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Establishing the validity of glycoursodeoxycholic acid as a coadjuvant of temozolomide therapy in gliomas

Dissertação apresentada para a obtenção do Grau de Mestre em Investigação Biomédica, pela Universidade Coimbra, Faculdade de Medicina

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Abbreviations

ABC-transporter	ATP-binding cassette transporter
AIC	Aminoimidazole-4-carboxamide
BTSC	Brain tumor stem cell
CNS	Central nervous system
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
DMEM	Dulbecco's modified eagle's medium
FBS	Fetal bovine serum
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
GSH	Glutathione
GUDCA	Glycoursodeoxicholic acid
HIF-1	Hypoxia inducible factor 1
IDH	Isocitrate dehydrogenase
LC3	Light chain 3
MAP2	Microtuble-associated protein 2
MGMT	Methylguanine methyltransferase
MMP	Metalloproteinase
MRP1	Multidrug resistance-associated protein 1
MTIC	5-(3-methyltriazen-1-yl)imidazole-4-carboxamid
NEP	Neuroepithelial progenitor

Abbreviations

NS	Neurosphere
NSC	Neural stem cell
PBS	Phosphate-buffer saline
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
RG	Radial glia
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Radiation therapy
SDS-PAGE	Sodium dodecyl sulfate-polyacrilamide gel
SDF-1	Stroma-cell-derived factor 1
SGZ	Subgranular zone
Sox2	Sex determining region Y-box2
SVZ	Subventricular zone
TBS	Tris-buffered saline
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone
WHO	World health organization

Abstract

Brain tumors are the second most common neoplasms in children and their incidence is also relatively high in the adult population, with gliomas accounting for the majority of cases. So far, the treatment protocols available for gliomas did not improve the prognosis, mainly due to a phenomenon known as multidrug resistance. Thus, research has now been aimed to identify the mechanisms leading to gliomagenesis and it was recently suggested that neural stem/progenitor cell or early-differentiated cell type lineages might be in the origin of glioma. Therefore, the main goals of the present work are: a) to identify which developmental stage, in the neural stem cell (NSC) differentiation process towards astrocytes, is most similar to the glioma phenotype and b) to find successful adjuvant molecules for temozolomide (TMZ), a chemotherapeutic agent.

The GL261 mouse glioma cell line was cultured until 7 days *in vitro* and some tumor-related factors were determined in glioma cells and compared with astrocytes differentiated from mouse neural stem/precursor cells (neurospheres, NS). Moreover, we also evaluated the potential beneficial effect of TMZ treatment in the presence of the bile acid glycoursodeoxycholic acid (GUDCA) or the multidrug resistance-associated protein 1 (Mrp1) inhibitor MK-571. Finally, we have assessed the effect of both GUDCA and MK-571 on the expression of CXCR4, a chemokine receptor.

The analysis of tumor-related factors showed that during GL261 maturation, there is a decrease on the expression of the vascular endothelial growth factor (VEGF) as well as on the activity of the matrix metalloproteinases MMP-9 and MMP-2, which is associated with an increase on S100B release. Also, the Mrp1 presents a peak of expression at 5 DIV. Although not as evident as we were expecting, the NS proliferating stage seems to be the phenotype most similar to glioma cells, suggesting that the origin of glioma might be somehow associated with NSC malignant transformation. Moreover, TMZ therapy appears to be improved by the synergistic effect of GUDCA or Mrp1 inhibition, since it was observed a further reduction on cell viability and cell cycle arrest at the G2/M phase. Conversely, GUDCA or MK-571 seem to improve the migratory ability of GL261, by the induction of increase of CXCR4 levels.

Efforts to enlarge knowledge about the pathways implicated on a malignant alteration during neural development as well as to further understand how to improve the current therapy, would allow a more specific targeting and consequently, an increased survival of glioma patients.

Keywords: GL261 glioma cells; glycoursodeoxycholic acid; MK-571; neural stem cells proliferation and differentiation; temozolomide; tumor-related factors

Resumo

Os tumores cerebrais são as segundas neoplasias mais comuns em crianças e a sua incidência é também relativamente elevada na população adulta, sendo os gliomas os mais frequentes. Até agora, os tratamentos disponíveis para gliomas não melhoraram o prognóstico, principalmente devido a um fenómeno conhecido como resistência a múltiplas drogas. Assim, atualmente a investigação tem como objetivo identificar os mecanismos que levam à gliomagénese e foi recentemente sugerido que células estaminais/progenitoras neurais ou no início da diferenciação podem estar na origem dos gliomas. Desta forma, os principais objetivos do presente trabalho são: a) identificar qual o estadio de desenvolvimento, no processo de diferenciação de células estaminais neurais para astrócitos, é mais parecido com o fenótipo de glioma e b) encontrar moléculas adjuvantes eficazes na terapia com temozolomida (TMZ), um agente quimioterapêutico.

As células de glioma de ratinho GL261 foram mantidas em cultura durante 7 dias *in vitro* e alguns fatores relacionados tumores foram determinados em células de glioma e comparadas com os astrócitos diferenciados a partir de células estaminais/progenitoras de ratinho (neuroesferas, NS). Além disso, foram igualmente avaliados os potenciais efeitos benéficos do tratamento com TMZ na presença do ácido glicoursodesoxicólico (AGUDC) ou de um inibidor da proteína associada à resistência a multidrogas (Mrp1), o MK-571. Finalmente, foi avaliado o efeito quer do GUDCA, quer do MK-571 na expressão de CXCR4, um recetor de quimocinas.

A análise dos factores relacionados com tumores mostrou que, durante a maturação das células GL261, há uma diminuição na expressão do factor de crescimento endotelial vascular (VEGF), bem como na actividade das metaloproteinases MMP-9 e MMP-2, associado a um aumento da libertação de S100B. Além disso, a Mrp1 apresenta um pico de expressão ao 5 DIV. Apesar de não ser tão evidente como esperávamos, o estadio de NS parece ser o fenótipo mais semelhante com as células de glioma, o que sugere que a origem dos gliomas pode estar de alguma forma associada à transformação maligna das CEN. Além disso, a terapia com TMZ parece ser mais eficaz pelo efeito sinergético do GUDCA ou inibição da Mrp1, uma vez que se observou uma redução na viabilidade celular e paragem do ciclo celular na fase G2/M. Por outro lado, o GUDCA ou MK-571 parecem melhorar a capacidade de migração das GL261, através da indução do aumento dos níveis de CXCR4.

Um melhor conhecimento sobre as vias implicadas em alterações malignas durante o desenvolvimento neural, bem como sobre novas formas de melhorar a actual terapia, permitirá o uso de esquemas terapêuticos mais direccionados e

específicos e, consequentemente, um aumento da sobrevivência dos pacientes que apresentam gliomas.

Palavras chave: células de glioma GL261; ácido glicoursodesoxicólico; MK-571; proliferação e diferenciação de células estaminais neurais; temozolomida; factores relacionados com tumores

CHAPTER I - INTRODUCTION

1. Brain tumors

The term “brain tumor” refers to a group of neoplasms, with high incidence both in children as well as in the adult population, and on these, they mainly occur in the elderly (Sutter et al., 2007). Each neoplasm has its own biology, diagnosis and treatment. However, the clinical presentation, diagnosis and initial treatment are similar for most tumors.

Nowadays, tumors of the central nervous system (CNS) are mostly classified by the World Health Organization (WHO) guidelines, which facilitate the communication throughout the world. A brain tumor can be primary, if the tumor starts in the brain and secondary (or metastatic), if it results from somewhere else in the body (ABTA, 2010).

1.1. Classification of gliomas

Gliomas, the most common form of primary brain tumors are characterized by a great heterogeneity both histologically and clinically, as well as by diverse grades of malignancy. The most recent classification of gliomas was described by WHO in 2007 based mainly on three parameters: cell type, malignancy grade and tumor location (Louis et al., 2007).

a) Classification Based on Cell Type

This type of classification is based on the histological characteristics of the cells, according to the phenotypic and morphologic similarities of the tumor cells with those of different types of glial cells, such as astrocytes, oligodendrocytes and ependymal cells. Thus, gliomas can be classified as astrocytomas, oligodendrogliomas, ependymomas and also oligoastrocytomas (or mixed gliomas). Among all these types of tumors, astrocytomas are the most common.

b) Classification Based on Malignancy Grade

Grading of tumors facilitate the treatment and the prediction of their outcome. The grade indicates its degree of malignancy and it is assigned based on the tumor’s microscopic examination using criteria: like similarity to normal cells (atypia), rate of growth (mitotic index), indication of uncontrolled growth, dead tumor cells in the center of the tumor (necrosis), potential for invasion and/or spread (infiltration) based on whether or not it has a definitive margin (diffuse or focal), and blood supply (vascularization) (Fig.I.1). When gliomas contain several grades of cells, the grade is

determined by the highest or most malignant grade of cells, even if most of the tumor is a lower grade kind.

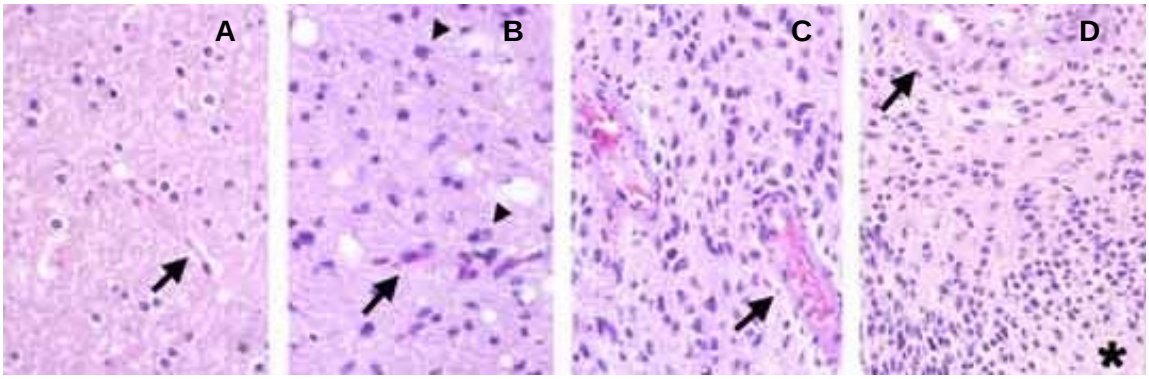


Fig. I.2. – Histophatologic progression of infiltrating astrocytoma to glioblastoma multiforme (GBM) according to the WHO classification. The normal brain white matter (A)**,** presents blood vessels (arrows) and cell density similar with an infiltrating astrocytoma grade II (B). Arrowhead shows that tumor cells of this grade II tumor can be found near the CNS parenchyma. Anaplastic astrocytoma (AA; grade III; C) is characterized by a higher number of cell and blood vessels density, which are often dilated or with thickness walls (arrows). AA cells also present an atypical morphology and some mitotic cells can be found. GBM (D) shows necrotic zones with pseudopalisading tumor cells (asterisk within necrotic center), which are usually surrounded by microvascular hyperplasia and vascular glomerules proliferation (arrow). From Brat et al. (2003).

Grade I gliomas are benign and are typically related with long-term survival. The tumors exhibit a slow grow and a limited cell proliferation potential and have an almost normal appearance when viewed through a microscope. Surgery alone might be an effective treatment for these tumors. Grade II tumors have a relatively slow growing rate, a low-level of proliferative activity and a slightly abnormal microscopic appearance. They are in general infiltrative and some can recur as a higher-grade glioma. Grade III tumors are, by definition, malignant. The cells of a grade III tumor are actively reproducing abnormal cells, which grow into nearby normal brain tissue. These tumors tend to recur, often as a higher grade. In most cases, it is necessary to receive adjuvant radiation and/or chemotherapy. The tumors of grade IV are the most malignant. In addition to the strange appearance, they have a high mitotic activity and can easily spread into the surrounding normal brain tissue. These tumors have also an enormous ability to form new blood vessels so they can maintain their rapid growth. Necrosis zones are usually associated with a rapid evolution and fatal outcome. The glioblastoma multiforme (GBM), sometimes referred only as

glioblastoma, is the most common between grade IV tumor (Louis et al., 2007; Ohgaki, 2009).

c) Classification Based on Tumor Location

Gliomas can also be classified regarding their location, whether above or below the tentorium, a membrane that separates the cerebrum (above) from the cerebellum (bellow). Hence, they are defined as supratentorial, which develop above the tentorium, and as infratentorial, which develop below the tentorium. The supratentorial and the infratentorial gliomas correspond to 70% of the tumors in adults and children (Louis et al., 2007).

1.2. Epidemiology of gliomas

The peak of gliomas onset is around 50-55 years, which makes them a strongly age-related pathology. The incidence of brain tumors tends to be highest in developed and industrialized countries. In Western Europe, North America and Australia there are about to 6-11 new cases of primary intracranial tumors per 100 man individuals and to 4-11 new cases in the women population (Ohgaki and Kleihues, 2005; Ohgaki, 2009). Ethnic differences in the vulnerability to develop of brain tumors cannot be excluded. Caucasians are more susceptible than African or Asian people. Some reports indicate that the incidence rate of gliomas is approximately twice in whites when compared to blacks. In addition, in Japan, gliomas are about half as frequent as in the United Sates of America (Ohgaki and Kleihues, 2005; Ohgaki, 2009).

With the exception of pilocytic astrocytoma (WHO grade I), survival of glioma patients is still poor and one of the factors for this is the older age at diagnosis. Mortality rates from CNS tumors are similar to the incidence rate, i.e., around to 4-7 cases per 100,000 persons per year in men and to 3-5 cases in women, throughout the geographical areas referred above (Ohgaki and Kleihues, 2005; Ohgaki, 2009).

1.3. Signaling pathways regulating gliomagenesis

It is recognized that morphological changes during the malignant transformation, reflect the sequential acquisition of genetic alterations. Although primary and secondary tumors differ on the genetic level in many ways, there are some common genetic abnormalities, which are considered as hallmarks of gliomas. So far, a variety of studies have identified DNA copy number alterations and mutations as

recurrent events on gliomagenesis, suggesting the involvement of tumor suppressor genes and oncogenes (Fig. I.2) in tumor initiation and progression (Furnari et al., 2007; Ohgaki, 2009).

The first studies identified the existence of mutations in the epidermal growth factor receptor (EGFR) gene, encoding the receptor for the epidermal growth factor (EGF) (Van Meir et al., 2010). Mutation in EGFR glioma enhances tumorigenic behavior by reducing apoptosis and increasing proliferation (Furnari et al., 2007). Later, further analysis identified the p53 tumor suppressor gene, as important for, which is involved in the regulation of cell cycle progression and apoptosis in response to a wide variety of stress signals, including DNA damage. Upon exposure to genotoxic agents, p53 is stabilized, accumulates in the nucleus, binds and transcriptionally regulates the promoters of potential effector genes. Since p53 function is critical for normal cell growth and development, its activity is tightly regulated by phosphorylation, which is the first step to induce stabilization of p53 (Carmo et al., 2011). This pathway is nearly invariably altered in sporadic gliomas and frequent in the beginning of secondary glioblastomas (Furnari et al., 2007; Carmo et al., 2011). The next genes discovered were the p16 cell cycle inhibitor, and the phosphatase and tensin homolog (PTEN), acting both as tumor suppressors. The p16 is responsible for the slow down of the cell cycle progression, whereas PTEN is a negative regulator of the phosphoinositide 3–kinase (PI3K) pathway, a major signaling pathway that stimulates cellular proliferation in response to growth factor stimulation (Westphal and Lamszus, 2011). Inactivation of PTEN is associated with increased angiogenesis, a parallel process in the progression of high grade gliomas (Furnari et al., 2007). In fact, PTEN mediates a variety of biological functions like apoptosis, inflammation and immunity (Westphal and Lamszus, 2011).

Recently, some mutations were identified at the level of the genes encoding isocitrate dehydrogenase 1 (IDH1) (and to a lesser extent IDH2) and retinoblastoma (RB) in lower grade gliomas and in a subset of glioblastomas. IDH1 mutation is associated with longer survival of patients with secondary glioblastoma and one consequence of its raised expression is an altered pattern of DNA methylation in gene promoter regions, leading to epigenetic silencing (Westphal and Lamszus, 2011). RB blocks proliferation by binding and sequestering the E2F family of transcription factors, which prevents the activation of essential genes for progression through the cell cycle (Furnari et al., 2007). Moreover, mutations in the ERBB2 gene have also been found as recurrent event in primary glioblastomas (Westphal and Lamszus, 2011).

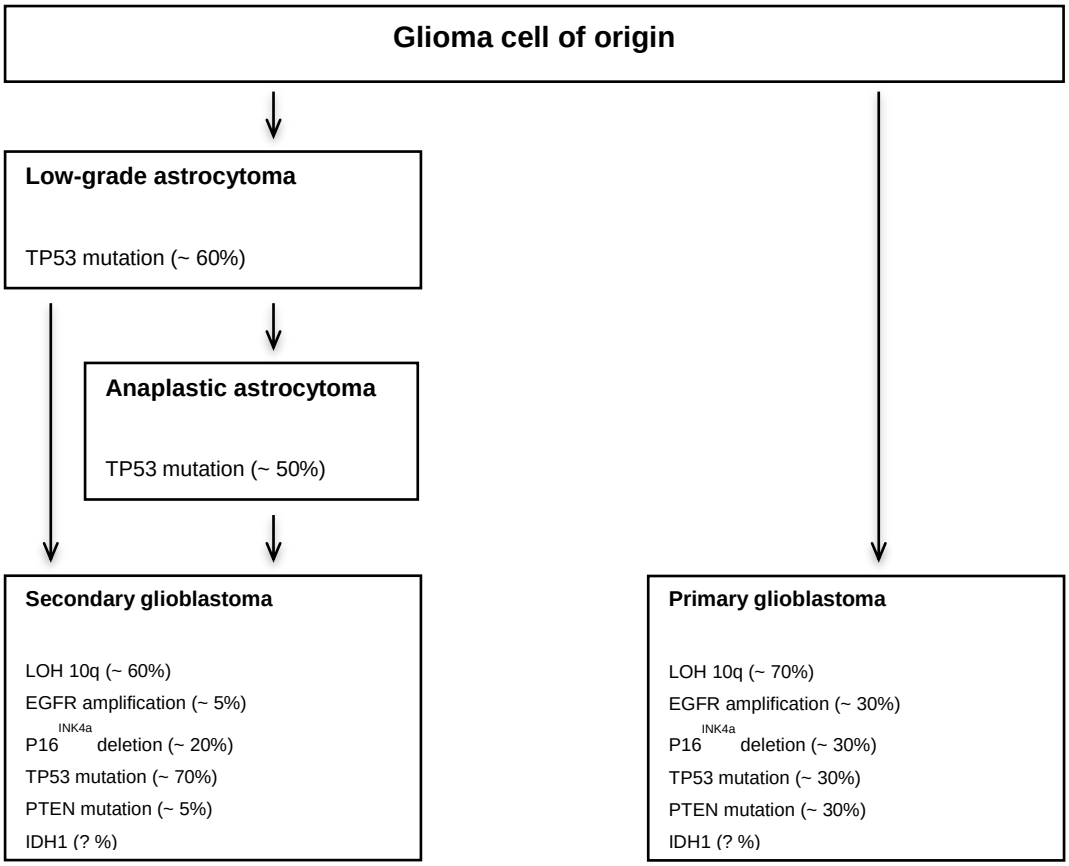


Fig. I.2. - Pathways that mediate the development of glioblastoma. Distinct molecular alterations correlate with the clinical development of gliomas. EGFR, endothelial growth factor receptor; IDH1, Isocitrate dehydrogenase 1; LOH, Loss of heterozygosity 10q; P16 ^{INK4a}, Cyclin-dependent kinase inhibitor 2A; PTEN, Phosphatase and tensin homolog; TP53, tumor protein 53. Adapted from Sulman (2009).

