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Response of Glioblastoma Cells to Doxorubicin and Methotrexate: Role of Methamphetamine

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Ana Paula Silva (Universidade de Coimbra), co-orientação da Doutora Célia Gomes (Universidade de Coimbra) e supervisão da FCTUC pela Professora Doutora Emília Duarte (Universidade de Coimbra).

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Abbreviations

ABC	ATP binding cassette
ADHD	Attention-deficit hyperactivity disorder
AIC	5-aminoimidazole-4-carboxamide
AJs	Adherens junctions
AQP	Aquaporins
ATS	Amphetamine-type stimulants
BBB	Blood-brain barrier
BCNU	Carmustine (1-3-bis(2-chloroethyl)-1-nitrosourea)
Bcl-2	B-cell lymphoma protein 2
Bax	BCL2 associated X protein
CDK	Cyclin dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A gene
$\mathbf{C_{max}}$	Maximum concentration that a drug achieves
CNS	Central Nervous System
CSF	Cerebrospinal fluid
CXCL10	Interferon gamma-induced protein 10
CXCL11	Interferon-inducible T-cell alpha
CXCL12/SDF-1	Stromal cell-derived factor-1
CXCL9	Gamma interferon
CXCR3	G protein-coupled receptor 9
CXCR4	C-X-C Chemokine receptor type 4
DA	Dopamine
DAT	Dopamine transporter
dTMP	Deoxythymidine monophosphate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DOX	Doxorubicine
ECATD	Estudo sobre o consumo de álcool, tabaco e drogas
ECF	Enhanced chemifluorescence
ECM	Extracellular matrix

EGFR	Epidermal growth factor receptor
eNOS	Endothelial cell nitric oxide synthase
EORTC	European Organization for Research and Treatment of Cancer
ESPAD	European school survey on alcohol and other drugs
ETC	Electron transport chain
FDA	Food and Drug Administration
GLU	Glutamate
IC_{50}	Median Inhibition Concentration
IDT	Instituto da droga e da toxicodependência
Ig E	Immunoglobuline E
IL-6	Interleukin 6
ISF	Interstitial fluid
JAK	Janus kinase
JAMs	Junctional adhesion molecules
JNK	c-Jun NH2-terminal kinase
MAPK	Mitogen-activated protein kinases
MDM2	Murine doble minute 2 gene
Mdr1	Multiple drug resistance 1gene
MeCCNU	Semustine (1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea)
METH	Methamphetamine
MGMT	Methylated-DNA-protein-cysteine methyltransferase
MMP	Metalloproteinase
MMP-2	Matrix metallopeptidase 2
MMP-9	Matrix metallopeptidase 9
MRI	Magnetic resonance imaging
MRP	Multidrug resistance-associated protein gene
MTIC	Methyl-triazeno-imidazole-carboxamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MTX	Methotrexate
NADH	Nicotinamide adenine dinucleotide
NCIC	National Cancer Institute of Canada
NO	Nitric Oxid
NOS	Nitric oxide synthase

O^6-meG	O^6-methylguanine
P-gp	P-glycoprotein
PI	Propidium iodide
PI3K/AKT/mTOR	Phosphatidylinositol 3- kinase/Akt/mammalian target of rapamycin
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog protein
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Nitric oxide synthase
SEM	Standard error of mean
STAT	Signal transducer and activator of transcription protein
Tjs	Tight junctions
TMZ	Temozolomide
TNFα	Tumor necrosis factor α
Topo IIα	Topoisomerase IIα
TP53	Tumor protein 53 gene
VEGF	Vascular endothelial growth factor
VMAT-2	Vesicular monoamine transporter-2
WHO	World Health Organization
ZO-1, 2, 3	Zonulae occludentes -1, 2, 3

Resumo

Os glioblastomas são os tumores cerebrais mais comuns, recorrentes e com uma alta taxa de mortalidade, principalmente devida à ineficácia das terapias usadas que incluem a combinação de cirurgia, radioterapia e quimioterapia. Os resultados insatisfatórios obtidos com o uso da quimioterapia atual, como a carmustina e a temozolomida, são fortemente atribuídos à resistência adquirida pelo tumor mediada principalmente pela glicoproteína-P e pela enzima metil-guanina metil-transferase (MGMT).

A doxorubicina (DOX) mostrou ser eficaz contra alguns tumores periféricos e gliomas malignos em estudos *in vitro*. No entanto, não é possível o seu uso para o tratamento de glioblastomas visto que este fármaco não atravessa a barreira hematoencefálica (BHE) devido à sua baixa lipofilicidade e elevado peso molecular. Também o metotrexato (MTX) é usado desde há muito tempo como um potente fármaco anticancerígeno mas também apresenta dificuldade em atravessar a BHE, sendo necessário uma administração sistémica em doses elevadas e potencialmente tóxicas a nível periférico, para se atingir uma dose efetiva no cérebro. Uma vez que as vias de administração dos fármacos quimioterapêuticos são tecnicamente difíceis e dolorosos para os pacientes, são necessários métodos mais práticos e seguros. Desta forma, e tendo em conta a capacidade que a metanfetamina (MET) possui para aumentar transitoriamente a permeabilidade da BHE, o objetivo deste trabalho foi avaliar se a MET interferia com os efeitos da DOX ou MTX, analisando especificamente a viabilidade celular, migração, quimiotaxia e ciclo celular.

Os resultados deste trabalho demonstraram que a MET na concentração de 1 µM não interfere na viabilidade, migração ou ciclo celular das células de glioblastoma humano, a linha celular U-118. Da mesma forma, esta droga não alterou os efeitos na viabilidade celular, migração, quimiotaxia e no ciclo celular induzidos por DOX ou MTX. Contudo, a MET por si só conseguiu diminuir a quimiotaxia das células de glioblastoma, sendo este efeito semelhante ao que foi induzido pela DOX ou MTX quando administrados individualmente. No que diz respeito ao efeito da DOX, este fármaco levou a uma diminuição da viabilidade celular, migração e quimiotaxia assim como a uma paragem no ciclo celular na fase G_2 e a uma diminuição na expressão da ciclina A. Também o MTX diminuiu a viabilidade, migração e quimiotaxia das células de

glioblastoma, do mesmo modo que afetou o seu ciclo celular levando a uma paragem das células na fase S.

Em suma, podemos concluir que a DOX e o MTX têm um efeito tóxico nas células de glioblastoma e que a METH, a uma concentração que aumenta a permeabilidade das células endoteliais, não interfere com o efeito da DOX ou MTX. Deste modo, consideramos que esta abordagem merece ser alvo de um estudo mais aprofundado de forma a melhor esclarecer o uso da MET como uma abordagem terapêutica em situações de tumores cerebrais.

Palavras chave: Glioblastoma; Metanfetamina; Doxorubicina; Metotrexato; Barreira hematoencefálica.

Abstract

Glioblastomas are the most common primary tumors of the brain with a high recurrence and mortality rate justified by the ineffectiveness of current therapeutics that include combinations of surgery, radiotherapy and chemotherapy. The unsatisfactory results with current chemotherapeutic agents, such as carmustine and temozolomide, have been strongly attributed to tumor cell resistance mainly mediated by P-glycoprotein and the enzyme methylated DNA-protein cysteine methyltransferase (MGMT).

Doxorubicin (DOX) showed to be effective against some peripheral tumors and malignant glioma tumors *in vitro.* However, it is not possible to use DOX for the treatment of glioblastomas since it cannot cross the blood-brain barrier (BBB) due to its low lipophilicity and high molecular weight. Also, methotrexate (MTX) has been used for long time as a potent anticancer agent but also shows limitations to cross the BBB. Consequently, to achieve an effective dose of MTX in the brain, high and potential toxic doses of MTX should be administered systemically. Since the conventional administration routes for chemotherapy drugs delivery are difficult and painful for the patients, more practical and safe delivery methods are needed. In this way, due to the ability of methamphetamine (METH) for transiently increase BBB permeability, the aim of the present work was to evaluate whether METH could interfere with DOX and MTX effects, focusing particularly on the cellular viability, migration, chemotaxis and cell cycle of glioblastoma cells.

