it will be important to confirm the properties of integrin during the tumor progression and also the survival pathways involved in integrin inhibition. Additionally, it will be necessary to check the shikonin effect has in U-118 cells is due to reduction of integrins expression and not the others mechanisms underlying, so we need to silencing the integrin expression using SiRNA for αV and β3 integrin subunits and compare with the results obtained to see what differences occur when compare with our results obtained in integrin expression when cells were treated with Shikonin. Moreover, to confirm if the U-118 glioma cells express the heterodimers αVβ3, we should make an immunoprecipitation. However, it will be very complicated to detect the heterodimers because they have similar molecular weight (130kDa and 135kDa for αV and β3, respectively).

Furthermore, its necessary to identify the pathways involved in the effect of Shikonin in U-118 cells motility that could explain the increase in apoptosis and the decreased in cell proliferation and migration observed in U-118 glioma cells. Some studies showed that the anti-tumor effects of Shikonin in Oral squamous cells were at least partly through the inactivation of the NF-κB pathway and subsequent activation of the protease caspases family (Min *et al*., 2008). The effect of Shikonin observed in our cell line could be mediated through

the inactivation of the NF-kB pathway and, consequently, by caspases activation or can be the result of ROS production (Gong & Li, 2011) and activated ERK signaling pathways, as shown by Chang *et al*., (2010). Moreover, we intend to evaluate the survival pathways involved in migration, invasion and proliferation processes during the inhibition of integrins which might help to develop potential new targeted therapies for glioma treatment such, FAK signaling including RAS, RAC, PI3K and PKC (Khwaja *et al*., 1997; Besson *et al*., 2002; Aoudjit & Vuori, 2012).

CHAPTER 6

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