Although histologically indistinguishable, GBM can occur in different age groups and present distinct genetics alterations affecting similar pathways. The understanding and identification of these alterations will assist a more correct diagnosis.

1.4. Tumorigenic properties

The tumorigenic properties that are the most responsible for the initiation and maintenance of tumor include tumor cell invasion, angiogenesis, resistance to therapy and autophagy, which will be further discussed.

1.4.1. Tumor cell invasion

The infiltrative nature of tumors makes curative surgical resection nearly impossible and contributes to the poor prognosis and short median survival of patients (Choe et al., 2002).

Invasion of tumor cells into adjacent brain structures occurs through the activation of matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases that mediate the degradation of protein components of the extracellular matrix (ECM) and of basement membranes (Choe et al., 2002; Hagemann et al., 2012). Degradation of the ECM by MMPs not only enhances tumor invasion, but also affects tumor cell behavior and leads to cancer progression. MMPs can be classified as collagenases (MMP1, MMP8 and MMP13), stromelysins (MMP3, MMP10, MMP11, MMP7 and MMP26), gelatinases (MMP2 and MMP9) and as membrane-type (MMP14, MMP15, MMP16, MMP17, MMP24 and MMP25) (Rao, 2003).

MMPs enhance tumor-cell invasion and migration by degrading ECM proteins, activating signal transduction cascades that promote motility and solubilizing extracellular matrix-bound growth factors, in particular by cleaving laminin-5 (Choe et al., 2002; Rao, 2003; Hagemann et al., 2012). In fact, it was observed that interference with MMP-9 and one of its upstream regulators by RNA interference lead to a reduction in tumor growth and invasion in a mouse model. MMP-9, MMP-2 and its activator MMP-14 are involved in migration and invasion of human GBM cells and the first clinical trials using the MMP inhibitor, marimastat, in combination with chemotherapy have recently been performed in GBM patients (Hagemann et al., 2012).

In addition, MMPs also play a central role in a number of physiological processes, such as cell growth and development (by cleaving and activating some growth factors, as the transforming growth factor-β), differentiation, angiogenesis (by increasing the bioavailability of pro-angiogenic growth factors) and apoptosis (Lu et al., 2010; Ponnala et al., 2011). Very recently, it was published a review that summarizes the currently available data on the expression of MMPs in human glioblastomas (Hagemann et al., 2012).

Besides MMPs, S100B is also related with tumor cell invasion. This protein is a member of a multigenic family of Ca^{2+}-binding protein of the EF-hand type, and is located diffusely in the cytoplasm and associated with membranes and certain cytoskeleton elements (Brozzi et al., 2009; Zhang et al., 2011). S100B has been implicated in the regulation of both intracellular and extracellular activities, such as

regulation of the state of microtubules assembly and type III intermediate filaments, some enzyme activities, and cell proliferation. High levels of S100B are found in certain cancer cells, reason why it has been proposed that it contributes to tumorigenesis by inhibiting the function of p53 protein and by regulating cell proliferation and differentiation by stimulation of kinases activation (Brozzi et al., 2009; Zhang et al., 2011). Being a chemotactic molecule, S100B protein stimulates microglia migration via RAGE-dependent up-regulation of chemokine expression and release (Bianchi et al., 2011). Thus, we can hypothesize that this molecule may perform a pivotal function in tumor cell invasion and metastasis.

Other molecules also associated to tumor cells invasion are chemokines, which are a family of chemotatic cytokines involved in multiple biological functions, as leukocyte migration, hematopoiesis, mitosis, apoptosis, survival, angiogenesis and tumor cell growth (Carmo et al., 2010; Calatozzolo et al., 2011). The most important chemokine associated with tumorigenesis process and metastasis is the stroma-cell-derived factor 1 (SDF-1/CXCL12) and its receptor, the CXCR4, a G-protein, which expression is upregulated by hypoxia, via hypoxia-inducible factor 1 (HIF-1α) and vascular endothelial growth factor (VEGF) (Calatozzolo et al., 2011). The activation of CXCR4 by CXCL12 regulates numerous essential processes such as cardiac and neuronal development, stem cell motility, and as a pro-angiogenic factor (Calatozzolo et al., 2011). Regarding brain tumors, it has been shown that both CXCR4 and CXCL12 were overexpressed when compared with normal tissue, predominantly in necrosis areas and angiogenesis (Calatozzolo et al., 2011), what is correlated with the infiltrative extension of the tumor (Carmo et al., 2010). *In vivo* and *in vitro* studies demonstrated that CXCL12 promotes tumor growth and inhibits apoptosis through Erk1/2 and Akt pathways and also mediated glioma chemotaxis (Calatozzolo et al., 2011). On the other hand, CXCR4 expression in malignant gliomas has been associated with poor prognosis in patients and mouse models (Calatozzolo et al., 2011). Thus, the importance that this CXCL12/CXCR4 axis has in tumorigenesis makes it a great therapeutic target in glioma treatment.

1.4.2. Angiogenesis

Studies support that angiogenesis, the formation of new vessels from pre-existing ones, is required for tumor growth. Thus, during the last three decades, intensive research has been performed to characterize the angiogenesis process and many angiogenesis-related factors or genes have been identified (Jouanneau, 2008).

Angiogenesis is characterized by a series of steps including degradation of the basement membrane, endothelial cell proliferation, invasion of the surrounding stroma and structural reorganization into a novel functional vascular network through the recruitment of perivascular supporting cells (Jansen et al., 2004). The complexity of the process implies the involvement of multiple regulatory factors, such as growth factors, adhesion molecules and matrix-degrading enzymes. Malignant gliomas exhibit many vessel-related pathological features (Yamanaka and Saya, 2009). These features include marked endothelial proliferation, and tortuous disorganized vessels of higher permeability, larger diameters and thicker basement membranes than vessels found in normal tissues. Aberrant microvasculature typically appears as glomeruloid tufts, proliferations of microvessels consisting of multilayered mitotically active endothelial and perivascular cells (Jain et al., 2007).

The most important growth factor in angiogenesis is VEGF, which has highly specific mitogenic and chemotactic activity on endothelial cells (Kargiotis et al., 2006). Up-regulation of VEGF seems to be triggered by hypoxia through HIF-1 and mediated by two mechanisms (Jansen et al., 2004; Kargiotis et al., 2006). Through multiple regulatory mechanisms, HIF acts as a delicate sensor leading to a rapidly cell response to changes in environmental levels of oxygenation (Kaur et al., 2005). First, hypoxia induces the activation of VEGF gene transcription through an HIF-dependent mechanism, mediated by HIF-1 binding to the VEGF promoter, resulting in increased gene transcription. The second mechanism upregulates VEGF mRNA levels by regulating mRNA stability. Regulation is finely tuned to the availability of oxygen because HIF-1a, the oxygen-sensitive subunit of the HIF-1 complex, is stable in hypoxia (Jansen et al., 2004). VEGF is predominantly located in the pseudopalisading cells surrounding hypoxic/necrotic foci, which is likely due to hypoxic induction. In fact, besides abundant microvessels, regional necrosis is another common pathological feature in glioma tissues and emerging evidence has suggested that hypoxia is an important modulator in the process of glioma angiogenesis (Jensen, 2009). Thus, due to obvious and aggressive vascular proliferation and very poor prognosis, many antiangiogenic drugs were rushed for approval in clinical trials for glioma patients. Unfortunately, under experimental and clinical conditions, antiangiogenic therapy has led to increased invasion and higher recurrence rates (Thurston and Kitajewski, 2008).

1.4.3. Resistance to chemoradiotherapies

Although chemo and radiotherapy remain the adjuvant treatments of brain cancer, these treatments fail to cure the majority of patients mainly due to chemoresistance. Several mechanisms may contribute to the development of therapeutic resistance, including cell intrinsic factors, selection of resistant genetic subclones, and microenvironmental factors.

ATP-binding cassette (ABC)-transporters are transmembrane proteins that utilize ATP hydrolysis to transport substrates from the intracellular to the extracellular compartment (Atkinson et al., 2009), acting as drug efflux pumps and decreasing the intracellular levels of various cytotoxic agents (Benyahia et al., 2004; Lebedeva et al., 2011). The multidrug resistance-associated protein 1 (MRP1/ABCC1) is a member of a subfamily of the ABC-transporters superfamily. It was discovered by Cole *et al.* (Cole et al., 1992), that described it as responsible for multidrug resistance of tumors (Begley, 2004). In addition to its ability to confer resistance in tumor cells, multi-resistance protein 1 (MRP1) is ubiquitously expressed in normal tissues and is a primary active transporter of glutathione (GSH), although it also transports unmodified xenobiotics that often require GSH (Fig. I.3) (Leslie et al., 2001). In untreated gliomas, an overexpression of MRP1 has been reported in about 70% of cases, with a higher expression in high-grade gliomas, particularly glioblastoma (Benyahia et al., 2004; Lebedeva et al., 2011).

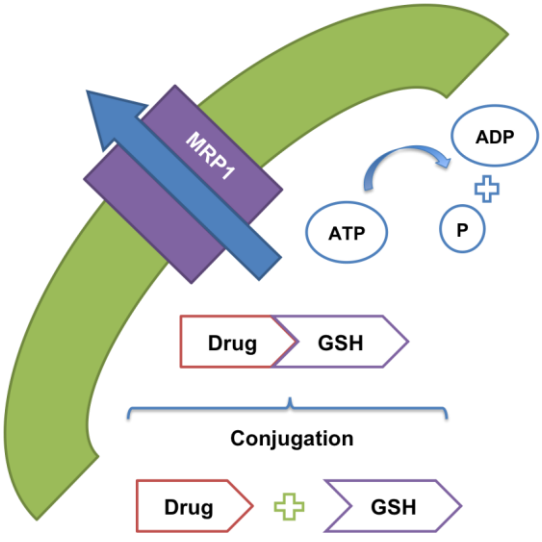


Fig. I.3. - Multidrug resistance mechanism of the multidrug resistance-associated protein 1 (Mrp1). Mrp1, a transmembrane protein, functions as an ATP-dependent efflux pump by carrying cytotoxic drugs out from brain cells mediated by the conjugation with glutathione (GSH), particularly in glioma cells. Adapted from Bredel et al. (2001).

Besides MRP1 phenotype, resistance to chemotherapy is often caused by elevated levels of enzymes involved in intracellular drug mechanism, including MGMT, as already described, contributing to resistance to alkylating agents.

1.4.4. Autophagy

Autophagy, also known as the programmed cell death type II, is a conserved process that degrades and recycles organelles and portions within cytosol. The intracellular molecules and organelles, such as endoplasmic reticulum, mitochondria, and peroxisomes, are sequestered into double-membrane structures called autophagosomes (autophagic vesicles) (Fan et al., 2010). The C-terminal fragment of microtubule-associated protein light chain 3 (LC3, which is essential for autophagy) is cleaved to a cytosolic form LC3-I, which is further converted to LC3-II, a 16-kDa protein that localizes into autophagosomal membranes. Autophagosomes then fuse with lysosomes, forming autophagolysosomes, which promote the degradation of intracellular contents by lysosomal enzymes (Fan et al., 2010; Lin et al., 2012). Autophagy thus enables the cell to eliminate and recycle proteins or organelles to sustain metabolism and can be recognized in part by formation of LC3-II punctae (Fan et al., 2010), since the amount of LC3-II correlates with the number of autophagosomes. Therefore, LC3-II is considered an autophagy marker (Lin et al., 2012).

This type of cell death is also highly adaptable and can be modified to digest specific cargoes to bring about selective effects in response to numerous forms of intracellular and extracellular stress. It is not a surprise, therefore, that autophagy has a fundamental role in cancer and that perturbations in autophagy can contribute to malignant disease. However, there are conflicting reports suggesting that autophagy can be both oncogenic and tumor suppressive, perhaps indicating that autophagy has different roles at different stages of tumor development. Recent data point out that this process may play a critical role in the benign to malignant transition that is also central to the initiation of metastasis (Macintosh et al., 2012).

1.5. Diagnosis and treatment

The classification of a tumor stage determines if it has spread beyond the site of its origin and this information often influences treatment recommendations and prognosis. The first steps of the diagnosis consist in making the medical history and a basic neurological exam, which analyzes diverse parameters, including memory.

Thereafter, if the result is suspicious, additional testing as scans, like Magnetic Resonance Imaging (MRI) and Computerized Tomography (CT) or Positron Emission Tomography (PET), x-rays or laboratory tests are performed. During and after treatment, it is recommended to repeat these tests in order to follow the evolution or stage of the disease (Omay and Vogelbaum, 2009).

Until recently, treatment decisions regarding malignant gliomas began only when diagnosis was established by standard histopathology only, what has been for the most cases inexact because of the diversity that exists within these tumors, even among those of the same grade and histologic type (Sulman et al., 2009). However, over the past 10 years, there has been an increasing use of molecular markers, such as methylation of the methylguanine methyltransferase (MGMT) promoter and mutations of IDH-1 (Sulman et al., 2009), in the assessment and management of adult malignant gliomas. Some molecular signatures are used diagnostically to help pathologists in the classification of tumors, whereas others are used to estimate prognosis for patients. Most important, those markers are used to predict response to certain therapies, thereby directing clinicians to a particular treatment while avoiding other potentially deleterious. It has also paved the way for the possibility of personalized medicine, in which a patient's tumor expression profile can be used to design a treatment specific to that individual's tumor with the greatest possibility of response (Sulman et al., 2009). Thus, large-scale genome-wide surveys have been used to identify new biomarkers that have been rapidly developed as diagnostic and prognostic tools (Jansen et al., 2010).

Prognosis and therapeutic approaches depend on the type of tumor, as well as on the location and its degree of malignancy. However, in most cases, therapy starts with surgical removal of the tumor follow by radio and chemotherapy. Since the mid-80s, various compounds for the treatment of gliomas have been studied, such as cisplatin and carmustine, but none of them have proved to be effective in increasing survival and/or improving patients outcomes (Parney and Chang, 2003). At the present, the chemotherapeutic drugs most used are nitrosourea, etoposide, cisplatin, vincristine and temozolomide (TMZ), being the first-line treatment with radiotherapy and concomitant chemotherapy with TMZ. These compounds were shown to be relatively effective used either as monotherapy or in combination with other agents like procarbazine, lomustine, resveratrol, irinotecan and bevacizumab, among others (Argyriou et al., 2009). In fact, Lin and colleagues described a synergistic effect of TMZ and resveratrol, which reduced tumor volumes by inhibiting autophagy of glioma cells after TMZ treatment, inducing apoptosis (Lin et al., 2012). This suggests that

combined therapy could improve the efficacy of chemotherapy for brain tumors (Lin et al., 2012).

1.5.1. Temozolomide as a chemotherapeutic agent

TMZ was developed in the 80s by the UK Cancer Research as one of a series of novel imidazotetrazinones, and it was initially developed with the intent to treat patients with malignant melanoma metastases in the brain. However, it also showed activity in relapsed GBM patients, encouraging further investigation. The first *in vivo* studies (phase I trial), in early 1990, confirmed the antitumoral potential of TMZ, which also exhibited less side-effects than the conventional chemotherapeutics drugs (Mason and Cairncross, 2005). In 1999, TMZ was approved by the FDA and, subsequently, the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) demonstrated an improved median survival, of the treatments with TMZ representing the first trials in which such improvement was seen, since those performed with radiation therapy (RT) in the mid-1970s (Villano et al., 2009). In 2002 and 2005 the results of phase II and phase III trials were, respectively, published (Stupp et al., 2002, 2005), showing the safety and efficacy of RT alone versus TMZ plus RT followed by TMZ monotherapy (Stupp regimen or EORTC-NCIC) in patients with newly diagnosed GBM. In the randomized phase III study, at a median follow-up of 28 months, the median survival was 14.6 months with RT plus TMZ compared with 12.1 months for RT alone. The 2- and 5-year survival were also improved (26,5% against 10,4%) (Stupp et al., 2005; Villano et al., 2009). Nevertheless, the prognosis of patients are still poor, once fewer than 3% of patients are still alive at 5 years after diagnosis (Ohgaki, 2009).