Our results demonstrated that 1 µM METH did not interfere in U-118 glioblastoma cell viability, migration and cell cycle. In the same way, METH did not alter the effects induced by DOX or MTX on cell viability, migration, chemotaxis and cell cycle. However, METH by itself impaired the glioblastoma cells chemotaxis, having similar effects to those observed with DOX or MTX alone. Regarding DOX effects in glioblastoma cells, this drug was able to decrease cell viability, migration and chemotaxis and led to a cell cycle arrest at G_2 phase and to a decrease on cyclin A expression. Similarly, MTX induced a decrease in glioblastoma cell viability, migration and chemotaxis and a cell cycle arrest in the S phase.

Overall, we can conclude that both DOX and MTX have a toxic effect in glioblastoma cells and that METH, in a concentration that increases endothelial cells

permeability, does not interfere with DOX or MTX effect. Thus, we consider that this approach should be explored in order to clarify the use of METH as a therapeutic approach involving brain tumors.

Key words: Glioblastoma; Methamphetamine; Doxorubicin; Methotrexate; Blood-Brain Barrier.

CHAPTER 1

INTRODUCTION

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Introduction

1.1. Brain tumors

1.1.1. Epidemiology

Brain tumors are relatively rare but with a high death rate among the diagnosed cases. Indeed, 74% of the detected cases lead to death. Worldwide, the incidence of primary brain tumors is about 7 per 100,000 individuals per year, being 2% of all primary tumors (Furnari *et al.,* 2007). In Europe, the standardized incidence of primary Central Nervous System (CNS) cancers ranges from 4.5 to 11.2 cases per 100,000 men and from 1.6 to 8.5 per 100,000 women (Ferlay *et al,* 2008). Brain tumors are the most common solid tumors of childhood and account for 20% of all malignancies in this age group (Cokgor *et al.,* 1998). Moreover, there is worldwide geographic variation in the incidence of brain tumors. For example, malignant brain tumors occur in Japan with less than half the frequency of that in Northern Europe. Countries that report a high incidence of malignant brain tumors include Australia, Canada, Denmark, Finland, Sweden, New Zealand, and the United States of America. On the other hand, areas of the world with a lower incidence, such as Philippines and India, have an incidence four times less than the high-incidence countries. Nevertheless, there are some difficulties to obtain exact geographic comparisons, especially between countries, because all over the world there are different diagnostic practices and incomplete brain tumor reports. In addition, higher incidence rates appear in countries with better access to health care and better medical cares (Wrensch *et al.,* 2002).

1.1.2. Risk factors

Concerning risk factors for brain tumors, exposure to therapeutic doses of ionizing radiation is the only established potentially modifiable brain risk factor. Evidence for an association with head injury, foods containing N-nitroso compounds, occupational risk factors, and exposure to electromagnetic fields are inconclusive (Fisher *et al.,* 2007). Other factor that could increase the risk for brain cancer is the use of cell phone, and several recent studies provide some evidence for an association between long term cell phone use and glioma. Specifically, Khurana and collaborators (2009) showed that the

use of cell phone for ≥10 years approximately doubles the risk of being diagnosed with a brain tumor on the same side of the head as that preferred for cell phone use. Additionally, there is suggestive evidence of an association between immunologic factors and gliomas. Patients with atopic syndrome have a reduced risk of gliomas (Linos *et al.*, 2007) and patients with glioblastoma who have elevated IgE levels appear to live longer than those with normal levels (Wrensch *et al.*, 2006). Gene polymorphisms that affect detoxification, DNA repair, and cell-cycle regulation have also been implicated in the development of gliomas (Fisher *et al.*, 2007). Approximately 5% of patients with malignant gliomas have a family history of gliomas. Some of these familial cases are associated with rare genetic syndromes, such as neurofibromatosis types 1 and 2, the Li−Fraumeni syndrome (germ-line p53 mutations associated with an increased risk of several cancers), and Turcot's syndrome (intestinal polyposis and brain tumors) (Fisher *et al.*, 2007). Brain tumors include a group of different tumor entities anatomically close to each other but diverse in terms of morphology, site, molecular biology and clinical behavior and presumably etiology (Crocetti *et al,* 2012). They can be divided into two major groups: intrinsic tumors arising from cells within the CNS or its coverings, and those which arise from a primary tumor outside the CNS, and invade the brain as a metastatic disease. The group of intrinsic tumors can be further divided into neuroepithelial tumors (arising from glial and neuronal cells or their precursors within the brain and spinal cord), and non-neuroepithelial tumors (arising from cells within blood vessels, arachnoidal cells, cranial and spinal nerve sheath cells, and blood vessels). Metastatic brain tumors, which are the most common intracranial tumors and occur in 15-40% of cancer patients, most commonly arise from malignant melanoma, lung, breast and kidney tumors (Ironside *et al.*, 2012). Nevertheless, the frequency of metastases has increased because it is now possible to detect small tumors due to the improvement achieved in magnetic resonance imaging techniques (Tosoni *et al.*, 2004). Also, the incidence of CNS metastases has increased in recent years, probably due to the longer survival of patients given aggressive treatments for primary tumors. Lung cancer is the main primary source of metastases to the CNS, causing brain metastases in 9.7-64% of the patients. On the other hand, in patients with breast cancer the incidence of brain metastases is lower (2-25%) and it appears to exist a direct relation between stage of disease and incidence of the metastases. Interestingly, the occurrence of brain metastases in aged patients (more than 70 years old) with breast and lung cancer is rare, and this may be related with a less aggressive disease in the elderly patients (Schouten *et al.*, 2002).

With an incidence between 4% and 20%, melanoma is other of the most common form of cancer that spread to the CNS. Metastases in CNS from colorectal cancer involving the, genitourinary tract and sarcoma are less frequent (1%) and the primary tumor is unknown in 15% of patients with this kind of metastases. Most brain metastases are due to hematogenous spread from the primary tumor, usually via arterial circulation. Inside the brain, metastases tend to localize in the area of the gray-white junction and in border zones of the major cerebral vessels. This particular distribution is due to the progressive decrease in the size of blood vessels in these areas acting as a trap for emboli. Neuroimaging studies have shown that brain metastases are multiple in two thirds of patients affected. It seems that histology influences the number of brain metastases since most colon, breast and renal cell tumors produce single lesions, whereas melanoma and lung cancer have a greater tendency to form multiple metastases (Tosoni *et al.*, 2004).

1.1.3. Pathology

Brain tumors are classified on a scale of I to IV defined by the World Health Organization (WHO), and it is based on tumor genetics and histopathological characteristics pointing the degree of malignancy and subsequent propensity for aggressive behavior (Louis *et al*, 2007). Thus, these tumors are classified on the basis of histopathology into the following major groups: gliomas, including grade I astrocytoma (are generally benign and frequently curable with complete surgical removal), grade II astrocytoma, anaplastic astrocytoma (grade III), glioblastoma (grade IV), oligodendroglioma, and ependymoma, that arise from neuroepithelial tissue; tumors of meninges (including meningioma and hemangioblastoma); and germ cell tumors and tumors of the sellar region (including pituitary tumors and craniopharyngioma), that arise from non-neuroepithelial tissue (Fisher *et al*, 2007).

Generally, the incidence rates of meningioma and glioblastoma among adults increase with advancing age. In children and adolescents, incidence rates of all non-germ cell histologies decrease through childhood and adolescence, whereas the incidence of germ cell tumors reaches a peak during the adolescent years (Crocetti *et al.*, 2012).