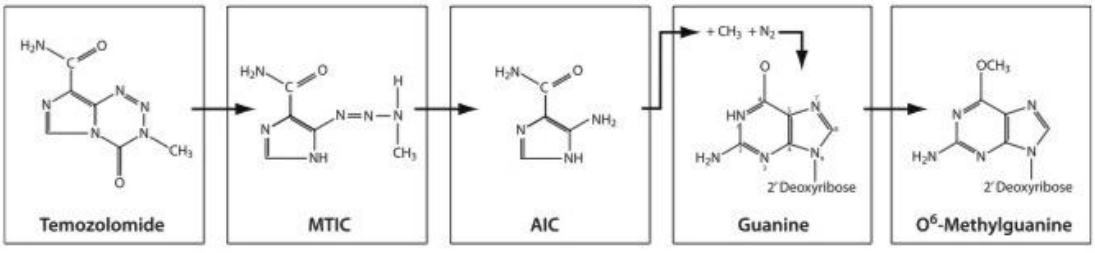


Fig. I.4. - Chemical structure of temozolomide (TMZ) and of its metabolites. After absorption, TMZ is spontaneously hydrolyzed at physiologic pH into the active metabolite MTIC (5-(3-methyltriazen-1-yl)imidazol-4-carboxamid), which is quickly converted to 5-aminoimidazole-4-carboxamide (AIC) and to the electrophilic alkylating methyldiazonium cation that transfers a methyl group to DNA. From Villano et al. (2009).

TMZ is a DNA alkylant agent, characterized by rapid and nearly complete oral absorption (Friedman et al., 2000; Villano et al., 2009). After absorption, the compound is spontaneously hydrolyzed at physiologic pH into the active metabolite MTIC (5-(3-methyltriazen-1-yl)imidazol-4-carboxamid) (Fig. I.4). Both TMZ and MTIC are able to cross the blood brain barrier. The active form of TMZ shows a plasma peak concentration within 30 to 90 min following uptake, and a half-life of 2 hours. MTIC is then quickly converted to 5-aminoimidazole-4-carboxamide (AIC) and to the electrophilic alkylating methyldiazonium cation that transfers a methyl group to DNA. Methylation of O_6 position of guanine (O_6-meG) explains the cytotoxicity of TMZ, which leads to inhibition of proliferation or cell death, late apoptosis, senescence (Gunther et al., 2003), autophagy, and cell cycle arrest (Fig. I.5) (Hirose et al., 2001; Carmo et al., 2011).

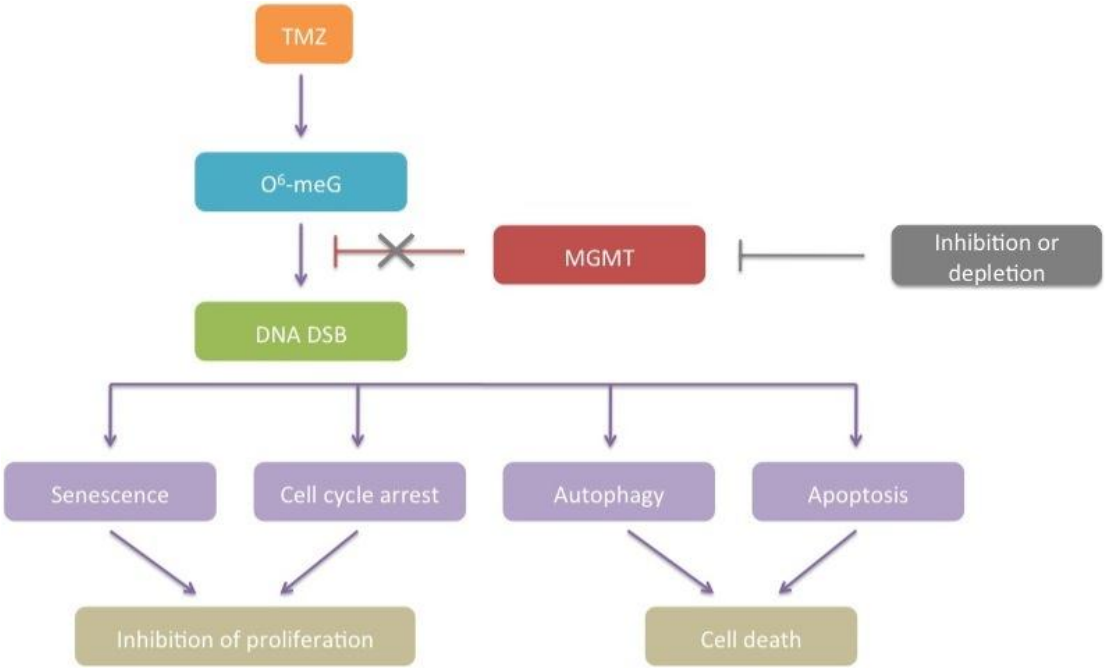


Fig. I.5. – Mechanisms of activity of temozolomide (TMZ) as an enhancer therapeutics. O6-meG (O_6 position of guanine) DNA adducts, as the DNA DSB (DNA double-straind breaks), are responsible for the cytotoxic effect of TMZ. MGMT (O6-methylguanine-DNA methyltransferase) repairs the lesions, resulting in resistance to TMZ. When MGMT is depleted or suppressed by methylation of the gene promoter, cytotoxicity of TMZ is enhanced.

The O6-G-alkylation is reversed by the O6-methylguanine-DNA methyltransferase (MGMT) and thus high levels of MGMT are thought to contribute

to the resistance to TMZ. MGMT repairs the O6-meG lesion by transferring the methyl group to its own cysteine residue. Methylated MGMT is then degraded. Thus, MGMT is considered a 'suicide' repair protein, and new MGMT must be synthesized in order to continue DNA repairing. Conversely, methylation of the promoter of the *MGMT* gene silences the gene, and would be expected to enhance the cytotoxicity of O6-meG lesions (Villano et al., 2009).

1.5.2. Ursodeoxycholic acid (UDCA) and its glyco- (GUDCA) and tauro- (TUDCA) conjugated species

Ursodeoxycholic acid (UDCA), the 7β-hydroxy epimer of chenodeoxycholic acid, is an endogenous bile acid that has been widely used for the treatment of hepatobiliary disorders since the mid-1980s (Lazaridis et al., 2001) and is also suggested to have a potential role in the treatment of non-liver diseases associated with increased levels of apoptosis, since as been considered an anti-apoptotic agent (Rodrigues and Steer, 2001). Following oral administration, UDCA is conjugated with taurine and glycine in the liver, originating tauroursodeoxycholic acid (TUDCA) and, mostly, glycoursodeoxycholic acid (GUDCA, 79,8%), respectively (Lazaridis et al., 2001; Rudolph et al., 2002). Thus, GUDCA is the conjugate form of UDCA with highest clinical relevance.

It was demonstrated that UDCA, as well as its conjugates act as cytoprotective agents, stabilizing cell and mitochondrial membranes and preventing cytochrome c release, consequently reducing cellular apoptosis (Guldutuna et al., 1993; Rodrigues et al., 2000; Silva et al., 2001). Additionally, UDCA is able to suppress the production of pro-inflammatory cytokines by inactivation of the NF-κB pathway in different cell types (Sola et al., 2003; Joo et al., 2004; Schoemaker et al., 2004; Shah et al., 2006). Our most recent findings showed that GUDCA suppresses the production of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β in astrocytes (Brito et al., 2008). Moreover, it was recently suggested that the cytoprotective mechanism of both UDCA and its conjugates, is mediated by a defense against oxidative stress, pointing to antioxidant properties of the molecule (Rodrigues et al., 2000; Lapenna et al., 2002; Serviddio et al., 2004; Perez et al., 2006).

There are no studies regarding the effect of GUDCA on glioma cells, but it has been described that UDCA was shown to prevent colon tumorigenesis and in addition to its antiproliferative effect, it induces tumor growth suppression, reinforcing its chemopreventive actions (Wali et al., 2002).

2. Neural stem cells (NSCs)

Stem cells have been described as cells with extensive proliferative potential, differentiation ability and self-renewal capability. NSCs can generate both neurons and glial cells (astrocytes, oligodendrocytes and microglia). Contrary to what was thought initially, NSCs exist also in the adult brain, playing an important role in neuronal plasticity (Temple, 2001).

2.1. NSC in the developing and adult brain

The CNS is formed over a short time in vertebrate embryogenesis and begins as a layer of neuroepithelial progenitors (NEPs) that rapidly form the neural tube (Merkle and Alvarez-Buylla, 2006). The development of the CNS includes several steps, such as the generation and differentiation of distinct cell lineages of neurons and glia, known to be descendents of multipotent NSCs. NEPs are cells specially located in the ventricular zone (VZ) of the neural tube, which has a great mitotic activity. Later, these cells move to the pial (external layer) of the neural tube as they progress through the mitotic cycle. In early embryonic phases, NEPs undergo mainly symmetric divisions, maintaining the stemness and increasing the stem cell pool, but then they divide asymmetrically to generate new stem cells that remain in the VZ, and intermediate progenitors (mostly neuronal precursors, but also glial) that migrate radially outward to its final position in the brain (Temple, 2001; Nicolis, 2007). At this point, the proliferating precursors cells originated may differentiate into more committed phenotypes, such as differentiated neurons or glial cells (astrocytes, oligodendrocytes or microglia). The differentiation of the neuroepithelial stem cells into neurons and glia proceeds in a temporal specific manner that is particular for each region of the developing neural tube. Before differentiate and in contrast with primary progenitors, intermediate progenitors may suffer one or more symmetrical divisions in the subventricular zone (SVZ), above the VZ, and the subgranular zone (SGZ) within the dentate gyrus of the hippocampus. Until birth, the SVZ increases in size and later decreases, persisting in adult life as SVZ (Merkle and Alvarez-Buylla, 2006).

In parallel to the onset of neurogenesis, radial glia (RG) cells appear to replace NEPs. RG cells function as stem cells for neurons (at early stages) and later for glia (Nicolis, 2007). Like NEPs, RG cells are a transiently population in the developing brain that divide in the VZ, still its differentiation potential is less broad than that of NEPs (Merkle and Alvarez-Buylla, 2006).

Within the adult mammalian brain, two major germinal regions are the SVZ along the walls of the lateral ventricles, and the SGZ in the hippocampus. The SVZ contains the principal concentration of dividing cells in the adult brain. This region is formed by type B cells (or astrocyte-like neural stem cells), which are characterized by slow division. Type B cells generate actively proliferating type C cells (transient amplifying progenitors), which in turn produce immature neuroblasts (type A cells) that migrate to the olfactory bulb, where they can differentiate into interneurons. Type B cells express the astrocyte marker glial fibrillary acidic protein (GFAP), and it was recently shown that the potential of type B cells is limited (Ihrie and Alvarez-Buylla, 2008).

The adult progenitors of the dentate gyrus are found in the SGZ of the hippocampus, where two types of cells can be identified according to their morphologies and expressions of molecular markers. Type 1 progenitors rarely divide, express GFAP and SRY (sex determining region Y)-box2 (Sox2), and have a radial process across the granular zone and ramify in the inner molecular layer. On the other hand, type 2 progenitors divide more frequently, display short processes and do not express GFAP. Some *in vivo* evidences demonstrated that type 1 progenitor cells can give rise to neuroblasts that mature to neurons and, at least some of them, have self-renew ability and generate both astrocytes and neurons (Miller and Gauthier-Fisher, 2009).

2.2. Applying NSC biology to glioma research: the brain tumor stem cells (BTSCs) hypothesis

Brain tumor stem cells (BTSCs) term to often describes a subpopulation of stem cells, with properties such as self-renewal, unlimited proliferative potential, slow rates of division, resistance to toxic xenobiotics, high DNA repair capacity and ability to generate partially differentiated progenies (Foreman et al., 2009) (Fig.I.6).

Divergent perspectives on the origin of a brain tumor fuel a debate that revolves around the theory BTSCs. This theory suggests that within a tumor, there is a small distinct cell population showing stem cell characteristics that are at the origin of the tumor, being responsible for tumor growth and maintenance. Corroborating this hypothesis, several groups studying brain tumors cells identified a minor population of cells in culture that are able to self-renew, form clonogenic neurospheres and differentiate into a broad range of cell types (Ignatova et al., 2002; Hemmati et al., 2003; Singh et al., 2003; Galli et al., 2004; Huhn et al., 2005). These brain tumor cells expressing the cell surface marker CD133/Prominin1 (1-35% of the cell

population), also evidenced the stem cell marker Nestin, as well as molecular markers associated with neural precursors such as Sox2, Bmi1, Notch, Emx2, Pax6 and Jagged1. Upon exposure to serum, these clonogenic neurospheres were able to differentiate into a mixed population of neurons (Tuj1+), astrocytes (GFAP+) and oligodendrocytes (PDGFR+), suggesting that they derived from a cell with multilineage differentiation capacity – a neural stem cell (Dirks, 2008).

Later, other groups extended these findings to medulloblastoma, by evidencing that they express NSCs proteins, including Sox2, Bmi1 and Musashi1 (Hemmati et al., 2003). These findings confirm that (1) brain tumors contain undifferentiated neural precursors, (2) stem-like cells possess some of the molecular features of NSCs and (3) $CD133^{+}$ cells can be used for the enrichment of tumor stem-like cells (Singh et al., 2003; Singh et al., 2004).

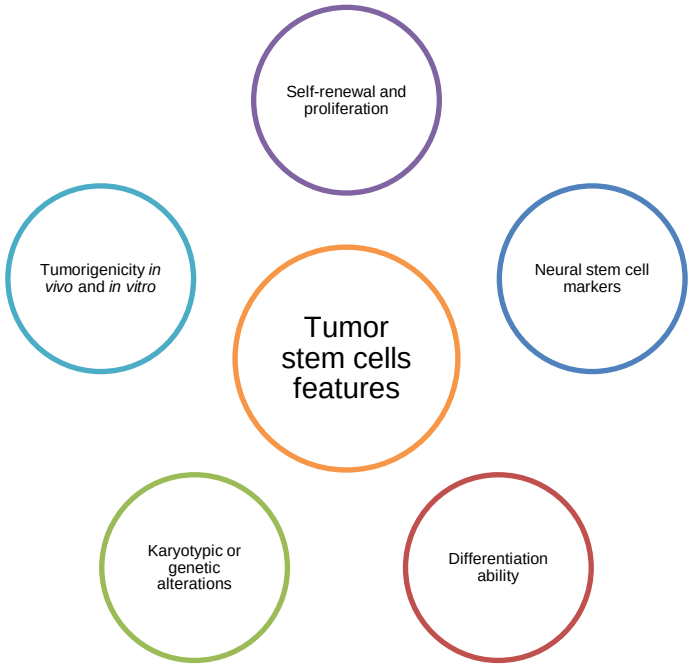


Fig. I.6. – Characteristics of brain tumor stem cells (BTSCs). BTSCs are characterized by (1) extensive self-renewal and proliferation ability, (2) expression neural stem cell (NSC) markers, (3) capacity to generate differentiate multilineage, (4) karyotypic or genetic alterations as well as tumorigenicity *in vivo* or *in vitro* (5).

Subsequently, studies *in vitro* through the injection of sorted CD133+ cells demonstrated that they could produce orthotopic tumors in the brain of NOD/SCID mice (an immunodeficient rodent), with common *in vivo* features of human GBM, such as extensive migratory and infiltrative capacity (Galli et al., 2004), whereas

injection of $CD133^-$ cells failed to form tumors (Singh et al., 2004). However, recent studies suggest a less clear distinction between the ability of $CD133^-$ and CD133+ cells to form orthotopic tumors (Bao et al., 2006; Beier et al., 2007). Although CD133 expression seems to be related to stemness, it might only indicate an intermediate, adaptive state of a cell rather than a phenotype. It has been reported that $CD133^-$ isolated from primary GBM were equally capable of forming orthotopic tumors as the CD133+ population (Beier et al., 2007). These findings suggest that CD133 is not a reliable marker for the tumorigenic capacity of stem like cells.

Currently, all stem-like glioma cells used in research are almost exclusively derived from glioblastoma and no defined cell type for its origin has emerged, most probably owing to the heterogeneity of the disease. Thus, the tumor brain stem cells fraction will require further purification.

2.2.1. The origin of BTSCs

Although there is an accumulating evidence that tumors contain a subpopulation with a tumor-initiating potencial, as described in the previous Section, the cell of origin of BTSCs has not been determined yet. At the present, it is unclear whether a tumor arises from NCSs, progenitor cells or differentiated cells that dedifferentiate into a stem-like state (Fig. I.7).

Traditional neuro-oncology postulated that the differentiated glial cells were the cells at the origin of gliomas. However, to undergo oncogenic events, mature glial cells would have to be proliferative and it is currently accepted that most brain cells do not divide, during adult life. Thus, numerous recent studies have been suggesting that these tumors may arise from the transformation of NPCs or the dedifferentiation of mature glial cells in response to genetic alterations (Llaguno et al., 2008).

The SVZ is one of the most important sources of neural stem/progenitor cells and this region is then believed to be in the origin brain tumors. Indeed, many tumors develop near this region (Sanai et al., 2005). In addition, corroborating the stem/progenitor cell origin of tumors are the similarities shared by normal stem cells and tumor stem cells, such as high mobility, extensive self-renewal and proliferation, expression of immature profiles and association with blood vessels (Sanai et al., 2005). Moreover, histological studies demonstrate the lack of the expression of differentiated cell markers (Dahlstrand et al., 1992). Finally, Holland and co-workers have found that undifferentiated cells may be more sensitive to transformation than differentiated cells (Holland et al., 2000). Using a retroviral system, they directed the expression of oncogenes to brain cells expressing GFAP or to cells expressing

Nestin, and they found that malignant glial tumors arise most efficiently after oncogene transfer to nestin-expressing cells. Taken together, these evidences point out the involvement of immature precursors cells in the development of the tumor phenotype, but it is not known which developmental stage, from NSC to early-differentiated cell type lineages, are more prone to malignant transformation.

On the opposite, the existence of cells in the adult brain capable of reverting to a less mature state in response to certain stimuli, supports the hypothesis of dedifferentiation of mature glial cells (Canoll and Goldman, 2008). These cells were known to dedifferentiate into transformed glia with stem cell-like properties through retroviral transfection (Bachoo et al., 2002).

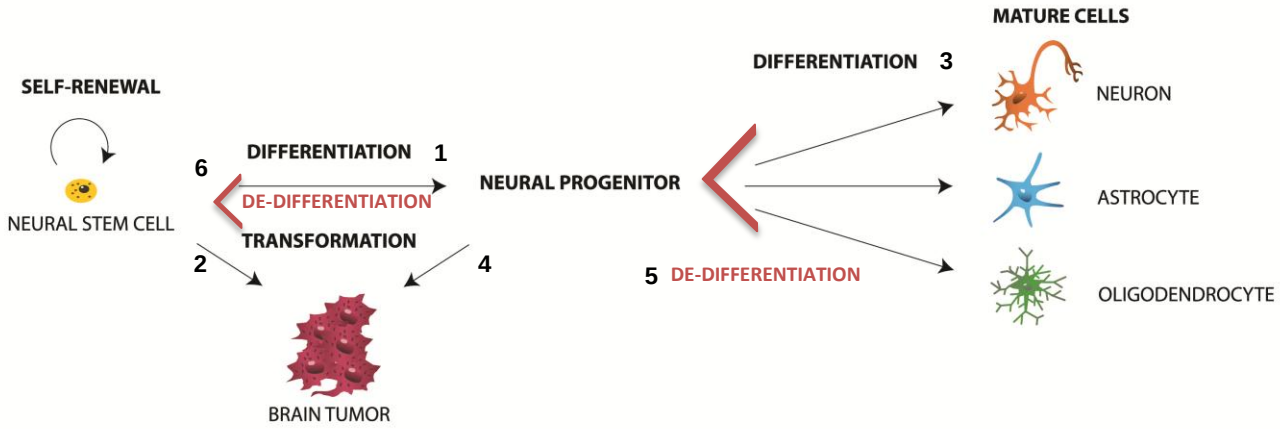


Fig. I.7. – Possible origins of brain tumor stem cells. Brain tumor stem cells (BTSCs) may be originated from different cell types and stages. A neural stem cell (NSC) can (1) differentiate into a neural progenitor cell or (2) suffer a transformation, originating a BTSC. In its turn, the neural progenitor may also (3) differentiate into mature cells (such as neurons, astrocytes or oligodendrocytes) or (4) undergo transformation into BTSCs. Notwithstanding, mature cells may (5) de-differentiate into neural progenitor, which consequently might (4) transform to a BTSC or (6) de-differentiate to a more immature stage (NSC), that still may suffer transformation to a BTSC. Adapted from http://www.igp.uu.se/Research/Cancer_and_vascular_biology/karin_forsberg_nilsson/?languageId=1

Thus, if we can identify the cell(s) at the origin of brain tumors, we will be better equipped to understand which molecular alterations may lead to cancer, and how we can target these by therapeutics or by modulators able to prevent their occurence. The cell that is transformed may have important influences on the behaviour of the neoplasm and therefore may also affect the patient prognosis.

2.2.2. Therapeutic perspectives

In addition to their relatively quiescence, BTSCs are also resistant to chemotherapy due to their enhanced capacity for DNA repair and ABC-transporter expression (Atkinson et al., 2009). In fact it was recently described that BTSCs highly express Mrp1 (Jin et al., 2010). Besides, these cells have a great infiltrative ability and may activate certain survival pathways to inhibit apoptosis (Carmo et al., 2011). Thus, new therapies targeting BTSCs may be developed if we want to prevent or eliminate recurrent and metastatic disease.

Molecular analysis of the BTSCs population may lead to the identification of novel pathways important for the proliferation, self-renewal and differentiation of these cells, oppening new targets for therapy, which should be able to modify the signaling pathways or the microenvironment favouring for their self-renewal (Singh et al., 2004; Xie, 2009). Yet, another possibility is disrupting the interactions between BTSCs and their niche that hopefully will slow tumor bulk malignancy progression can be slowed. Also, the promotion of differentiation, particularly if terminally differentiated cells types can be generated, may be another useful strategy.

Due to the infiltrative nature of BTSCs, it is difficult to specifically deliver therapeutic agents to these cells. One option it would be to harness the potential of normal NCSs, which exhibit strong tropism for brain tumor cells when transplanted into the host brain. Thus, intracranial transplantation of NSCs carrying therapeutic agent might effectively eliminate BTSCs and inhibit tumor growth (Xie, 2009).

Efflux pumps, like ABC-drug transporters, may also be targeted it a therapy of chemotherapy and adjuvant chemosensitizers could be used with the aim of alter the activity of some ABC transporters, leading to better clinical outcomes (Dean et al., 2005).

Tumor cells have been found to be in a state of redox imbalance with a more oxidizing environment, and show an increased ability to withstand oxidative stress (Ogasawara and Zhang, 2009). Recently, several signaling pathways involved in different cell processes, such as self-renewal, proliferation and differentiation, have been recognized as being under redox regulation (Hernandez-Garcia et al., 2010). In fact, some authors have already hypothesized that the highly drug resistance of BTSCs can be due to the use of their redox regulatory mechanisms to escape the cell death by several anticancer agents (Blum et al. 2009; Hill and Wu, 2009; Ogasawara and Zhang, 2009; Boman and Huang, 2008; Morel et al., 2008; Tang et al., 2008). Thus, given the significance of redox environment in BTSCs, we can hypothesize that molecules with antioxidant potential, such as GUDCA can be used as coadjuvants of classical chemotherapy (Szatmari et al., 2006).

Thus, potential avenues for therapeutic intervention may require combinations of targeted therapies against both stem-like and less tumorigenic cancer cells, as well as directed to inhibition of resistance mechanisms in cancer stem cells. The potential stem/progenitor glioma origin and the presence of stem-like cancer cells also paves the way for new therapeutic avenues such the use of therapy that promotes differentiatiom, to retard the growth of malignant astrocytomas.

CHAPTER II - OBJECTIVES

Chapter II - Objectives

The main goals of the present work are a) to identify novel cues to the cellular pathways implicated in gliomagenesis and b) to find a successful adjuvant molecule for TMZ therapy that may decrease chemoresistance and/or alter cell environment.

More specifically, our first aim is to identify which developmental stage, from NSC to immature/early differentiated glia, is more susceptible to malignant transformation. To accomplish this goal, we will use the mouse glioma cell line GL261, which will be first characterized regarding to the expression of neural phenotypes, as well as primary cultures of NSC, growing as neurospheres, which will be induced to differentiate into astrocytes. Then, we will identify the neural developmental stage more similar to glioma cells by comparing the expression of some tumor-related markers such as multidrug resistance, angiogenesis potential, autophagy ability, migratory and invasion capability.

In addition, we will also explore the validity of some new molecules as coadjuvants in TMZ therapy. Thus, we will evaluate the effect of TMZ, alone in association with GUDCA or with MK-571 (an Mrp1 inhibitor) on the viability and proliferation of glioma cells, as well as on their cell cycle progression. We will finally, explore the effect of GUDCA and MK-571 at the level of some migration-related present in of glioma cells.

CHAPTER III – Materials and methods

1. Cell cultures

1.1. GL261 mouse glioma cell line

The Gl261 was a kind gift from Dr Geza Safrany, from the National Research Institute for Radiobiology and Radiohygiene, in Hungary. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with 38.9 mM glucose, 11 mM sodium bicarbonate, 1% penicillin/streptomycin and 10% feral bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), at 37ºC and 5% CO_2 conditioned atmosphere during 7 days. The medium was changed every two days and cells were passaged when the cells reached confluence. After 3, 5 and 7 days *in vitro*, cells plated in coverslips were fixed with freshly prepared 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) during 20 min and used for immunocytochemistry assays. The ones that were in the wells without coverslips were used for flow cytometry studies or lysed for western blot. Growth medium was removed, centrifuged and stored at -80^{o}C to evaluate the release of MMPs and S100B.

1.2. Primary neurosphere culture of mouse brain cortex at E15 and induction of astrocyte differentiation

Animal care followed the European Legislation on Protection of Animals Used for Experimental and Scientific Purposes (EU directive L0065, 22/07/2003) in order to ensure their well-being and minimize animals use and suffering.

Cortical neural precursors were isolated from embryonic day (E) 15. Briefly, pregnant female mice at gestational stage E15 were euthanized by asphyxiation with CO_2. The fetuses were rapidly decapitated and after removal of meninges and white matter, the neocortices were collected in 9 ml of Hank's Balanced Salt Solution (HBSS, Invitrogen) and mechanically fragmented. After chemical dissociation with trypsin-EDTA 5% (Sigma-Aldrich, St. Louis, MO, USA) and deoxyribonuclease I bovine (DNAse I, 1 U/ml, Sigma-Aldrich), the suspension was incubated for 30 min at 37ºC, with occasional mixing. Following trypsinization, cells were washed three times with HBSS and resuspended in 5 ml of RHB-A^{TM} medium (Stem Cell Sciences, Cambridge, UK). Once resuspended, cells were mechanically dissociated using a Pasteur pipette performing around 20 passages. Approximately 1x10^{6} cells/ml were plated into 24-well uncoated tissue culture plates in culture medium supplemented with growth factors (10 ng/ml, recombinant murine epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, NK, USA), to form free-floating neurospheres, maintained at 37ºC in a humidified atmosphere of 5%

CO_2, during 48 h. After this period, astroglial differentiation was induced by using 10% FBS during 7 days. In neurospheres and in cells with 3 and 7 DIV under differentiating conditions, cell lysates were collected for western blot analysis and their growth medium was removed, centrifuged and stored at -80^{o}C to evaluate the release of MMPs and S100B.

2. Characterization of the mouse glioma cell line GL261

2.1. Characterization of the GL261 cells by immunocytochemistry

To characterize the glioma cell line, fixed cells were incubated in a 0.1M glycine (Merck) solution during 10 min and then permeabilized with 0.1% Triton X-100 (Roche Diagnostics, Indianapolis, USA) solution for other 10 min. Following three rinses with PBS, coverslips were blocked using 10% FBS in Tween 20-Tris buffered saline (TBS-T, 0.05% Tween 20, Merck; 20 mM Tris-HCL, 500 mM NaCl, pH 7.5) for 30 min at room temperature (RT). Coverslips were incubated overnight, at 4°C, with anti-microtubule associated protein (MAP)-2 antibody (mouse, 1:100, Millipore, Billerica, MA, USA), anti-glial fibrillary acidic protein (GFAP) antibody (rabbit, 1:500, Millipore); anti-nestin antibody (mouse, 1:200, Millipore), anti-glutamate transporter (GLAST/EAAT1) antibody (mouse, 1:500, AbCam, Cambridge, UK), anti-(sex determining region Y)-box 2 (Sox2) antibody (rabbit, 1:500, Millipore) and anti-vimentin antibody (mouse, 1:25, Santa Cruz Biotechnology, CA, USA). Following three rinses with TBS-T, coverslips were incubated with FITC-labelled anti-mouse IgG (horse, 1:227, Vector Labs, Burlingame, CA, USA) and Alexa 594-labelled anti-rabbit IgG (goat, 1:1000, Invitrogen), during 90 min at RT. After rinsed, coverslips were incubated with Hoechst dye 33258 (Sigma-Aldrich) during 2 min for cell nuclei staining. Following a final rinse in TBS-T and dehydration with methanol (Merck), coverslips were mounted using DPX (BDH Prolabo, Bangkok, Thailand) and stored at 4°C. Finally pairs of U.V. and fluorescence images of ten random microscopic fields (original magnification: 252x) were acquired per sample. Immune-positive cells for each cell type and total cells were counted to determine the percentage of positive nuclei. The resultant values were presented as percentage of positive cells for each staining.

2.2. Characterization of the GL261 cells by flow cytometry

To characterize the glioma cell line by flow cytometry, cells were trypsinized with 0,1% Trypsin-EDTA in PBS and collected. Then cells were centrifuged at 500 *g* for 5 min at 4ºC and washed once with Phosphate buffered saline (PBS). After that, cells

were fixed with 4% PFA for 20 min in ice and centrifuged. Cells were blocked using 10% FBS TBS-T for 20 min at RT. Following centrifugation, cells were incubated during 30 min at RT, with the antibodies mentioned in section 4.2. Cells were incubated with FITC-labelled anti-mouse IgG (1:227) and Alexa 594-labelled anti-rabbit IgG (1:1000), during 30 min at RT. After centrifugation, cells were rinsed once and ressuspended in PBS. Finally, cellular suspension was plated in a 96-wells plate and analysed by flow cytometry (Guava – Easy Cyte HT model, Millipore). Results were expressed as percentage of positive cells for each one of the antibodies analyzed.

3. Charaterization of tumor-related factors

3.1. MMPs activity

To compare the activity of MMPs of the GL261 cell line and with each of the neural developmental stages, aliquots of glioma cells supernatants (3, 5 and 7 DIV), NSC and differentiating astrocytes at 3 and 7 DIV were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography in 0.1% gelatin/10% acrylamide (Sigma-Aldrich; Merck) gels under non-reducing conditions. After electrophoresis, gels were washed for 1 h with 2.5% Triton X-100 (in 50 mM Tris pH7.4; 5 mM $CaCl_2$; 1µM $ZnCl_2$) to remove SDS (VWR-Prolabo) and renature the MMPs species in the gel. Then the gels were incubated in the developing buffer (50 mM Tris pH7.4; 5 mM $CaCl_2$; 1µM $ZnCl_2$) overnight to induce gelatine lysis. For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma-Aldrich) and destained in 30% ethanol/10% acetic acid/H2O. Gelatinase activity, detected as a white band on a blue background, was quantified by computerized image analysis and normalized with total cellular protein.

3.2. S100B assay

The expression of S100B in glioma cells (at 3, 5 and 7 DIV), NSC and differentiating astrocytes at 3 and 7 DIV was assessed by ELISA. Supernatants were incubated with monoclonal antibody anti-S100B (1:1000, Sigma-Aldrich) in carbonate-bicarbonate buffer (50mM, pH 9.5) per well at 4^{o}C overnight. After three washes with wash buffer (0.1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.05% Tween(Merck)), supernants were blocked (2% BSA in PBS) for 1 h at RT. To each sample was added 50mM Tris buffer (pH 8.6, with 0.2mM $CaCl_2$), followed by three washes with wash buffer. Supernants were incubated with polyclonal antibody anti-S100 (1:5000 in 0.5% BSA with 0.2 mM $Cacl_2$ in PBS, Dako, Dernmark, A/S) for 30 min at 37^{o}C and, then, incubated with antibody anti-rabbit peroxidase conjugated

(1:5000 in 0.5% BSA in PBS, Santa Cruz Biotechnology) under same conditions. Following washes with both wash buffer and PBS, it was added substract solution (Sigma Fast OPD in H_2O, Sigma-Aldrich) for 30 min at RT and under light protecting. At last, absorbance was measured at 450 nm.

3.3. Expression of tumor-associated factors

To compare some tumor-associated factors of the GL261 cell line with the different neural developmental stages, total cell extracts of both glioma cells (3, 5 and 7 DIV), NSC and differentiating astrocytes at 3 and 7 DIV, were obtained by lysing cells in ice-cold Cell Lysis Buffer (Cell Signaling, Beverly, MA, USA) plus 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich) for 10 min, on ice, followed by sonication. The lysate was centrifuged at 14 000 *g* for 10 min, at 4ºC, and the supernatants were collected and stored at -80ºC. Protein concentrations were determined using the Bradford assay. Equal amounts of protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). After transfer, blotted membranes were blocked for 1 hour at RT with 4% milk in TBS-T (in case of Mrp1 expression evaluation) and in 5% BSA (Sigma-Aldrich) in TBS-T (for determination of VEGF, β-actin and LC3) and incubated overnight at 4ºC with anti- multidrug resistance protein 1 (Mrp1)-A23 antibody (1:750, Sigma-Aldrich), anti-vascular endothelial growth factor (VEGF) antibody (1:200, Santa Cruz Biotechnology), anti-β-actin antibody (1:5000, Sigma-Aldrich) and anti-protein light chain 3 (LC3) antibody (1:2000, Cell Signaling) in respective blocking solution. After washing with TBS-T, the membranes were incubated with secondary antibody anti-rabbit (horse, 1:5000, Santa Cruz Biotechnology) or anti-mouse (goat, 1: 5000, Amersham Biosciences), as appropriate, in blocking buffer for 1 h, at RT. After washing membranes with TBS-T, chemiluminescent detection was performed by LumiGLO® (Cell Signaling) and bands were visualized by autoradiography with Hyperfilm ECL. The relative intensities of protein bands were analyzed using the Quantity one® 1-D densitometric analysis software (Bio-Rad, Hercules, CA, USA).

4. Cell treatments

Glioma cells were first treated (or untreated, control) with TMZ (50, 100 and 250 µM, Sigma-Aldrich) during 24, 48 and 72 h. After incubation, cell viability was evaluated in order to ascertain the most efficient TMZ incubation conditions to be subsequently used. After this first trial, glioma cells were then incubated with TMZ alone or in the presence of MK-571 (25 µM, Sigma-Aldrich), or GUDCA (50 µM,

Calbiochem Darmstadt, Germany) at selected exposure conditions. After incubation, it was determined the cell viability, proliferation, cell cycle progression and cell death by apoptosis. The success of GUDCA or MK-571 co-incubation was evaluated by comparing the results with those obtained with TMZ alone.

Glioma cells with 3, 5 and 7 DIV were also incubated with MK-571 (25 µM), or GUDCA (50 µM) at the previously selected exposure time, to explore the effect of these molecules on some migration-related factors of glioma cells, such as CXCR4 expression.

4.1. Cell viability

Cell viability was determined by evaluating [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium] (MTS) reduction in the presence of phenazine methosulfate (PMS), which forms a formazan product that is released to the culture medium, with an absorbance maximum at 490 nm.

A combined MTS/PMS solution (1:20, with stock solution at 2 mg/ml and at 0.92 mg/ml, respectively) was freshly prepared and after the cell treatment, supernatants were removed and cells incubated for 45 min, at 37°C, in a dilution of 1:10 in culture medium. At the end of incubation, the absorbance of the medium was read at 490 nm using an ELISA plate reader.

4.2. Cell cycle progression

For determination of cell cycle progression, the cells were analyzed by flow cytometry. At the end of incubation period, cells were collected, washed with PBS, centrifuged at 500 g for 10 min, and then the pellet was resuspended in a fixative solution with glycine:ethanol (3:7, volume/volume) solution for 30 min at 4ºC. After centrifugation at 500 g for 10 min, cells were washed with PBS and the pellet resuspended and incubated for 10 min in the dark, at RT, in a solution of PBS containing 10µL/mL propidium iodide (PI, Invitrogen, Paisley, UK) and 10µL/mL RNAse. The PI fluorescence was measured on a FACScan flow cytometer (BD FACSCalibur^{TM}) and the data were gated to exclude cell debris and aggregates.