1.2. Glioblastomas

1.2.1 Classification

From all types of malignant brain tumors, the most common and lethal is glioblastoma multiforme also called glioblastoma, a grade IV astrocytoma (Louis *et al*, 2007). The incidence of these tumors has increased over the past two decades, especially in older people (Fisher *et a*l, 2007). In spite of surgery and adjuvant therapy advancements, the prognosis remains unfavorable, with a median survival of 12-18 months (Hou *et al*, 2006). Specifically, the median survival is about 10-15 months for glioblastoma and up to 30-50 months for anaplastic astrocytoma, despite maximal surgical resection, postoperative radiotherapy and chemotherapy (Nieder *et al.*, 2009). This type of tumors can emerge in any region of the CNS, but is more frequent in subcortical white matter of cerebral hemispheres. The hallmark histopathological features of this tumor include nuclear atypia, necrosis, hypercellularity, and microvascular proliferation (Ironside *et al*, 2012). Malignant gliomas typically contain both neoplastic and stromal tissues, which contribute to their histologic heterogeneity and variable outcome. Molecular studies, such as gene-expression profiling, allow a better classification of these tumors and separation of the tumors into different prognostic groups (Collins *et al.*, 2007).

1.2.2. Molecular pathology

Glioblastomas can develop *de novo* or through progression from low-grade or anaplastic astrocytomas, which are called primary or secondary glioblastomas, respectively. These subtypes of glioblastoma represent distinct disease entities that evolve through different genetic pathways, affect patients at different ages, and are likely to differ in prognosis and response to therapy. Primary glioblastomas are more frequent in older patients and typically show epidermal growth factor receptor gene *(EGFR)* overexpression, *PTEN* (phosphatase and tensin homolog) mutations, *CDKN2A* (cyclin-dependent kinase inhibitor 2A gene) (*p16*) deletions, and less frequently, *MDM2* (murine doble minute 2 gene) amplification. Secondary glioblastomas develop in younger patients and often contain *TP53* mutations as the earliest genetic detectable alteration. There are more subtypes of glioblastomas with intermediate clinical and genetic profiles, and a good example is the giant-cell glioblastoma that clinically and genetically occupies a

hybrid position between primary and secondary glioblastomas (reviewed by Kleihues *et al*., 1998). These mutations in the glioblastoma genetic profile also contribute for their chemotherapeutic resistance since some of these mutations are in genes that protect cells against cell death. For instance, the PI3K/AKT/mTOR pathway that regulates several normal cellular functions critical for tumorigenesis, including cellular proliferation, growth, survival and mobility, is usually deregulated in malignant gliomas, probably due to receptor tyrosine kinases activation and/or PTEN loss, which regulates negatively the PI3K/AKT/mTOR pathway (Hu *et al.*, 2005) (Figure 1).

Genetic mutations in glioblastomas induce the overexpression of specific growth factors that induce the formation of new blood vessels, a process called angiogenesis. The expression level of vascular endothelial growth factor (VEGF) is directly correlated with tumor grade and malignancy, and can be 10 fold more expressed in high grade gliomas compared with low grade (Arko *et al.*, 2010). Due to the formation of new vessels, glioma cells are able to increase their oxygen and nutrient supply which increase the chance of tumor cell survival. In fact, malignant gliomas are highly-vascularized solid tumors and have a great invasive nature. Several studies have suggested that gliomas are capable of moving throughout the brain because of chemical modulation of the surrounding extracellular matrix (ECM). This migration is thought to occur *via* secretion of matrix metalloproteinases, such as MMP-9, that causes degradation of the ECM (Chetty *et al.*, 2012).

Recent studies indicated that glioma samples are characterized by increased expression of some chemokines receptors (Oh *et al*., 2001, Maru *et al*., 2008, Zhou *et al.*, 2002). Chemokines are the most important and best known group of chemotactic proteins and exert a direct role in regulation of leukocyte trafficking during immunosurveillance and inflammation. Nevertheless, chemokines participate in many other physiological processes, such as immune system cell differentiation and homeostasis, and in both angiogenesis and development (Gerard *et al.,* 2001). Regarding tumors, chemokines are known to address intratumoral trafficking of immune cells, and to modulate tumor proliferation, metastasis and angiogenesis (Vandercappellen *et al.,* 2008). The most known chemokines receptors with an increased expression in human glioma are CXCR3 (G protein-coupled receptor 9 or CD183) (Maru *et al*., 2008) and CXCR4 (Sciaccaluga *et al.,* 2010), which belong to the CXC chemokine receptor subfamily. CXCR3 has three endogenous ligands, monokine induced by gamma interferon (MIG or CXCL9), interferon gamma-induced protein 10 (IP-10 or CXCL10) and interferon-inducible T-cell

alpha chemoattractant (ITAC or CXCL11), while CXCR4 has the chemokine stromal-derived factor-1 (SDF-1 or CXCL12) as a ligand (Bonavia *et al.*, 2003). Studies with astrocytoma and glioblastoma cells suggested that the increased expression of chemokine receptor/ligand pair, CXCR3/CXCL10, has an important role in the proliferation, growth and increase DNA synthesis of these cells types (Maru *et al.*, 2008; Liu *et al.*, 2010). Meanwhile, the CXCR4 expression on brain tumor cells at high levels has negative prognostic significance (Bian *et al.*, 2007). The studies of Carmo and others (2010) demonstrated that the activation of CXCR4 by CXCL12 promoted proliferation, survival, migration and chemotaxis of glioma cells, confirming that CXCL12/CXCR4 signaling pathway may contribute to the growth and invasive characteristics of glioblastomas. Moreover, it is due to this malignant invasive nature that surgical resection of gliomas is not so efficient. Even though surgical resection has been shown to improve patient survival, some glioblastomas spread and evade critical areas of the brain that are unable to be surgically removed, because of the risk of compromising the neuronal function of patients. These residual tumor cells are then able to invade other brain tissues, causing the high incidence of glioblastoma recurrence (reviewed by Furnari *et al.*, 2007).

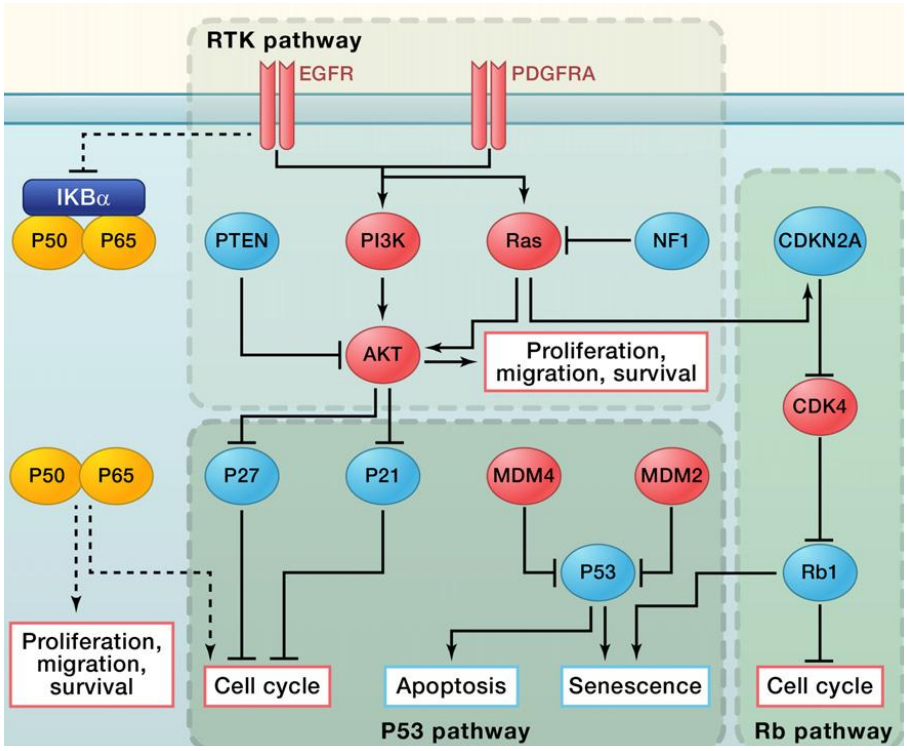

Figure 1 - Key deregulated pathway in human gliomas. Many different genetic and molecular alterations in human gliomas lead to modifications of the same signaling pathways that result in brain tumor growth and progression. Red indicates oncogenes that are overexpressed or amplified in glioblastoma samples, and blue indicates tumor suppressor genes that are somatically mutated or deleted (except for P27 and P21) (Adapted from Chen *et al.*, 2012).

1.2.3. Recurrence

Glioblastoma recurrence is inevitable, despite extensive surgical intervention. Indeed, after a median patient survival time of 32 to 36 weeks, it has been reported that 90% of patients with glioma would experience tumor recurrence at the original tumor site or near the original tumor (2 to 3 cm from the border of the original lesion). It is usual to define glioblastoma recurrence as a change from an interval of time with tumor absence followed by a complete loss of tumor control. The treatment approaches for recurrent glioblastomas are hindered by the lack of uniform definition and criteria of recurrence, institutional variability in treatment, and the heterogeneous nature of recurrence in glioma spatial location and acquired resistance to chemotherapy (Hou *et al.*, 2006).

Glioblastomas assume a challenge in neuro-oncology field not only because of their, infiltrative nature, propensity for recurrence and resistance to conventional therapies (Furnari *et al*, 2007) but also because of blood-brain barrier (BBB) that limits the entrance of systemically administered chemotherapeutic drugs into the brain (Parney *et al.*, 2003).

1.3. Therapeutic approaches for glioblastomas

1.3.1. Surgery

The classic treatment of glioblastomas includes, on the most part of the cases, surgery followed by radiotherapy and then chemotherapy. Surgical removal of the tumor, not only improves the patient survival time but is crucial for the acquisition of tumor tissue samples that are used to confirm the histological type and malignancy of the tumor. Surgery can also reduce intracranial pressure and mass effect, improving patients' neurological status and the efficacy of adjuvant therapy (Hou *et al.*, 2006). However, surgery seems to be more effective in first-line treatment. When recurrence occurs re-operation is a possible option, but there is an increased risk of surgery-related morbidity and mortality (reviewed by Niyazi *et al.*, 2011).

1.3.2. Radiotherapy

Radiotherapy substantially improves survival and is generally administered with a total dose corresponding to the upper limit of a safe dose. However, cognitive dysfunction and radionecrosis may occur with this regimen, especially when concomitant

chemotherapy is given. Other techniques, such as brachytherapy, adjunction of radiosensitisers, or hyperfractionation have demonstrated no benefits in comparison with standard radiotherapy (reviewed by Behin *et al.*, 2003). As happen with the surgery, oncologists have been highly reluctant to re-treat local recurrences in the brain, although radiation is proven to be effective in primary glioblastomas. This reluctance is based on the assumption that CNS tissues are not capable of repair radiation injuries (reviewed by Niyazi *et al.*, 2011). So, due to the possibility of severe side effects there is a limited use of radiation in re-treatment settings of recurrent malignant gliomas (reviewed by Niyazi *et al.*, 2011).

1.3.3. Chemotherapy

Regarding the treatment options for glioblastomas, chemotherapy is the most versatile strategy because possesses greater range of choices. Chemotherapeutic drugs are usually administered following surgical resection of tumor and irradiation of glioblastoma lesions. Drugs could be given systemically and others could be given *in situ*, directly in the tumor.

Over the past 30 years, no significant changes in the standard treatment of malignant gliomas have been observed. In 1980, the possible treatments were: semustine (MeCCNU), radiotherapy, carmustine (BCNU) plus radiotherapy, or semustine plus radiotherapy. Patients who received radiotherapy alone or in combination with a nitrosourea (carmustine or semustine) had a significant improvement in overall survival when compared to patients treated with other combinations. In 1996, the FDA (Food and Drug Administration) approved a polyanhydride biodegradable polymer wafer containing BCNU (1-3-bis(2-chloroethyl)-1-nitrosourea), known as Gliadel®, for the treatment of recurrent gliomas. Patients with recurrent tumors and those that made primary resections for newly diagnosed tumors who had wafers placed at the time of their second surgeries, were found to have approximately 2 months of survival benefit (Brem *et al.*, 1995; Westphal *et al.*, 2003). However, the most significant advance in malignant glioma therapy for newly diagnosed tumors, since radiation therapy, has been the administration of temozolomide (TMZ). Temozolomide is an oral alkylating agent approved by the FDA in 1999 after the European Organization for Research and Treatment of Cancer (EORTC) and National Cancer Institute of Canada (NCIC) phase III trial demonstrated an improved median survival. So, TMZ received FDA approval for refractory anaplastic astrocytoma, followed by a first-line indication for glioblastoma (Stupp *et al.*, 2005, 2009).

More recently, other experimental therapies have been raised. Irinotecan, a topoisomerase 1 inhibitor, provide a viable treatment option for tumors resistant to temozolomide. It is also an alternative choice for recurrent malignant gliomas despite the controversy regarding its ability to pass through the blood-brain barrier (BBB). However, as a single agent, irinotecan showed disappointing results in the treatment of recurrent malignant gliomas. In 2009, FDA has granted accelerated approval for single bevacizumab, the humanized monoclonal antibody against VEGF, for the use in patients with glioblastoma that has progressed despite previous therapy. Furthermore, bevacizumab has generally been used in combination with cytotoxic agents. However, the efficacy of bevacizumab and irinotecan combination to treat glioblastoma remains to be clarified (Xu *et al.*, 2010).

There is a range of chemotherapeutic drugs that have been investigated against the different types of malignant gliomas, *in vivo* and *in vitro*, but the drugs that are most clinically relevant to current malignant gliomas are summarized in Table 1.

Table 1- Summary of current treatments for malignant gliomas (adapted from Wen and Kesari, 2008).

Type of tumor	**Therapy**
Newly diagnosed tumors	
Glioblastomas (WHO grade IV)	Maximal surgical resection plus radiotherapy, or plus concomitant and adjuvant Temozoline (TMZ) or carmustine wafers (Gliadel).
Anaplastic astrocytomas (WHO grade III)	Maximal surgical resection, with the following options after surgery (no accepted standard treatment): radiotherapy plus concomitant and adjuvant TMZ or adjuvant TMZ alone.
Recurrent tumors	Reoperation in selected patients, carmustine wafers (Gliadel), conventional chemotherapy, *e.g*. lomustine, carmustine, PCV lomustine, procarbazine, vincristine, carboplatin, irinotecan, etoposide, bevacizumab plus irinotecan, or experimental therapies.

Despite the well known advantages of chemotherapeutic drugs, there are also some limitations. The oldest drug used for glioblastoma and recurrent malignant tumors, carmustine (BCNU), has several side effects especially because of its cytotoxicity. The main effect of BCNU is mediated by the cross-link of DNA strands by its chloroethylation of a nucleophilic site on one DNA strand and displacement of a chloride ion on the other strand. Chloroethylation produces ethyl bridges between these two DNA strands. This cross-linking causes the disruption of DNA that in the end hinders RNA synthesis (Bota *et al.*, 2007). Traditional methods of delivering BCNU to the tumor site, such as intravenous perfusion or oral administration, cause debilitating side-effects including hematological depression, cytotoxic effects on kidney, liver, lungs and CNS, brain edema and seizures (Wang *et al.*, 1999; Guerin *et al.*, 2004). Also, because of the short half-life of BCNU, there is an insufficient drug exposure and distribution throughout the brain (Bota *et al.*, 2007). The development of localized delivery of BCNU directly to the glioblastoma tumor is also unattractive to patients. In order to increase the exposure of the tumor to the drug (Brem *et al.*, 2001), Gliadel® wafer is administered as an intracranial implant and requires surgical intervention for placement, and the wafers are very thin and fragile. Broken wafers contribute to increased intracranial edema and a potential toxic release of chemotherapeutic drug leading to fatal clinical complications (Arifin *et al.*, 2009).