4.3. Expression of CXCR4

To evaluate CXCR4 expression, total cell extracts of GL261 cells (3, 5 and 7 DIV) were obtained by lysing cells in ice-cold Cell Lysis Buffer plus 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min, on ice, followed by sonication. The lysate was centrifuged at 14 000 *g* for 10 min, at 4ºC, and the supernatants were collected and stored at -80ºC. Protein concentrations were determined using the

Bradford assay. Equal amounts of protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. After transfer, blotted membranes were blocked for 1 h at RT in 5% milk TBS-T and incubated overnight at 4ºC with anti-CXCR4 antibody (rabbit, 1:1000, AbCam), and anti-β-actin antibody (1:5000) in respective blocking solution. After washing with TBS-T, the membranes were incubated with secondary antibody anti-rabbit (1:5000) or anti-mouse (1: 5000), as appropriate, in blocking buffer for 1 h, at RT. After washing membranes with TBS-T, chemiluminescent detection was performed by LumiGLO® and bands were visualized by autoradiography with Hyperfilm ECL. The relative intensities of protein bands were analyzed using the Quantity one® 1-D densitometric analysis software (Bio-Rad, Hercules, CA, USA).

Chapter IV – Results and discussion

1. Characterization of the mouse glioma cell line GL261

In this Chapter of Results we have decided to continuously evaluate and moderately discuss the significance of the values obtained to better understand the relevance of the values achieved in each point, due to the novelty of the approach we have programmed to follow.

The application of suitable experimental models to glioma research, which ideally should harbor key features of the human disease, is necessary for the identification of more specific targets and development of novel and target-directed therapies. One of the most widely used for preclinical and translational research is glioma 261 (GL261) cell line (Wu et al., 2008). These cells carry point mutations in the K-ras and p53 genes (Szatmari et al., 2006) and exhibit, as other glioma cell lines, populations of cells that have characteristics of cancer stem cells, such as the CD133+ cells (Wu et al., 2008), as well as a sub-population of cells more sensitive to ATP (Tamajusuku et al., 2010). The choice of the GL261 cell line, instead of the other currently used cell line C6 derived from rat glioma cells and representing astrocyte-like cells (Swarnkar et al., 2012), was based on data evidencing that C6 gliomas are slightly invasive and only induce moderate vascular alterations, whereas GL261 tumors dramatically alter the brain vessels in the glioma region (Doblas et al., 2010), a property that we were interested to explore in the present work. Moreover, GL261 cells were recently considered, between several tested rodent glioma models, the one showing the greatest alterations in glioma metabolites (e.g. glutamate, lactate, total choline and creatine, glutamine-, aspartate, guanosine, mobile lipids and macromolecules, among others) (Doblas et al., 2012).

To assess the different stages of differentiation and the several cell types that may constitute the GL261 cell line, we started by using specific antibodies against proteins that are characteristic of undifferentiated and differentiated cells, at three different time points – 3 days *in vitro*, 5DIV and 7DIV. In order to characterize and evaluate the content in undifferentiated proliferating cells, it was used antibodies against Nestin and Sox2. Vimentin was used to stain early astrocyte progenitors and antibodies against βIII-Tubulin and MAP2 to identify neuronal cells. The astrocytic population was evaluated through the use of antibodies specific for GFAP and GLAST.

Initially, the characterization was performed by using immunocytochemistry (Fig.IV.1A.). We have observed that although all glioma cells were positive to the antibodies tested, it can be observed a different staining pattern. In fact, it seems that the markers more related to differentiated cells, such as GFAP and MAP2, were particularly evident in the cytoplasm and thus we can clearly visualize cell

ramifications, while GLAST is located near nuclei. The markers more related to undifferentiation, such as Nestin and Vimentin, stained cytoskeleton, whereas Sox2 labeled the perinuclear zone. However, this method enabled us to more accurately quantify the different phenotypes of GL261 cells along the time in culture, since all the cells were labeled.

Thus, the characterization proceeded by using the flow cytometry. This method is often used, not only for being a faster one, but also because of its higher specificity. The evaluations were optimized and the data obtained by flow cytometry confirmed that, in fact, the cellular composition of the GL261 cell line was not the same along the culture time window.

As shown in Fig. IV.1B, and despite only one assay has been performed, it can be observed that the expression of Vimentin increased 15,2% from 3 to 5 DIV and then remained constant until 7DIV, whereas the expression of both Sox2 and βIII-Tubulin decreased from 3 to 7 DIV, the decrease was more marked for Sox2 (40%) than for βIII-tubulin (23%). Because Sox2 is a glioma stem cell marker (Allen et al., 2012) data indicate that the stem cell representation in the GL261 cell line declines significantly and continuously (Fig. IV.1B) along the time in culture. Being Sox2 an undifferentiated cell marker, we can speculate that the early GL261 3DIV, may correspond to a more stem cell-like population and thus, with higher proliferation ability. In fact, undifferentiated phenotypes, this is, stemness phenotypes, have been associated to self-renewal capacity with implications toward possible roles in brain tumorigenesis (Shiras et al., 2003; Gangemi et al., 2009). Gangemi *et al* have denoted that Sox2 silencing in GBM cancer stem cells drive to proliferation inhibition and the loss of the tumorigenicity in immunodeficient mice, demonstrating the fundamental role for maintenance of the self-renewal capacity of neural stem cells when they have acquired cancer properties. Therefore, it is speculated that SOX2, or its immediate downstream effectors, would then be an ideal target for glioblastoma therapy.

βIII-tubulin is a neuronal differentiation marker aberrantly expressed in astrocytic gliomas (Katsetos et al., 2003) and linked to malignant changes in glial cells (Katsetos et al., 2007). Its overexpression has been related with chemoresistance (Zheng et al., 2012) and accumulation of βIII-tubulin was observed around the G2/M stage of the cell cycle of tumor cells (Shibazaki et al., 2012). The increased expression of βIII-tubulin in GL261 cells asserts, thus, a link between its aberrant expression and a disruption of microtubule dynamics usually observed during the transformation of a low-grade to a high-grade glioblastoma (Katsetos et al., 2011). Therefore, we may assume that GL261 even at 7DIV differentiation still have glioma

tumorigenesis, tumor progression and malignant transformation characteristics of glioblastoma multiform (Katsetos et al., 2009).

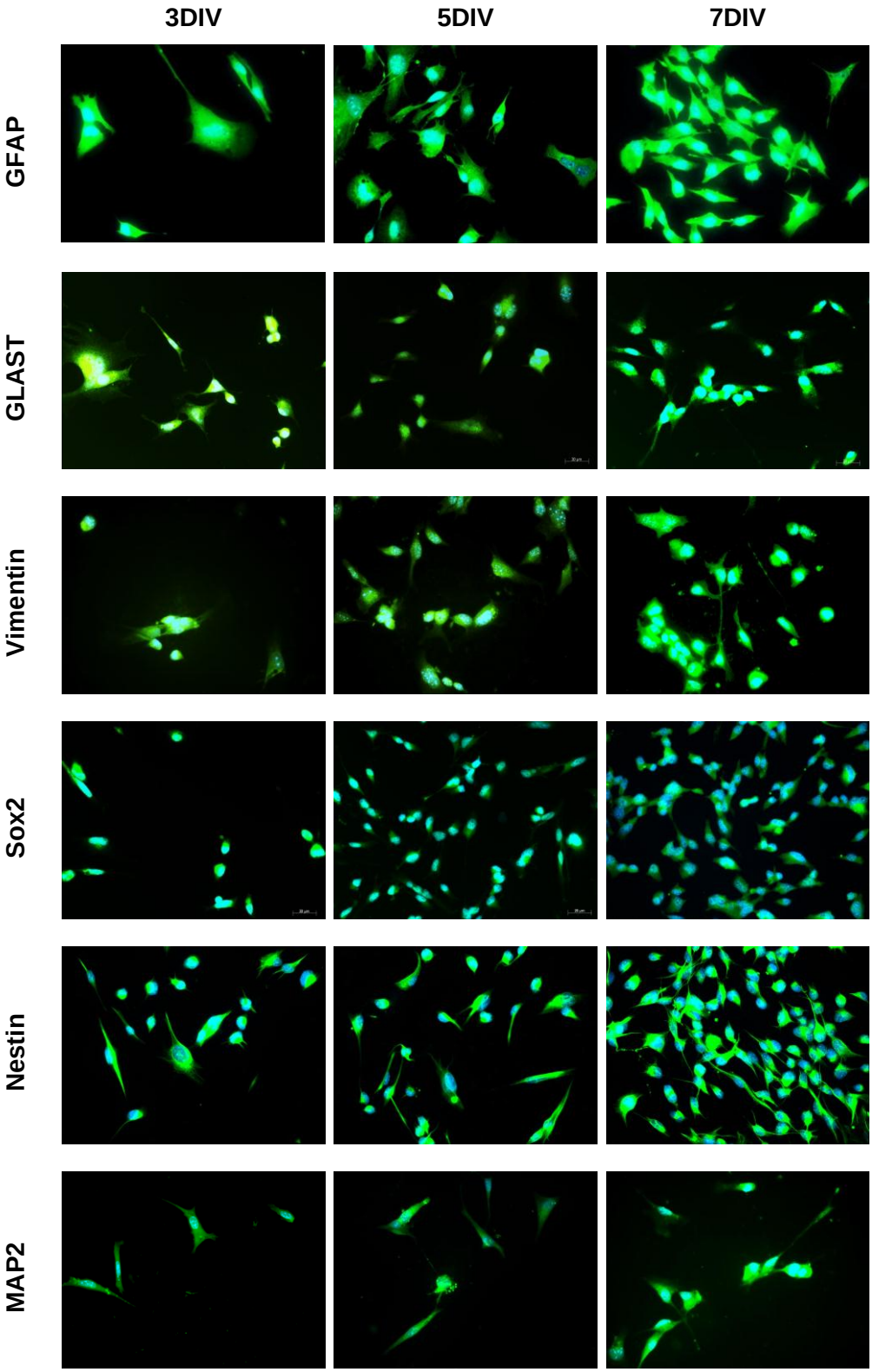
Vimentin, a mesenchymal marker (Ma et al., 2012), is a primordial component of the cytoskeleton and the nuclear envelope (Wang et al., 2010) that has been used as a molecular marker for glioblastoma multiform and astrocytoma (Yang et al., 1994; Mennel and Lell, 2005). The increase in Vimentin at both 5 and 7DIV (Fig. IV.1B) may result from increased cell migration abilities as its up-regulation is associated with tumor invasiveness (Jan et al., 2010; Thakkar et al., 2011).

GFAP is widely expressed in astroglial cells, neural stem cells, astroglial tumours, such as glioblastoma. It is consider a diagnostic marker of glioblastoma multiforme because GFAP presence is associated with more aggressive and invasive potentials (Jung et al., 2007). These authors observed that the most uniform GFAP staining was in well-differentiated grade II astrocytomas. We found that the expression of GFAP was almost the same along the time in culture (~70% of positive cells), with a 10% decrease at 7DIV. Once GFAP expression is related to the differentiation status of astrocytes (Jung et al., 2007) we can hypothesize that cell proliferation in our case is not so much elevated. Interestingly, many high-grade gliomas also seem to lose GFAP expression (Jacque et al., 1978; van der Meulen et al., 1978; Jacque et al., 1979; Velasco et al., 1980; Tascos et al., 1982) (Rutka et al., 1997). In addition, GFAP-negative cells proliferate more rapidly than GFAP-positive cells in the same tumor (Hara et al., 1991; Kajiwara et al., 1992). These *in vivo* findings allow demonstrate that the loss of GFAP expression could represent secondary loss of a differentiation marker or alternatively, it could be a step in tumor development (Wilhelmsson et al., 2003). Thus, we may speculate that GL261 at 7DIV have increased its aggressiveness and invasive potentials in comparison to 3DIV cells.

Finally, GLAST and MAP2 are expressed unevenly, with increased levels at 5 DIV. GLAST is an astroglial glutamate transporter that was shown to be present in glioma cells (Baber and Haghighat, 2010) at similar levels to those of astrocytes, although its mislocation was noticed as an intrinsic feature of glioma cells (Ye et al., 1999). Variations in the Wnt-1 oncogene expression (Palos et al., 1999; Jimenez et al., 2003) may be in the origin of the observed GLAST fluctuation between GL261 from 3 to 7DIV, and deserve to be evaluated in the future.

MAP2 is another early neuronal marker that was shown to be also present in glioma cell lines and biopsies (Yan et al., 2011). In fact, MAP2 expression was demonstrated to occur transiently in migrating immature glial cells and indicated as corroborating the glial origin of the gliomas (Blumcke et al., 2001).

A)



B)

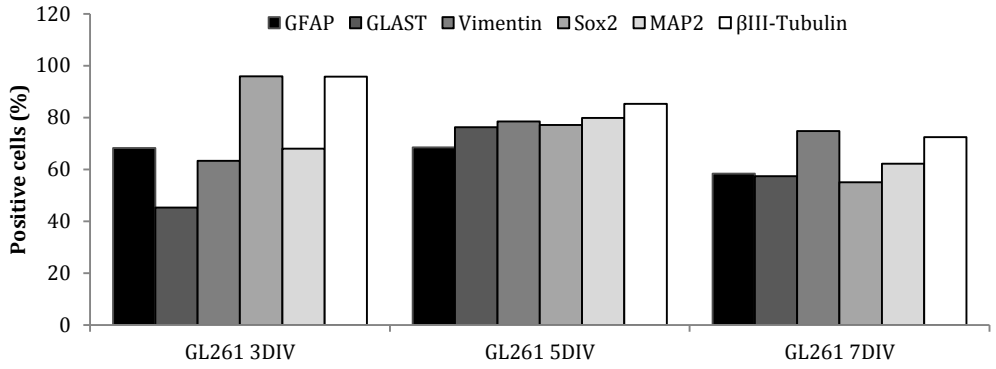


Fig. IV.1. Characterization of the glioma cell line GL261. Glioma cells were maintained in DMEM supplemented with 1% PenStrep and 10% FBS as previously described in Methods. Cells were fixed at 3, 5 and 7 days *in vitro* and processed for immunocytochemistry (A), where nuclei were stained with Hoechst dye (blue), or toflow cytometry analysis (B). Representative images (A) and data obtained from a single independent experiment (B). GFAP, glial fibrillary acidic protein, GLAST, glutamate aspartate transporter, MAP2, microtubule-associated protein 2. Scale bar: 20 μm.

Overall, these results indicate that the sub-fractions of cells that constitute the GL261 cell line and attest the tumor heterogeneity feature (Deleyrolle et al., 2012) change in accordance with the time of cells in culture. Also, indicate that independently of the time in culture these cells exhibit a primary tumor phenotype and highlight their value to explore the origin of gliomas and for preclinical modeling of novel anti-glioblastoma therapeutic agents. However, taking in account the results of a sole experiment, careful should be taken in the appreciation of the results just presented, and new series should be undertaken in the near future to validate them. In the next section, we decided to compare some important biological glioma like properties of GL261 cells at the 3 different culture temporal windows with 3 steps of neural precursors cell differentiation.

2. Characterization of common features between GL261 glioma cells and differentiating astrocytes from neural stem cells

NSCs, due to their longevity, self-renewal, high motility and sustained proliferative capacity, are believed to be in the origin of the glioma (Ignatova et al., 2002; Sanai et al., 2005) and their pluripotency the cause of the cellular diversity of the tumor (Tan et al., 2006; Louis et al., 2007). Therefore, it is plausible that in a particular point of NSC differentiation they are at an increased risk for malignant transformation. This risk may be associated with a phenotype that will most resemble equivalent one in glioma cells. To explore this resemblance, we

characterized and compared some features associated to brain tumors, in glioma cells, as well as on primary cultures of NSC, growing as neurospheres, which were induced to differentiate into astrocytes. This way, we propose to identify the neural developmental stage more similar to glioma cells by comparing the expression of some tumor-related factors such as multidrug resistance, angiogenesis potential, autophagy ability and invasion capability.

2.1. Invasion ability

One of the most important characteristics of tumor cells is their ability to invade the surrounding tissue. This feature is associated to the presence of proteins, like MMPs and S100B.

Interestingly, the activity of MMPs, both MMP-2 and MMP-9, decreased along the different time points in GL261, reaching very low values at 7DIV (0.3- and 0.2- fold *vs*. GL261 3DIV $p<0.01$, respectively). The activity of these gelatinases increased from neurospheres to 3 and 7 DIV, thus approaching the levels observed at GL261 3DIV. Conversely, the value obtained in neurospheres was close from the one observed in GL261 at 5 and 7DIV. Thus, it seems that Sox2 and βIII-tubulin, which were more expressed at GL261 at 3DIV may be related with an increased MMP expression and mobility. Intriguingly, in a recent study Oppel et al. (Oppel et al., 2011) reported that the knockdown of Sox2 impaired the invasive proteolysis-dependent migration of glioma cells also reducing the expression level of pro-MMP1 and pro-MMP2, and that Sox2 plays a role in the maintenance of a less differentiated glioma cell phenotype. In addition, silencing of MMP-2 evidenced to reduce stem cell migration and tropism towards the tumor cells (Bhoopathi et al., 2011). Further, the expression of active MMP-2 and MMP-9 was indicated to enhance with the growth of malignant gliomas (Zhao et al., 2007) and their down-regulation evidenced to reduce glioma stem cell proliferation (Reddy et al., 2011) and invasion (Annabi et al., 2008; Silveira Correa et al., 2010). To emphasize that βIII-tubulin positive immunoreactivity, although less documented, was also related with a cell active migration (Katsetos et al., 1998). Finally, based on these results we can speculate that GL261 3DIV and 3 and 7DIV differentiating astrocytes are those with most invasive ability.