A major limitation of chemotherapy with BCNU involves a resistance mechanism (Bredel, 2001) mediated by a DNA-repair enzyme, MGMT (methylated-DNA-protein-cysteine methyltransferase). This DNA-repair protein is inherent to a majority of human brain tumors, and protects tumor cells from the cytotoxic effects of alkylating agents (Silber *et al.*, 1993). MGMT also contributes to resistance to TMZ. Following oral absorption, TMZ is hydrolyzed in aqueous solution to methyl-triazeno-imidazole-carboxamide (MTIC). MTIC is rapidly converted to the inactive 5-aminoimidazole-4-carboxamide (AIC) and to the electrophilic alkylating methyldiazonium action that transfers a methyl group to DNA. The DNA-methyl adducts are responsible for the cytotoxicity. Alkylation of the O6 position of guanine accounts for only about 5% of DNA adducts, but is primarily responsible for the cytotoxic effects of TMZ. The N7 of guanine and the N3 of adenine represent the majority of DNA-methyl adducts. The O^6-methylguanine (O^6-meG) lesion leads to DNA double strand breaks and subsequent cell death via apoptosis and/or autophagy. MGMT repairs the O^6-meG lesion, and thus high

levels of MGMT are thought to contribute to resistance to TMZ. Chemotherapeutic resistance is not the only limitation of using TMZ. TMZ has dose-limiting toxic side-effects that could be seen as leucopenia, seizures and myelosuppression, nausea, constipation and headache (reviewed by Villano *et al.*, 2009).

In general, resistance to chemotherapy in brain tumors is complex and may involve multiple mechanisms besides the one related with MGMT and alkylating agents. These may include: ATP-dependent efflux of cytotoxic agents by transmembrane transport proteins encoded by the multiple drug resistance 1 (*Mdr1*) gene and the multidrug resistance-associated protein-1 (*MRP1*) gene; DNA damage caused by quantitative alteration topoisomerase IIα (Topo IIα) expression; deficiency in DNA mismatch repair pathways and increased nucleotide excision repair as a result of an altered activity of poly(ADP)-ribose polymerase; enhanced detoxification of alkylating agents by the glutathione (GSH)-linked enzyme system; an impaired ability to commit apoptosis; and changes in the expression status of resistance-markers due to elevated levels of activity of protein kinase C (PKC) (Bredel, 2001).

Some drugs are used in combination with radiotherapy or with the traditional chemotherapy in order to minimize drug resistance and maximize tumor cell kill. One of those drugs is Doxorubicin.

1.4. Doxorubicin

Doxorubicin (DOX) or Adriamycin, is an anthracycline antibiotic with strong antitumor action against several tumors, such as lymphoblastic leukemias, sarcomas, lymphomas, carcinomas of the head and neck, and cancers of the breast, pancreas, stomach, liver, ovary, lung and prostate. DOX is produced by the *Streptomyces peucetius varieta caesius* and blocks DNA and RNA synthesis by inhibiting topoisomerase II (Lesniak *et al.*, 2005). The primary mechanism of action of DOX appears to be the poisoning of the enzyme topoisomerase II which results in double-strand DNA breaks, leading to apoptosis. More recently, it was also demonstrated that DOX forms covalent adducts with DNA and these lesions are more cytotoxic than those induced by topoisomerase II impairment. Adducts are formed predominantly at 50-GC-30 sites in DNA where the DOX sugar group (daunosamine) is covalently linked to the N-2 amino group of guanine via an aminal (N–C–N) bond. The central carbon atom in the aminal bond is derived from formaldehyde, hence formaldehyde is an absolute requirement for

adduct formation. The resulting drug–DNA monoadduct is further stabilized through intercalation and hydrogen bonding with the second strand of DNA. Apoptosis resulting from doxorubicin–DNA adduct formation does not depend on topoisomerase II status, reflecting an independent mechanism of cell kill and highlighting that formaldehyde availability switches the mechanism of DOX action from topoisomerase II impairment to the formation of more cytotoxic DNA adducts (Swiff *et al.*, 2006; Ugarenko *et al.*, 2010) (Figure 2).

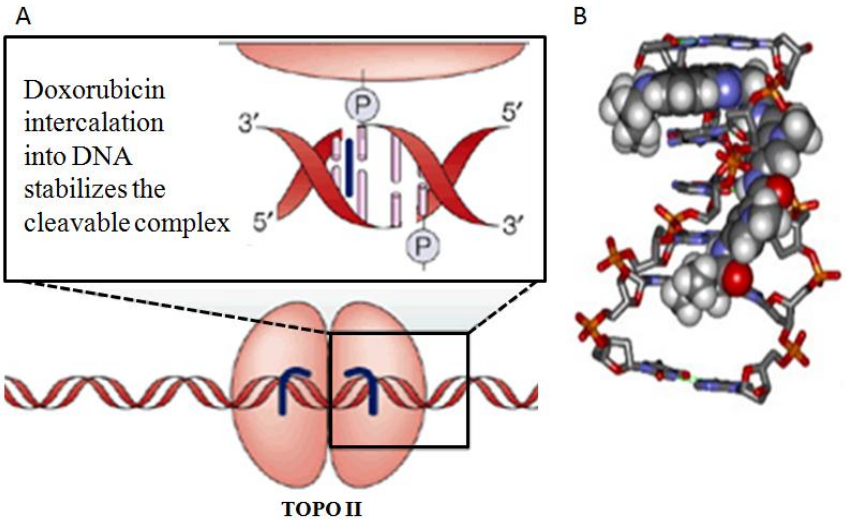

Figure 2 - Mechanisms of action of doxorubicin: (A) Doxorubicin blocks DNA and RNA synthesis by inhibiting topoisomerase II (Adapted from Hurley, 2002), as well as by (B) forming adducts with DNA (Zhang *et al.*, 2010).

In vitro studies demonstrated that DOX is efficiently toxic to highly invasive human glioma cell lines, inducing cell death by apoptosis. DOX also showed to inhibit tumor invasion on a chorioallantoic membrane assay involving embryonated chicken eggs and highly invasive human glioma cell lines (Stan *et al.*, 1999). Despite doxorubicin's clinical effectiveness in the treatment of many malignant tumors, clinical trials involving systemic administration of the drug have demonstrated very limited efficacy in the treatment of gliomas (Hau *et al.*, 2004). Very high doses of DOX must be administered systemically to exert its therapeutic benefit, which limits its usefulness because of the cardiotoxic and myelosuppressive side-effects induced by such a high doses of DOX.. The low lipophilicity and high molecular weight of DOX essentially prevent the delivery of the agent across the BBB (Lesniak *et al.*, 2005). Thus, the limited efficacy of DOX when administered systemically can be explained by its poor penetration through the

BBB, as well as by its extrusion by P-glycoprotein (P-gp) (Neuwelt *et al.*, 1981; Ohnishi *et al.*, 1995; Lesniak *et al.*, 2005).

Different methods have been used to facilitate the transport of drugs into the brain. These methods include neurosurgical based invasive brain drug delivery which have, however, several limitations. So, most of the methods involve the use of osmotic biologically active agents (Bellavance *et al.*, 2008). Additionally, some other drug delivery systems have been studied for BBB delivery, such as liposomes, microcapsules and osmotic pumps (Maysinger and Morinville, 1997). *In vivo* studies using animal models of malignant glioma suggest that DOX binds to nanoparticles and cross the intact BBB reaching therapeutic concentrations in the brain (Steiniger *et al*., 2004). To obtain better targeting efficiency, targeted liposomes and immunoliposomes have been proposed (Gong *et al.*, 2011). However, even these methods show disadvantages, such as high systemic doses for BBB permeabilization, osmotic pumps failure that leads to potential toxic dumping, refilling of pumps that can originate infections, and parenchymal delivery which may cause a reaction at the delivery site (Maysinger and Morinville, 1997).