The release of S100B into the extracellular medium revealed to increase with the time in culture, with the highest values in both GL261 and differentiating astrocytes at 7DIV (Fig. IV.3). S100 proteins are known to be involved in proliferation, differentiation and migration/invasion among other aspects (Donato et al., 2012). A recent study demonstrated that the transfection of S100B promotes cell invasion and migration and can be related with the development of brain metastasis (Pang et al.,

2012). However, the majority of the data published were related with the invasion property of lung cancer cells in the brain (Hu et al., 2010; Jiang et al., 2011).

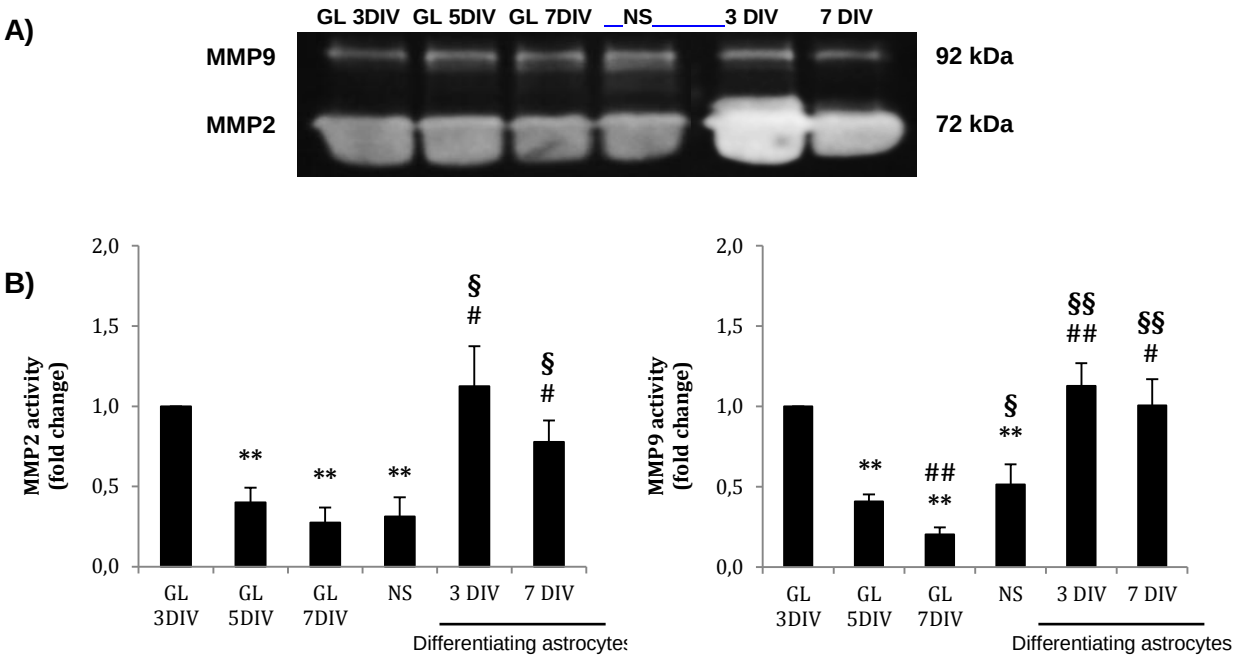


Fig. IV.2. Metalloproteinase (MMP)-2 and MMP-9 activities in GL261 glioma cells at 3, 5 and 7 days *in vitro* and in differentiating astrocytes from neurospheres (NS) during 3 and 7 DIV. Cells were cultured as indicated in methods. Cell supernatants were collected for quantification of MMP activity. A) MMP-2 and MMP-9 were identified by their apparent molecular mass of 72 and 92 kDa, respectively. Representative results from one experiment are shown. B) Graph bars represent the intensity of the bands that were quantified by scanning densitometry, standardized to respective protein quantification and expressed as mean ± SEM from at least three independent experiments. Results are and presented as fold change compared to GL261 3DIV (considered as 1). Data obtained from at least three independent experiments. **p<0.01 and *p<0.05 *vs*. GL261 3DIV; $^{\#\#}$p<0.01 and $^{\#}$p<0.05 *vs*.. GL261 5DIV; §§p<0.01 and §p<0.05 *vs*. GL261 7DIV.

Concentrations in glioma cells (~0.5 to 7 µM, from 3DIV to 7DIV) are several times higher than in neurospheres (~0.1 nM) or differentiating astrocytes (~0.2 µM), thus indicating a substantial difference between both types of cells, that surely deserves further investigation. Correspondingly, it is described that at nanomolar concentrations, as the ones observed in neurospheres, S100B exerts neurotrophic properties for normal brain development (Rothermundt et al., 2003). The increase of S100B during the astrocyte differentiation process may be related to the fact that S100B expression also characterizes a terminal maturation stage of cortical astrocytes, since astrocytes do express S100B in the mature nervous system (Brozzi et al., 2009). Contrastingly, at micromolar concentrations, similar to the ones found

in our glioma cells, S100B could be participate in the pathophysiology of some brain diseases, including brain cancer. These concentrations, revealed to increase cellular proliferation (Leclerc et al., 2007) and Brozzi et al. (Brozzi et al., 2009) suggested that S100B may contribute to reduce the differentiation potential of cells of the astrocytic lineage, beyond the contribution to enhance migration capability, suggests that this protein might contribute to maintaining a neoplastic, invasive phenotype. In fact, Vos *et al* related high levels of S100B with shorter survival in a relatively high proportion of patients with GBM (Vos et al., 2004).

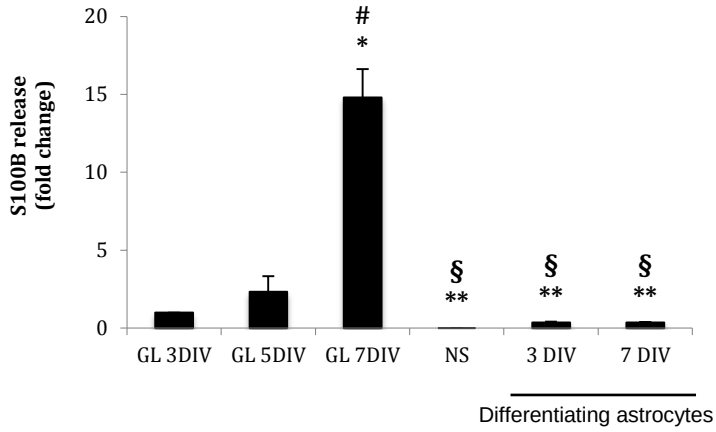


Fig. IV.3. S100B release from GL261 glioma cells at 3, 5 and 7 days *in vitro* and in differentiating astrocytes from neurospheres (NS) during 3 and 7 DIV. Cells were cultured as indicated in methods. The conditioned media was collected, and S100B released into the medium was determined by ELISA, with monoclonal antibody anti-S100B. Quantitative analysis of S100B release was expressed as fold increase *vs*. GL261 3DIV (considered as 1); **p<0.01 and **p<0.05 *vs.* the GL261 3DIV; $^{\#}$p<0.05 *vs.* GL261 5DIV; §p<0.05 *vs.* GL261 7DIV.

Overall, when comparing these developmental phenotypes to glioma cells, the most close to GL261 3DIV are the differentiating astrocytes, regardless the concentration values be significantly different, as it was also observed for MMPs.

2.2. Angiogenesis

Glioblastoma is characterized by its capacity to induce neovascularization, driving continued tumor growth, due to its high content in VEGF and autocrine signaling (Lee et al., 2011). As previously referred, angiogenesis might be triggered and enhanced by the release of VEGF, which is a protein regulated by hypoxia through HIF-1. In Fig. IV.4, a decrease in VEGF expression during the time in culture was noticed in both glioma cells and astrocytes differentiated from neurospheres. In the first case, there was a 50% reduction from GL261 at 3 DIV to 5 DIV (p<0.01). Similar expression was also observed in neurospheres and differentiating astrocytes at 3

DIV, followed by a decrease at 7 DIV (i.e. a change from 0.6- to 0.3-fold, p<0.01). However, it should be emphasized that these results correspond to two experiments (n=2) and thus, further data should be acquired in order to corroborate these observations, also using GL261 cells with 7 DIV.

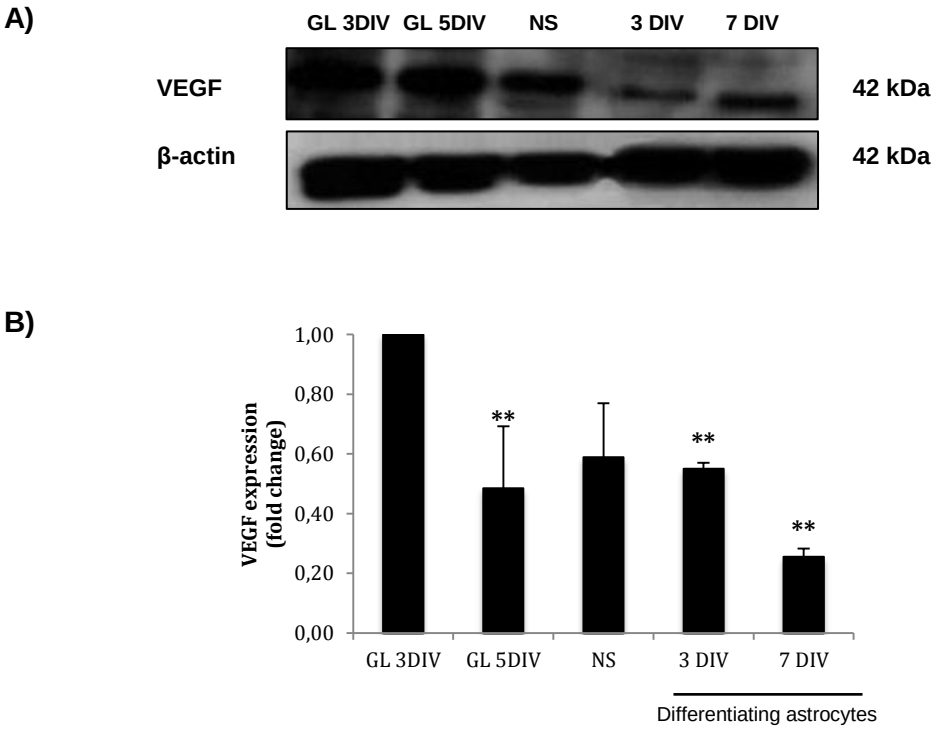


Fig. IV.4. VEGF expression in GL261 glioma cells at 3, 5 and 7 days *in vitro* and in differentiating astrocytes from neurospheres (NS) during 3 and 7 DIV. Cells were cultured as indicated in methods. Total cell lysates were subjected to SDS-PAGE followed by Western blotting with antibody specific for VEGF. A) Representative results from one experiment are shown. B) Graph bars represent the intensity of the bands, which was quantified by scanning densitometry, standardized with respect to β-actin protein and expressed as mean ± SEM fold change compared to glioma cells. The values indicate the fold change obtained when compared with GL261 at 3DIV (considered as 1). **p<0.01 *vs*. GL261 3DIV.

Interestingly, it was recently observed by immunohistochemistry that the percentages of tumors expressing VEGF (96%) and MMP-9 (75%) are in the glioma high-grade group, exhibiting higher levels than in the low-grade group (67% and 24%, respectively) and correlated to the invasion of glioma (Liu et al., 2011). Our results evidence the higher malignancy of GL261 at 3DIV with equivalent increased values of MMP-9 (see Fig. IV.2). Moreover, it deserves to be noted that both neurospheres and differentiating astrocytes at 3 DIV still contain elevated levels of VEGF and similar to those presented by GL261 cells at 5 DIV, thus evidencing a close affinity. Elevated expression of VEGF in NSCs has been documented and it has been unveiled an intrinsic relationship between angiogenesis and NSC, where

up-regulation of VEGF lead to the increase of Nestin positive cells (Mani et al 2005, Sun et al 210). Moreover, inhibition of the VEGF signaling was shown to reduce the migration and to induce differentiation (Kaus et al., 2010; Joo et al., 2012).

2.3. Multidrug resistance

The drug resistance of tumors is one of the main causes to treatment fail and to the progress of the disease over the years. There are diverse reasons why tumor cells can resist to chemotherapeutic drugs, and the transporter Mrp1 is one of them. Mrp1 revealed to be significantly up-regulated in cancer stem-like cells (Jin et al., 2008) and in CD133+ human brain glioma stem cells (Bi et al., 2007), besides being more expressed in the high grade glioma (Calatozzolo et al., 2012), reason why it has been suggested that chemosensitization of cells with Mrp1 inhibitors may favor the treatment of gliomas (Peignan et al., 2011).

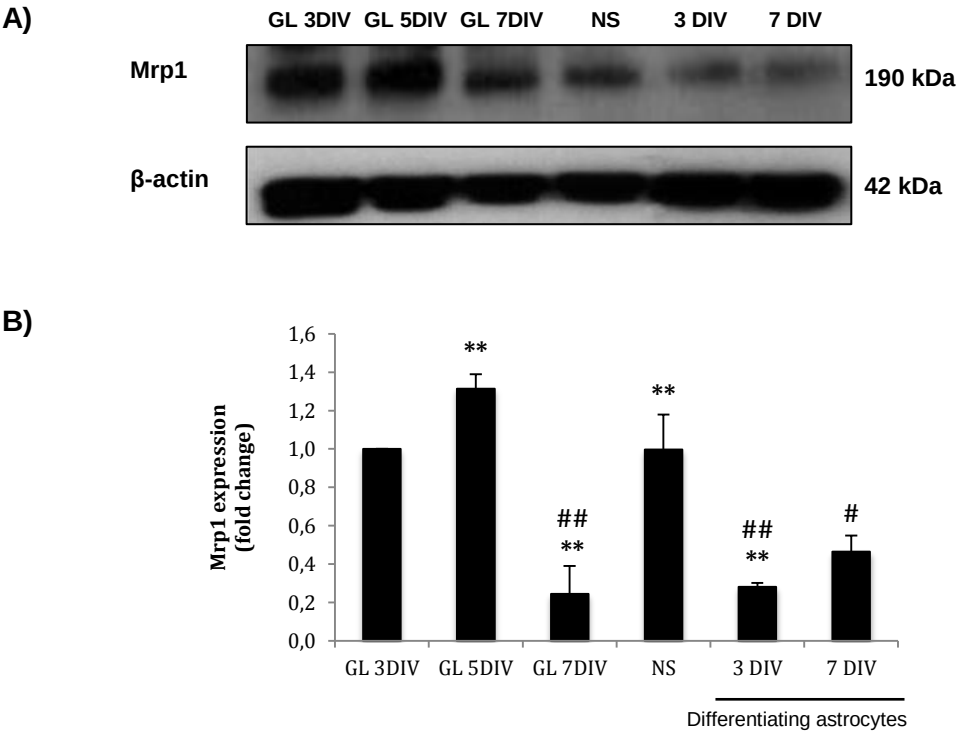


Fig. IV.5. Mrp1 expression in GL261 glioma cells at 3, 5 and 7 days *in vitro* and in differentiating astrocytes from neurospheres (NS) during 3 and 7 DIV.). Cells were cultured as indicated in methods. Total cell lysates were subjected to SDS-PAGE followed by Western blotting with antibody specific for Mrp1. A) Representative results from one experiment are shown. B) Graph bars represent the intensity of the bands quantified by scanning densitometry, standardized with respect to β-actin protein and expressed as mean ± SEM fold change compared to glioma cells, from at least 3 experiments. The values indicate the fold change obtained when compared with GL261 at 3DIV (taken as 1). **p<0.01 *vs*. GL261 3DIV; $^{\#\#}$p<0.01 and $^{\#}$p<0.05 *vs.* GL261 5DIV.

The results of the analysis of Mrp1 expression depicted in Fig. IV.5. show that despite the existence of a 1.3-fold increase from day 3 to day 5 of glioma cells ($p<0.01$), there is an unexpected marked decrease from 5 to 7 DIV (0.2-fold *vs*. GL261 3DIV, $p<0.01$). Moreover, results obtained in neurospheres were those presenting a higher similarity to GL261 cells at both 3 and 5 DIV. However, we can also consider that a matched correspondence still exist between GL261 at 7 DIV and astrocytes in differentiation from NS, although with significant lower levels of Mrp1 (decline to 0.3- and 0.5-fold) that the first group above mentioned.

Overall, it seems that the increased values of Mrp1 are associated with greater cell proliferation ability, as other ongoing studies also suggest. Further studies on Mrp1 should include the evaluation of Mrp1 activity (Lee et al., 2012), in order to investigate if an increased expression of this protein is related with a higher ability to make the efflux of some drugs, and thus, to an increased chemoresistance. As this multidrug resistance phenomenon is considered to be the major barrier to patient survival, a chemotherapeutic scheme that include Mrp1 modulators, may be imperative to overcome the conventional drug resistance in patients with relapsed GBM.

2.4. Autophagy

Autophagy is a process that promotes sequester and degradation of bulk cytosolic proteins and damaged organelles by the lysosome. It has been shown that some drugs used in chemotherapy may activate autophagy instead of apoptosis in malignant glioma cells (Kanzawa et al., 2004). Therefore, the expression of LC3, one of the autophagosome-membrane proteins, was analyzed. Autophagic activity can be analyzed by Western blot through by the ratio of lapidated LC3-II that studs the inner and outer autophagosoma membrane to unmodified LC3-I.

The results depicted in Fig. IV.6 show that LC3 is constitutively expressed in all cell types, GL261, NS and differentiating astrocytes. Due to limitation of time and some identification band problems only data from one experiment are shown.

Nevertheless, there is indication that LC3 lipidation increase along differentiation of astrocytes from neurospheres, which showed the lowest levels. Autophagic activity in differentiating astrocytes evidenced a 1.3-fold increase when compared to GL261 5DIV. This is not without precedent once the autophagic activity in glioma stem/progenitor cells was shown to be significantly lower than that in neural stem/progenitor cells. However, the autophagic activity markedly increased if glioma stem/progenitor cells are induced to differentiate by fetal calf serum (Zhao et al., 2010).

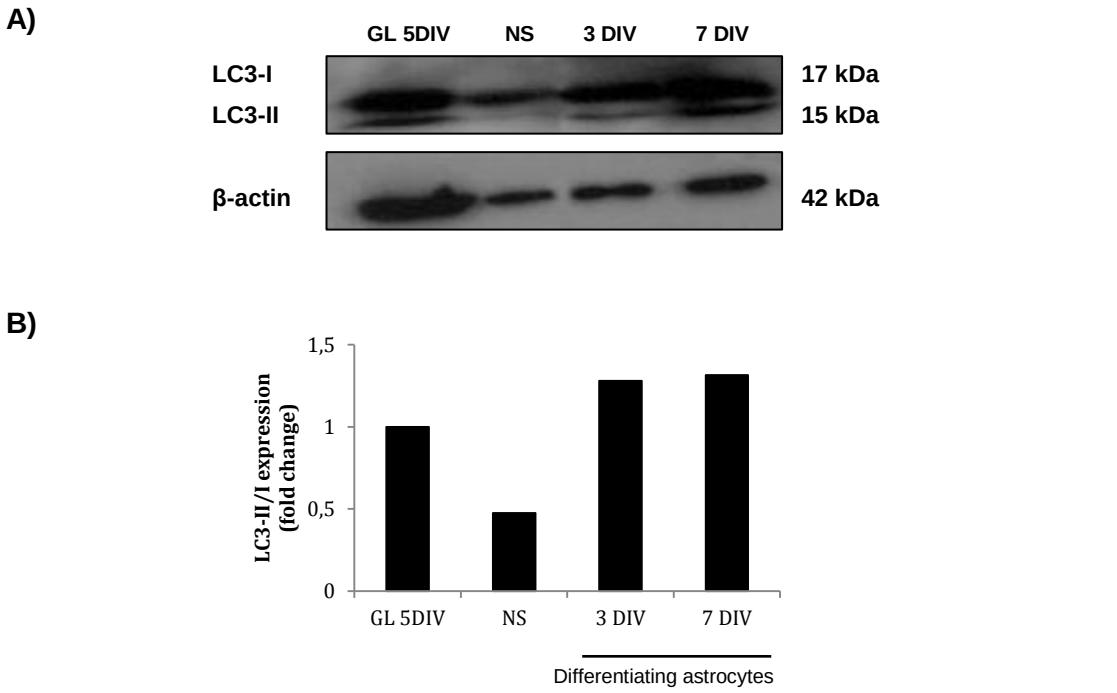


Fig. IV.6. LC3II/I expression in glioma cells at 5 days *in vitro* and in differentiating astrocytes from neurospheres (NS) during 3 and 7 DIV. Cells were cultured as indicated in methods. Total cell lysates were analyzed by Western blotting with antibody specific for LC3 and then quantified and treated as described before. A) Representative results from one experiment are shown. B) Graph bars represent the values indicating the density proportion of each protein compared with GL261 5DIV (taken as 1). Results are from one experiment.

Nevertheless further data should be obtained, as these results were acquired from one single experiment, and also it should be used the missing GL261 at 3 and 7 DIV, in order to look for the effect of the time in culture on this cell property, based on the findings above indicated. Enhanced LC3-II/LC3-I expression in differentiating astrocytes from neurospheres is in agreement with other studies suggesting that neural stem/progenitor cells activate autophagy to fulfill their high energy demands (Vazquez et al., 2012). Autophagy was similarly indicated to play an essential role in the regulation of self-renewal, differentiation, tumorigenic potential and radiosensitization of glioma-initiating cells. Moreover, it is suggested that induction of autophagy promotes the differentiation of these cells and their susceptibily to radiotherapy (Zhuang et al., 2011; Palumbo et al., 2012; Teres et al., 2012). However, it deserves to be noted that $CD133^+$ glioblastoma cells, considered as a small fraction of cells with features of primitive neural progenitor cells and tumor-initiating function in brain tumors, show defective autophagy, which probably relates

with their resistance to TMZ (Fu et al., 2009). Thus, we may considerer that this important glioma subpopulation should be a target to combined therapy using TMZ and inducers of this signaling pathway, in order to improve the survival of patients with glioma. Nevertheless, caution should also be undertaken as other studies have revealed that the inhibition of autophagy favors TMZ-induced apoptosis in glioma cells, and that agents targeting mitochondria or endoplasmic reticulum may be potential anticancer strategies (Lin et al., 2012). This is a very controversial subject deserving clarification.

3. Effects of a combined anticancer strategy on GL261 cell viability and cell cycle

Despite the new insights into the molecular pathogenesis of glioblastoma, several aspects are not full elucidated and treatments fail to cure the majority of patients. With standard therapy, which consists of surgical resection with concomitant TMZ in addition to radiotherapy followed by adjuvant TMZ, the median duration of survival is 12-14 months. Therefore, therapeutic schemes clearly deserve to be improved, and novel molecular targets and inhibitory agents has become a focus of research for glioblastoma treatment. Thus, the second main objective of this thesis was to find a successful adjuvant molecule for TMZ therapy that would enhance TMZ therapeutics potential. For that we started to evaluate the effect of TMZ in GL261 cell line, at the cell viability and cell cycle levels, followed by the analysis of conjoint association effects of some other molecules, such as GUDCA and an Mrp1 inhibitor, the MK-571.

3.1. Effect of TMZ on glioma cells viability

GL261 glioma cells were treated (or untreated, control) with TMZ (50, 100 and 250 µM) during 24, 48 and 72 h. After incubation, cell viability was evaluated by the MTS assay, in order to determine the most efficient TMZ incubation conditions to be subsequently used in the following studies. We have observed that TMZ treatment induced a significant decrease in glioma cells viability, which occurred in a dose- and time-dependent manner (Fig. IV.7.). Thus, the effect of TMZ was particularly evident at the highest TMZ concentration, with an incubation period of 72 h. At these conditions, TMZ 250 µM was able to induce a 40% decrease on cell viability ($p<0.05$) as compared to the respective control cells.

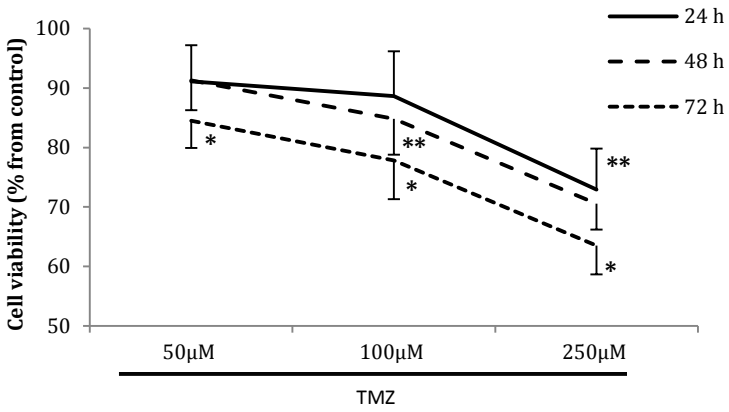


Fig. IV.7. Effect of temozolomide (TMZ) addition in glioma cells viability. Glioma cells were incubated with crescent concentrations of TMZ (50, 100 e 250 µM) during three different incubation periods (24, 48 and 72 h). Cell proliferation was determined by evaluating [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) reduction in the presence of phenazine methosulfate (PMS), and absorbance of the medium was then red at 490 nm. Data are expressed as percentage ± SEM of the value obtained for the control at each incubation period. **p<0.01 and *p<0.05 *vs.* control.

Based on the results achieved we selected to the following assays a TMZ concentration of 250 µM, and the lowest and the highest incubation periods (24 and 72 h), to easily compare the effects produced by the combined therapy.

3.2. Effect of TMZ, GUDCA and TMZ+GUDCA on glioma cells viability and cell cycle

It was recently demonstrated that TMZ-treated glioma cells undergo ROS/ERK-mediated apoptosis and autophagy, during which autophagy serves to protect glioma cells from TMZ-induced apoptosis. Furthermore, resveratrol, an antioxidant molecule, augments the effect of TMZ by reducing autophagy and increasing apoptosis both *in vitro* and *in vivo* (Lin et al., 2012). Overall, those studies suggested that the administration of resveratrol combined with TMZ could be a potential treatment strategy for patients with brain tumors. Therefore, we sought that was also very interesting to explore if the antioxidant GUDCA, a molecule with anti-proliferative, anti-apoptotic and anti-inflammatory properties (Akare et al., 2006), which has already been shown to prevent colon tumorigenesis (Wali et al., 2002), could also be a successful adjuvant molecule for TMZ therapy.

After the first trial for the conditions to be used, GL261 were then incubated with TMZ alone (250 µM) or in the presence of GUDCA (50 µM) during 24 and 72h. As observed in Fig. IV.8, treatment with GUDCA alone caused no changes in cell viability at both time points, as it was expected. Also anticipated was the significant decrease induced by TMZ, a little more pronounced when time of incubation changed from 24 (19%, $p<0.01$) to 72 h (31%, $p<0.01$). A parallel study accomplished with the U-118 MG grade III human glioma cell line obtained a reduction of 43% in the cell proliferation at 72 h and with 250 µM TMZ (Carmo et al., 2011). However, and this is relevant, the incubation of TMZ in the presence of GUDCA largely enhanced the effect of TMZ alone, further reducing the cell in 31% at 24 h and in 17% at 72h ($p<0.05$).

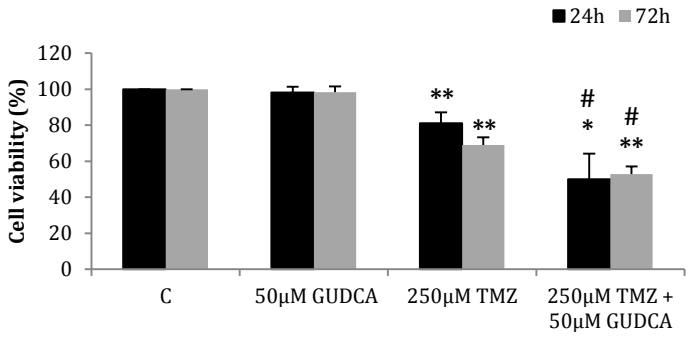


Fig. IV.8. Cell viability of GL261 cells in the absence (control) or in the presence of temozolomide (TMZ), glycoursodeoxycholic acid (GUDCA), and TMZ+GUDCA. Glioma cells were treated with 250 µM TMZ or 50 µM GUDCA alone or in combination during 24 h and 72 h. To assess cellular viability and functionality, we used the MTS reduction assay. Data are expressed as percentage ± SEM of the value obtained for the control at each incubation period. **p<0.01, *p<0.05 *vs.* control; #p<0.05 *vs.* TMZ.

The chemoresistance of GL261 after 72 h of TMZ treatment with more than 60% cells alive, may result from the subpopulation of cells that are known to be responsible for propagation of glioblastoma growth after chemotherapy (Chen et al., 2012). Increased sensitization by combined therapy as here observed with GUDCA was already indicated for other compounds, such as a blocker of base excision repair (Montaldi and Sakamoto-Hojo, 2012), inhibitors of histone deacetylases (Ryu et al., 2012), antiangiogenic (Den et al., 2012), autophagic (Lin et al., 2012) and non-apoptotic inducers (Overmeyer et al., 2011), inhibitors of Mrp1 activity (Peignan et al., 2011), among many other proposals. We may then assume that dual (or even triple) targeting directed delivery systems are promising strategies against glioma.

Given the beneficial effects evidenced by the combined treatment TMZ+GUDCA, we thought to next assess the effect of these treatments on the cell cycle and to

determine whether the reduced cell viability was related to an increase in apoptosis. The cell cycle analysis was performed by flow cytometry and in this technique the cells to be analyzed must be very well dissociated, avoiding the formation of cell aggregates. However, our cells formed these cellular bulks very easily, hindering the readings and leading to the absence of results, which happened very frequently, particularly concerning the untreated (control) cells. Therefore, in the absence of some control values, it was very difficult to ascertain the role of TMZ in the cell cycle of GL261 cells, which was never described. However, this effect was already studied by other and it was described that different cell lines possess distinct TMZ effects on cell cycle (Zhang et al., 2010), depending on their resistance this drug. In fact, while Hirose *et al* indicated that TMZ led to cell cycle arrest in G2, whit an accumulation of cells at this phase, Carmo *et al* reported that TMZ did not induced cell cycle arrest nor in G1 or G2 (Carmo et al., 2011).

Due to the above described method limitations, and in the absence of trusting control values, we decide to present our results in a way that we can ascertain about the cell cycle effect of TMZ+GUDCA co-incubation (Table IV.1.). As in cell viability, the more significant results were obtained at the 72h incubation period. At this timepoint, we have observed that TMZ plus GUDCA has induced cell cycle arrest at the G2 phase, since there is an accumulation of cells at this check point (2.7-fold increase *vs.* TMZ alone, $p<0.01$). Consequently, the number of cells at the S phase have further decreased has compared to TMZ (0.6-fold, $p<0.05$). Interestingly, similar results were obtained by Yuan *et al*, that found that the combination of resveratrol and TMZ significantly resulted in G2/M cell cycle arrest in a model of GBM (Yuan et al., 2012). Since GUDCA is also an antioxidant molecule, we may speculate that it may share some of the already mechanisms by which resveratrol increase TMZ efficacy.

Nonetheless, cell cycle of GL261 cells after treatments with either GUDCA or TMZ alone or TMZ in the presence of GUDCA were characterized by a low apoptotic fraction (data not shown), which prove that the reduction of cell viability is not related with apoptosis. Due to the described limitations of the used cell cycle analysis method, further assays should be performed using other procedures to analyse the cell cycle, such as the expression of some cell cycle related proteins.

Table IV.1. Cell cycle analysis of GL261 cells incubated in the presence of temozolomide (TMZ), glycoursodeoxycholic acid (GUDCA) and TMZ+GUDCA, during 24 and 72.

Incubation conditions		Cell cycle phase (fold change from TMZ)		
		G0/G1	S	G2/M
24h	TMZ	1.00±0.37	1.00±0.01	1.00±0.01
	GUDCA	0.97±0.02	0.89±0.01**	0.61±0.05**
	TMZ+GUDCA	0.79±0.01	0.98±0.001	1.09±0.02
72h	TMZ	1.00±0.18	1.00±0.13	1.00±0.49
	GUDCA	1.46±0.10	0.74±0.05*	0.66±0.15
	TMZ+GUDCA	1.03±0.08	0.63±0.07*	2.66±0.44*

Glioma cells were treated with TMZ (250 μM) alone or in the presence of GUDCA (50 μM) during 24 and 72 h. Results are expressed as mean fold change from TMZ±SD). **p<0.01 and *p<0.05 *vs.* TMZ.

Overall, we can postulate that GUDCA can be useful as an adjuvant molecule for TMZ, particularly when used in therapeutic schemes with longer administration periods.

3.3. Effect of TMZ, MK-571 and TMZ+MK571 on glioma cells viability and cell cycle

Mrp1 transporters have been associated to drug efflux and resistance to chemotherapy in high-grade gliomas, showing an average expression of 51.3% in glioma specimens. Moreover, as no changes were detected between primary or recurrent gliomas, it is suggest that chemoresistance is mostly intrinsic (Calatozzolo et al., 2005), therefore, strategies to decrease the expression of the MRP gene have been enlightened (Matsumoto et al., 2004). In line with this, the inhibitor indomethacin has shown to significantly increase the cytotoxic effect of etoposide, and even more that of vincristine (Benyahia et al., 2004). MK-571 is a specific Mrp1 inhibitor, which may improve TMZ treatment, as it did for vincristine and etoposide (Peignan et al., 2011). However, these authors did not observe a beneficial effect by the combination of MK571 and TMZ when working with T98G and G44 GBM cell lines and suggest that TMZ may not be a substrate for Mrp1. However they only used TMZ at 100 μM and MK-571 (20 μM) for 24 h. As we showed in Fig. IV.7, this concentration of TMZ only slightly decreased the cell viability of our GL261 cell line. Therefore, we decided to test the combined effects of TMZ+MK-571 in our model. GL261 cells were treated (or untreated) with 250 μM TMZ alone or TMZ in the presence of MK-571 (25 μM) during 24 and 72 h, as pre-established. Once again, like GUDCA, MK-571 appears to be innocuous for the cells (Fig. IV.9), but caused a

significant reduction in cell viability when associated to TMZ. Thus, the effect caused by TMZ treatment in the inhibition of MTS reduction by GL261 cells, already previously noticed, was here potentiated in a dose- and time-dependent manner in the presence of MK-571 at 24 h (~8% more *vs*. TMZ alone, p<0.05) increasing at 72h (~14% more, *vs*. TMZ alone, p<0.05).

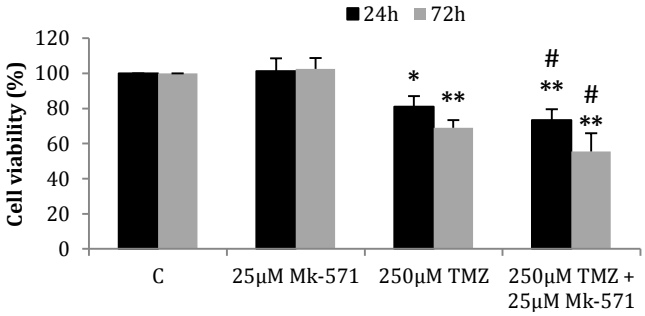


Fig. IV.9 Cell viability of GL261 cells in the absence (control) or in the presence of temozolomide (TMZ), Mrp1 inhibitor (MK-571) and TMZ+MK-571. Glioma cells were treated with 250 µM TMZ or 25 µM MK-571 alone, or in combination during 24 and 72 h. To assess cellular viability and functionality, we used the MTS reduction assay. Data are expressed as percentage ± SEM of the value obtained for the control at each incubation period. . **p<0.015 and *p<0.05 *vs.* control; $^{\#}$p<0.05 *vs.* TMZ alone.

Once more, after cell viability assay, we have studied the effect of MK-571 and TMZ co-incubation in the cell cycle, using the same approach used for GUDCA studies, regarding the presentation of the results (Table IV.2). Similarly to GUDCA, higher and more significant variations were obtained at the 72h incubation period. We have observed that TMZ and GUDCA co-incubation provoked cell cycle arrest at the G2/M phase, which is observed by the accumulation of cells (2.8-fold increase *vs.* TMZ alone, p<0.01). Consequently, the number of cells at the S phase have further decreased has compared to TMZ (0.6-fold, p<0.01). Once more, this additive effect of MK-571 is not related with an increase in apoptotic cells (data not shown).