1.5. Methotrexate

Methotrexate (MTX) is widely used as a chemotherapeutic agent for the treatment of many cancers, demonstrating consistent activity against a number of malignant tumors such as cervical and ovarian carcinoma, gastric and pancreatic carcinoma and leukemia (Hazard Substances Data Bank, 2005). MTX has also been found to have a successful therapeutic role in non-neoplastic diseases (*e.g.* psoriatic arthritis) as an anti-inflammatory and immunosuppressive agent (Dalmarco *et al.*, 2007). MTX is taken up by the cells *via* carriers, such as reduced folate carrier and folate receptor (Dixon *et al.*, 1994) and, once inside the cell, MTX acts as a potent competitive inhibitor of dihydrofolate reductase (DHFR). Inhibition of DHFR results in a depletion of the intracellular reduced folate pools required for the biosynthesis of purines and thymidines (Figure 3). Additionally, MTX is an antimetabolite that can penetrate the BBB when given in high doses by the intravenous route. Numerous studies of MTX or MTX-based chemotherapy in combination with whole-brain radiation therapy have been conducted in patients with primary CNS lymphoma, and these studies demonstrated that this combination results in the extension of progression-free survival when compared with historical series treated with whole-brain radiation therapy alone (Boiardi *et al.*, 1999).

More recently, intravenous and intrathecal administration of MTX have largely replaced cranial irradiation for treatment of children with acute lymphoblastic leukemia (Brugnoletti *et al.*, 2009).

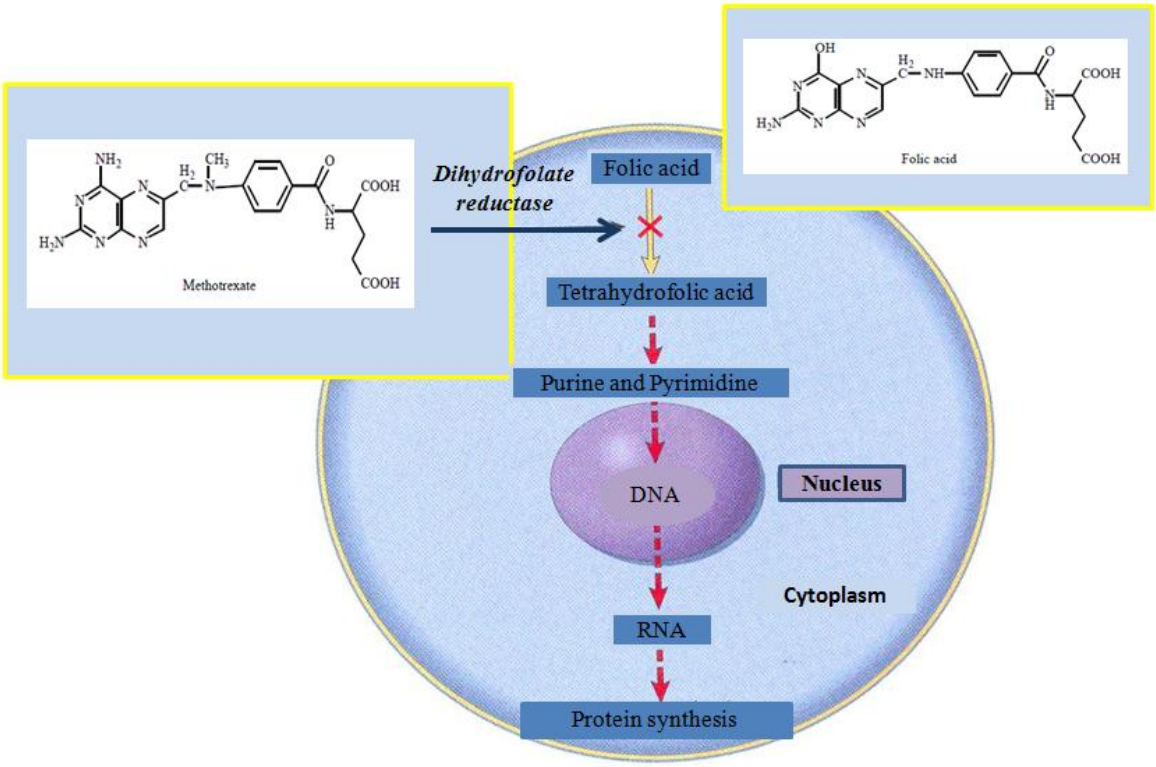

Figure 3 - MTX mechanism of action. MTX acts as a folic acid antagonist that binds to the active catalytic site of dihydrofolate reductase (DHFR). Folic acid is required in the synthesis for pyrimidine and purine nucleotides and for DNA synthesis. By inhibiting the enzyme DHFR, MTX prevents nucleic acid synthesis which leads to cell death (based on McGuire, 2003).

Despite the above mentioned positive role of MTX treatment, this is often accompanied by strong side effects, such as nausea, vomiting, diarrhea, stomatitis, gastrointestinal ulceration, and mucositis (Frei *et al.*, 1975). In cases of inadvertent intrathecal overdosage of MTX, severe neurotoxicity occurred, manifested as prompt burning or numbness in the lower extremities, stupor, seizures, and/or respiratory insufficiency. Moreover, brain damage or fatal necrotizing leukoencephalopathy may occur, but a complete recovery has been reported following aggressive therapy (Hazard Substances Data Bank, 2005).

MTX has a good potential as a chemotherapeutic drug against malignant gliomas since it shows acceptable results in CNS tumors, such as primary CNS lymphoma. However, there are still several risks related with the routes of administration and the dose limiting effects.

Since DOX and MTX have limited penetration into the brain, the administration route is a serious concern for its effectiveness as chemotherapy for brain tumors. Additionally, both drugs are substrates of the P-gp (Varma *et al.*, 2004) that is a great obstacle to CNS chemotherapy. Overall, BBB limits the entrance of chemotherapeutic drugs into the brain and the presence of P-gp both in endothelial cells and tumor cells will ultimately prevent the accumulation of drugs in the tumor. Both conditions are, indeed, a major impediment to the entry of many therapeutic drugs into the brain.

1.6. Blood-brain barrier

The blood-brain barrier (BBB), which is one of the blood-neural barriers, is present in almost all brain regions, except in the brain ventricular system (Cardoso *et al.*, 2010). BBB is a selective barrier formed by the endothelial cells surrounded by basal lamina, and by astrocytes and pericytes. Both astrocytic endfeet and pericyte processes wrap the abuminal capillary surface and provide physical support and stability to the BBB (Figure 4). In recent years, the concept of a BBB has been significantly extended to the concept of a neuro(glial)vascular unit, which best describes the dynamic communication between brain endothelium, neurons, astrocytes, pericytes and microglia at the interface between the blood and brain parenchyma compartments (Hawkins and Davis, 2005). A healthy brain relies on all of the cells of the neurovascular unit to function properly and communicate with each other in order to maintain normal cognitive functions.

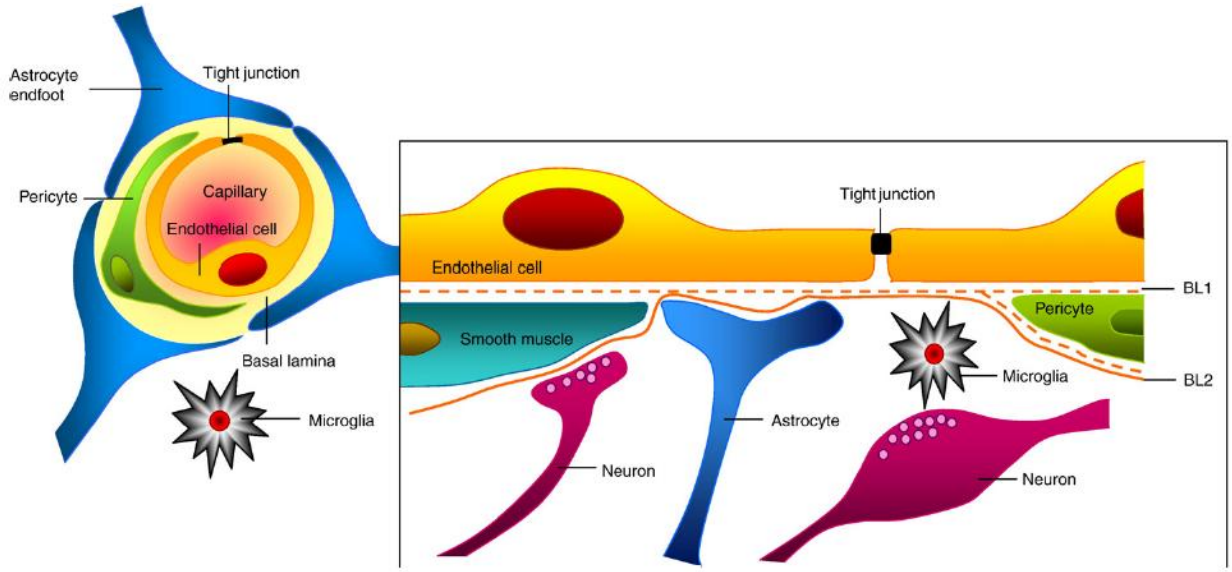

Figure 4 - Neuro(glial)vascular Unit. The cerebral endothelial cells have intercellular junctions that confer the barrier property of the BBB. Pericytes are distributed discontinously along the length of the cerebral capillaries. Both the cerebral endothelial cells and the pericytes are enclosed by, and contribute to, the local basement membrane which forms a distinct perivascular extracellular matrix (basal lamina 1, BL1), different in composition from the extracellular matrix of the glial endfeet bounding the brain parenchyma (BL2). Foot processes from astrocytes form a complex network surrounding the capillaries. Axonal projections from neurons contain vasoactive neurotransmitters that regulate local cerebral blood. Microglial cells have an immunovigilant function in the barrier (Adapted from Abbott *et al*., 2006).

BBB acts as a physical barrier since the complex tight and adherens junctions between adjacent endothelial cells force most molecular traffic to take a transcellular route across the brain endothelium, rather than moving paracellularly through the junctions. Small gaseous molecules, such as O_2 and CO_2 can diffuse freely through the lipid membranes, and this is also a route of entry for small lipophilic agents, including drugs like ethanol. The presence of specific transport systems on the luminal and albuminal membranes regulates the transcellular traffic of small hydrophilic molecules, which provides the transport by specific receptor-mediated transcytosis, or by the less specific adsorptive-mediated transcytosis (reviewed by Abbott *et al.*, 2006). Moreover, extremely strait tight junctions are a key characteristic of the BBB and significantly reduce permeation of polar solutes through paracellular pathway between the endothelial cells from the blood plasma to the brain extracellular fluid (Figure 5).

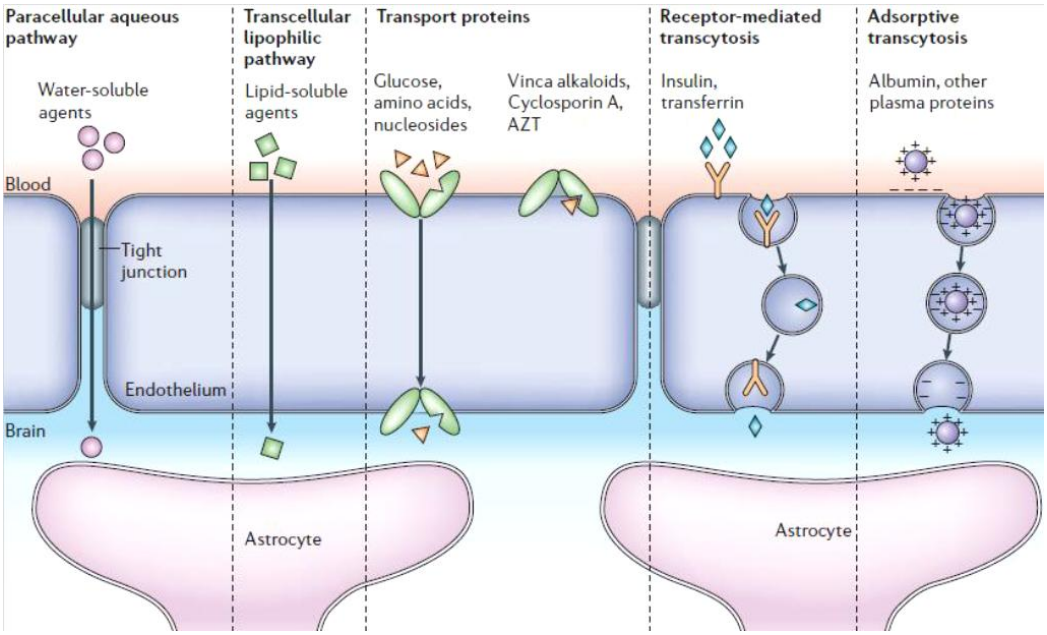

Figure 5 - Transport mechanisms at the blood-brain barrier. The transporters of proteins and transcellular pathway are essential to suppress the nutritional needs. The paracellular transport is importat for ionic homeostasis. Transcytosis may occur at a low level but under physiological conditions (adapted from Abbott *et al.*, 2006).

The junctional complexes between endothelial cells include tight junctions (TJs) and adherens junctions (AJs). TJs consist of a complex of proteins spanning the intercellular cleft (occludin and claudins), and junctional adhesion molecules (JAMs). Claudins are the first "seal" of TJs and occludin confer the high electrical resistance to endothelial cells. These are transmembrane proteins that are linked to a number of cytoplasmic scaffolding and regulatory proteins, such as ZO-1, ZO-2, ZO-3 and cingulin (Reviewed by Abbott *et al.*, 2010). In AJs, cadherin proteins span the intercellular cleft and are linked into the cell cytoplasm by the scaffolding proteins α, β and γ catenin. The AJs hold the cells together giving the tissue a structural support. Both TJs and AJs are essential for barrier properties and alterations of these proteins may lead to BBB dysfunction (Figure 6).

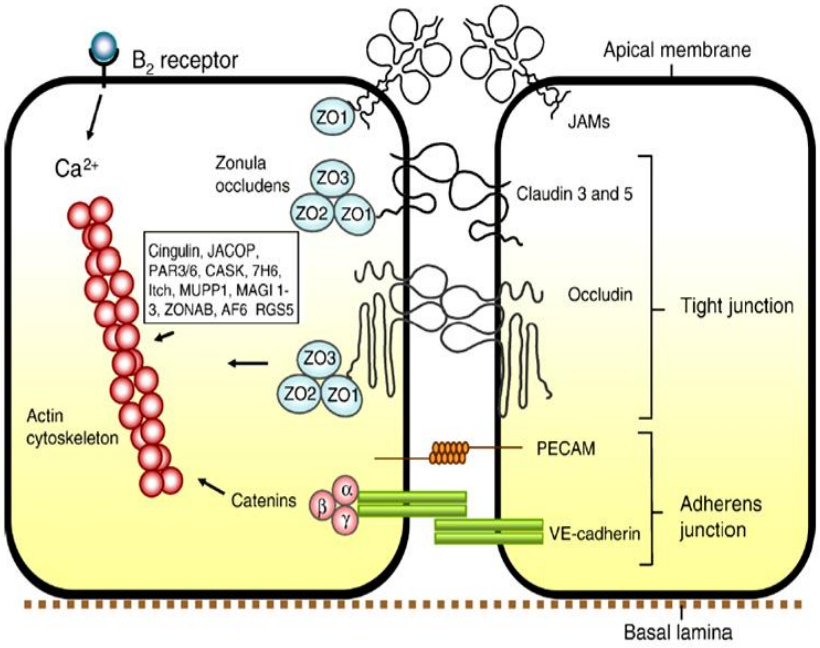

Figure 6 - Structure of brain endothelial junctions. The tigh junctional complex comprises junctional adhesion molecules (JAMs), occludin and claudins. The claudins and occludin are linked to the scaffolding proteins ZO-1, ZO-2 and ZO-3, which in turn are linked via cingulin dimers to the actin/myosin cytoskeleton within the cell. In the adherens junctions (AJs), VE-cadherin proteins are linked to the actin cytoskeleton by the scaffolding proteins α-, β- and γ-catenin (Adapted from Abbott *et al*., 2010).