Overall, these results suggest that inhibition of Mrp1 transporter may enhance TMZ therapy, its efficacy, particularly when used in therapeutic schemes with longer administration periods, as postulated for GUDCA.

Gliomas comprise of significant cell heterogeneity that contains a number of cancer stem-like cells that may contribute to the resistance to treatment. These cells are phenotypically similar to the normal stem cells of the corresponding tissue of origin, but they exhibit dysfunctional patterns of self-renewal and differentiation (Jin et al., 2010) and have more growing ability during chemotherapy than that of glioblastoma cells.

Table IV.2. Cell cycle analysis of GL261 cells incubated in the presence of temozolomide (TMZ), MK-571 and TMZ+GUDCA, during 24 and 72.

Incubation conditions		Cell cycle phase (fold change from TMZ)		
		G0/G1	**S**	**G2/M**
24h	**TMZ**	1.00±0.37	1.00±0.01	1.00±0.01
	MK-571	0.97±0.02	0.85±0.02**	0.79±0.01**
	TMZ+MK-571	0.75±0.02	1.00±0.09	1.14±0.24
72h	**TMZ**	1.00±0.18	1.00±0.13	1.00±0.49
	MK-571	1.60±0.07**	0.63±0.03**	0.70±0.27
	TMZ+MK-571	1.04±0.09	0.62±0.11**	2.81±0.36**

Glioma cells were treated with TMZ (250 μM) alone or in the presence of MK-571 (25 μM) during 24 and 72 h. Results are expressed asmean fold change from TMZ±SD). **p<0.01 and *p<0.05 *vs.* TMZ.

As it was already demonstrated that these glioblastoma stem-like cells have a higher Mrp1 expression than Mrp1 (Jin et al., 2010), we can speculate that the inhibition of this ABC transporter may be an important tool to decrease this chemoresistance phenomenon, which will open important avenues regarding glioma therapy.

However, it should be interesting to further investigate the role of Mrp1 in the GL261 cell line. This way, further assays should include the study of the Mrp1 activity in glioma cells and the respective effect of TMZ and TMZ plus MK-571, to ascertain about the level of Mrp1 inhibition activity achieved by MK-571. Further studies on Mrp1 modulation can also include Mrp1 silencing in glioma cells.

4. Effect of GUDCA and MK-571 in tumor cell migration

Briefly, the results obtained in the previous assays showed that either GUDCA or the Mrp1 inhibitor, MK-571, in addition to TMZ, enhanced the efficacy of TMZ treatment alone. Taking this in account, we considered that it would be important to elucidate which mechanism is underlying the potentiation of TMZ therapy by these compounds. For that, we proceed to analyze the expression of CXCR4, known to promote motility and proliferation of glioma cells (Carmo et al., 2010), in an attempt to clarify if it modulates cell migration. For this, GL261 cells with 3, 5 or 7 DIV were treated with either GUDCA or MK-571, during 24 and 72 h, using the same concentrations of previous assays (Fig. IV.10).

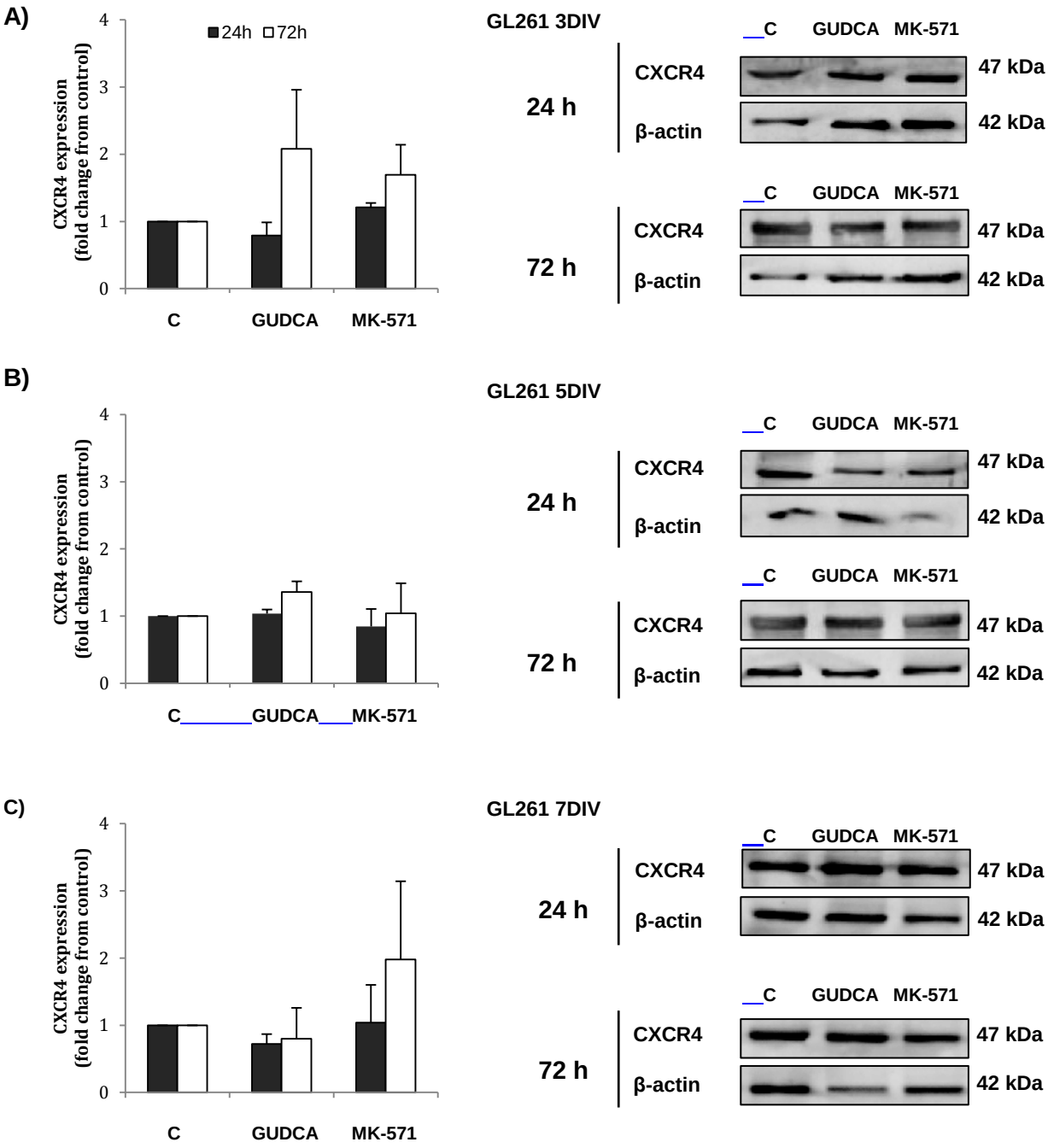


Fig. IV.10. CXCR4 expression in glioma cells at 3, 5 and 7 days *in vitro* in the absence (control) or in the presence of either glycoursodeoxycholic acid (GUDCA) ot the Mrp1 inhibitor MK-571. Glioma cells were not-treated or treated with 50 μM GUDCA or 25 μM MK-571 during 24 and 72 h. Total cell lysates were analyzed by Western blotting with an antibody specific for CXCR4. Results of analysis of 3, 5 and 7 DIV (A, B and C, respectively) are shown by representative results from one experiment.and graph bars representing the intensity of the bands quantified by scanning densitometry, standardized with respect to β-actin protein and expressed as mean ± SEM fold change from respective control (accepted as 1).

We have observed that CXCR4 was constitutively expressed in GL261 cells, as demonstrated in other cell glioma lines (Carmo et al., 2010). Both compounds, GUDCA and MK-571 seem to induce the expression of CXCR4, mainly after 72 h incubation. While GUDCA evidence a preferential induction on glioma cells at 3 DIV, decreasing thereafter (from 2.1-fold to 0.8-fold *vs.* control), MK-571 suggests more efficacy on GL261 at 7 DIV. Although only two experiments were performed, both GUDCA and MK-571 revealed to have no effect if used for 24 h, but indeed induced (despite not significantly) an increase in the expression of CXCR4. Therefore, none of the compounds tested showed to be CXCR4 antagonists, what is usually looked for glioma therapy (Terasaki et al., 2011; Fareh et al., 2012; Yu et al., 2012). However, due to other beneficial effects, they can be given for periods that do not surpass the 24 h. Anyway, since only 2 assays were performed we still should confirm the results acquired and determine whether the chemokine to this receptor, the CXCL12 (also stroma-derived factor 1. SDF-1) is actually released. This feature is relevant once it was demonstrated an alternative receptor, the CXCR7 with a 10 times higher affinity for SDF-1 (Balabanian et al., 2005) and also controls cell proliferation and migration (Odemis et al., 2012).

Moreover, there are studies showing that CXCL12 alone cannot induce glioma formation, and that CXCR4 inhibition does not attenuate gliomagenesis in a mouse model of Neurofibromatosis-1 (NF1)-associated optic pathway glioma (OPG) (Sun et al., 2010). Re-evaluation of the roles of GUDCA and MK-571 on CXCR7 and CXCR4 will be then important as targets to be modulated by therapy to glioma. Additionally it will be interesting to evaluate the effects of both compounds on MMPs activity once we saw that they were increasingly expressed at 3 DIV by GL261 cells (Fig. IV.2) and its action is mediated through the SDF-1/CXCR4 axis (Bhoopathi et al., 2011).

CHAPTER IV – Concluding remarks

Chapter VI - Concluding remarks

The study of tumorigenesis and the evaluation of new therapies for glioma require accurate brain tumor models. Cultures of malignant cells represent an excellent and permanent material for studying the biology of these tumors as, for example, specific antigens characterization, bioactive factors produced, determination of cellular proliferation, as well as heterogeneity of genotypic and phenotypic characteristics (Machado et al., 2005). Our GL261 glioma cell line characterization demonstrated that the cells express both undifferentiated (as Vimentin, Nestin, Sox2, as well βIII-Tubulin) and differentiated proteins (as GFAP, GLAST and MAP2), which levels change during the time in culture (Fig. V.1). This is in agreement with the cellular heterogeneity found in other glioma cell lines containing cells at different stages of differentiation (Shiras et al., 2003; Gangemi et al., 2009; Zhang et al., 2011) and to what was observed in human glioma (Bonavia et al., 2011). Therefore, this cell line has shown suitable characteristics for cellular and molecular studies, as well as for new trearment strategies for glioma, reason why it was used in the present study.

In this study, we have evaluated the GL261 cells characteristics throughout time in culture, which is unusual to find in the literature, but it might be fundamental to better understand in which way cell markers and certain essential features progress along tumor cell differentiation. In fact, we hypothesize that a good cell characterization will allow the correlation of the grade of maturation of the cells with the grade of malignancy. This information will be very useful for a more directed therapeutic targeting, especially if we want to use a cell model that can mimic the stages with highest resistance.

Regarding the evaluation of tumor-related factors either in glioma cells or in NS and respective differentiating astrocytes, though not as evident as it was expected, the results suggest that the initial stage, the proliferating NS, is the phenotype that presents more similarities with tumor cells concerning the majority of the evaluated tumor factors (Table.V.1). Thus, this may indicate that gliomagenesis could be related with malignant transformation of NSC, as suggested by many authors (Shiras et al., 2003; Gangemi et al., 2009), which is not surprising due to the similarities between both populations. However, based on some contradictory results along the maturation process of glioma cells, future research on this tumorigenic process is needed in order to confirm such hypothesis. Thus, our initial thoughts were to induce a glioma phenotype in both NSC and differentiating astrocytes to look which produced phenotype would be closer and acquire the same tumorigenic properties of glioma cells. This cell transformation could be performed by silencing both for the RNA of the lipid phosphatase PTEN and the tumor suppressor factor p53 by using

the respective siRNA, as it is described that this double inactivation cooperates to induce high-grade malignant gliomas (Zheng et al., 2008).

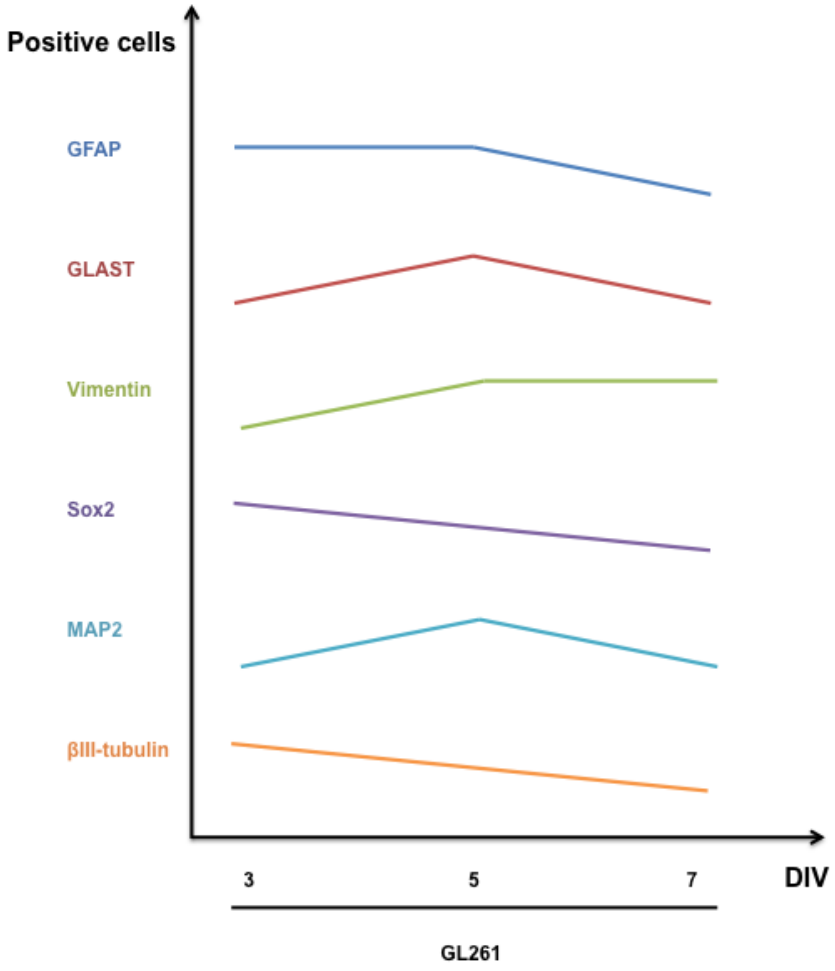


Fig. V.1. Summary of GL261 cell line characterization. Schematic representation of the cellular markers expression along time in culture (3, 5 and 7 *days in vitro* – DIV). Throughout time, there was a variable expression of these markers. Sox2 and βIII-tubulin expression decreased from 3 to 7 DIV, while vimentin increased till 5 DIV and then remained constant. GFAP (glial fibrillary acidic protein) suffered a reduction from 5 to 7 DIV, while GLAST (glutamate aspartate transporter) and MAP2 (microtubule-associated protein 2) revealed a peak expression level at 5 DIV.

Treatment of glioma cells with TMZ in the presence of GUDCA or MK-571 greatly enhanced the effect of TMZ alone, causing a further loss of cell viability, specially at 72 h. In addition, at the same conditions, it was observed an accumulation of cells at G2/M phase, corresponding to a cell cycle arrest at this checkpoint. Moreover, both co-incubation schemes showed low apoptotic levels, which indicates that the decrease of cell viability is not correlated with this type of cell death. Overall, our results suggest that GUDCA and MK-571 can act as adjuvants of TMZ therapy, particularly when used in therapeutic schemes with longer administration periods.

Intriguingly, either GUDCA or MK-571 seems to improve the migratory ability of GL261, by the induction of increase of CXCR4 levels, although with distinct patterns.

Table. V.1. Characterization of common features between GL261 glioma cells and neurospheres induced to differentiate into astrocytes during 3 and 7 days *in vitro*.

	GL261				Differentiating astrocytes	
	3DIV	5DIV	7DIV	NS	3DIV	7DIV
MMPs	++	+	+	+	+++	++
S100B	++	++	+++		+	+
VEGF	+++	++		++	++	+
Mrp1	++	+++	++	++	+	+
LC3		++		+	++	++

Similar to NS Similar to 3DIV differentiating astrocytes Similar to 7DIV differentiating astrocytes

The expression of the tumor-related factors is represented from the lowest (+) to the highest expression (+++). The green, red and blue squares represent, for each evaluated factor, the phenotype more similar to neurospheres (NS) or to 3 and 7 DIV differentiating astrocytes. The analysis of tumor-related factors showed that during GL261 maturation, there is a decrease on the expression of the vascular endothelial growth factor (VEGF) as well as on the activity of the matrix metalloproteinases MMP-9 and MMP-2, which is associated with an increase on S100B release. Also, the Mrp1 presents a peak of expression at 5 DIV. Overall, but not as evident as we expected. NS is the phenotype that present the highest similarities with GL261 cells. LC3, light chain 3, LC3II/LC3I ratio; Mrp1, multidrug resistance-associated protein 1; MMPs, matrix metalloproteinases; VEGF, vascular endothelial growth factor.

Due to these contradictory findings, the therapeutic potential of GUDCA and Mrp1 modulation should be further investigated by using *in vivo* studies. Thus, in a near future we propose to use these molecules in association with TMZ in an *in vivo* model of glioma, where tumors will be generated by intracerebral implantation of the GL261 glioma cells in rats (Doblas et al., 2010). We anticipate that these therapeutic schemes will inhibit tumor cell growth and prevent vascular alterations in early stages of glioma progression.

Interestingly, the factors with highest impact face to non-glioma cells were S100B, VEGF and Mrp1, in agreement with their fundamental role in glioma development. Taking this in account, these factors may be potential therapeutic targets and, subsequently, it would be interesting to further evaluate the synergistic effect of TMZ and GUDCA or MK-571 on these tumor related features.

The overall goal is to translate these results into the clinical practice, which will reduce the resistance to the commonly used chemotherapeutic drugs, increasing the survival of brain tumor patients.

Chapter VI - References

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