The BBB has several roles, including the brain supply with essential nutrients and to mediate efflux of many waste products. It restricts ionic and fluid movements between the blood and the brain, allowing specific ion transporters and channels to regulate ionic trafficproducing a brain interstitial fluid that provides an optimal medium for neural function (Abbott, 2004). More importantly, the BBB protects the brain from fluctuations in ionic composition that can occur after a meal or exercise, which would disturb synaptic and axonal signaling (Abbott *et al.*, 2006). The barrier helps to keep separate the pools of neurotransmitters and neuroactive agents that act centrally (in the CNS) and peripherally (in the peripheral tissues and blood), so that similar agents can be used in the two systems without crosstalk. Because of its large surface area and the short diffusion distance between neurons and capillaries, the endothelium has the predominant role in regulating the brain microenvironment.

There are several events that affect the BBB integrity altering the tight junctions, and in some cases the increased BBB permeability is a consequence of the pathology, as occurs in ischemic stroke and traumatic brain injury, whereas in other cases BBB opening may be a precipitating event, such as multiple sclerosis that is characterized by chronic

neuroinflammation. Also, some types of brain tumors characterized by edema such as glioblastoma appear to disturb the BBB function (Hawkins and Davis, 2005).

1.7. Blood-brain barrier and glioblastomas

In malignant gliomas, such as glioblastoma, the BBB appears to be leaky, and this was visualized in some studies involving immunohistochemistry of plasma proteins using biopsy samples (Seitz *et al.,* 1987), as well as by heterogeneous contrast enhancement in neuroradiological examination (Roberts *et al.,* 2000). Some glioblastoma vessels, in particular those with endothelial hyperplasia, show abnormal morphological features of endothelial cells, like fenestrations, prominent pinocytotic vesicles, lack of perivascular glial endfeet, and loss and/or abnormal morphology of tight junctions (Engelhard *et al.,* 1999; Liebner *et al.,* 2000). Accordingly, barrier-related molecular alterations were observed in immunohistochemical studies that revealed some transmembrane tight junction components down-regulated in glioblastoma microvessels, including occludin and claudin-3 (Papadopoulos *et al.,* 2001; Wolburg *et al.,* 2003). Also, claudin-5 and the intracellular tight junction component ZO-1 were additionally down-regulated in hyperplasic vessels (Liebner *et al.,* 2000; Sawada *et al.,* 2000). Abnormal expression of these tight junction components has been associated with the loss of the BBB integrity, as described for claudin-5-deficient mice (Nitta *et al.,* 2003). However, this alterations use to occur at the tumor centre in contrast to what happens near the infiltrating edges of the tumor, where the BBB is still functional (de Vries *et al*., 2006). On the other hand, the BBB is usually preserved in low-grade (WHO grade II) gliomas (reviewed by Wolburg *et al.,* 2012), making the delivery of chemotherapeutic drugs to these areas challenging.

There are some hypotheses that may explain the pathogenesis of BBB dysfunction in malignant gliomas. One of the hypothesis is that dedifferentiated glioma cells have lost the ability of inducing BBB features in endothelial cells, making the excessive *de novo* vascular proliferations typical of glioblastomas functionally aberrant. Another hypothesis involves the fact that glioma cells might secrete factors that actively open or degrade initially intact BBB tight junctions. The finding of structural and functional BBB impairment even in the invasion area and in peritumoral brain (Dinda *et al.,* 1993; Bertossi *et al.,* 1997) suggests the involvement of factors that negatively interfere with normal BBB functions.

Highly invasive tumors own a great capacity of invasion and migration into the normal neural tissue with subsequent dispersion of isolated tumor cells far from the tumor core. Related with this, the expression or/and activity of different members of the MMP family can be increased in several glioblastoma cell lines and also in primary cultures of human samples (Hagemann *et al.*, 2010). MMPs are proteolytic enzymes capable of degrading the extracellular matrix and consequently break down the connection between cells, including endothelial cells. MMP-9 acts as an important oncogene that improves invasiveness of cancer cells and high level of MMP-9 confers a poor prognosis in several cancers (Yang *et al.* 2011). A recent study hypothesized that MMP-9 increased activity may explain reduction on tight junctions proteins levels, like claudin-5, occludin and ZO-1 proteins, that are essential to maintain BBB structure and function (Martins *et al.*, 2011). This fact could be one explanation for the loss of barrier functions in patients with high-grade gliomas (reviewed by Wolburg *et al.*, 2012). Indeed, Wang and collaborators (2003) found a positive correlation between MMP-2 and MMP-9 expression and the different glioma WHO grades (I-IV). Using glioma tissue samples and by immunohistochemistry, the authors observed a weak expression of MMP-2 and MMP-9 in low-grade malignant glioma, in contrarily to the high-grade malignant glioma which showed a strong expression of both MMPs (Wang *et al.*, 2003). There are some glioblastomas clinical features, such as high aggressiveness that can be related with some changes in the BBB, but in contrast this increased permeability could facilitate the penetration of chemotherapeutic drugs into the tumor. However, there is no study clearly showing the BBB alterations in different tumor grades and even more important if these changes are sufficient to allow the entrance of antitumor drugs that usually do not cross the BBB. Of course that the success of this approach will always depend on the glioblastoma stage since the treatment of advanced tumor with a characteristic necrotic centre appears to come too late for the patient.

Another clinical feature of the glioblastomas involves the intracerebral pressure due to edemas. Brain edema is an abnormal state that consists of an increase of brain water content. The water content is controlled by a family of water channels denominated aquaporins (AQPs), and the most expressed AQPs in the brain are type 1, 4 and 9. Brain edema can be generally divided in two types: cytotoxic and vasogenic edema (Figure 7). In cytotoxic edema occurs cell swelling, associated with a reduced brain extracellular space volume and intact BBB. Water flows from the vasculature into the intracellular brain compartment when the Na^+/K^+ ATPase fails or extracellular [Na^+] falls. Under this

conditions water moves from the blood through endothelial cells and astrocyte foot process membrane, via AQP4, into brain. Usually, this type of edema is produced by early phase cerebral ischaemia, hypoxia and hyponatremia. In vasogenic edema, hydrostatic forces cause extravasation of a protein-rich exudate from plasma, through leaky BBB into brain extracellular space. Here, water moves from blood down a hydrostatic pressure gradient through a leaky BBB into brain extracellular space, and so independently of AQP4. Brain tumour and brain abscess produce vasogenic edema (Tait *et al*, 2008; reviewed by Francesca *et al.*, 2010). Importantly, AQP4 was described several times to be up-regulated in brain tumors including glioblastomas (Saadoun *et al.*, 2002), and, another study showed that phosphorylation of AQP4 by PKC leads to a decrease in water permeability and a decreased in glioma cell invasion. On the other hand, when PKC was inhibited the water permeability increased, as well as cell invasion potential (McCoy *et al.*, 2010). Also, it was shown that inhibition or silencing of AQP4 cause a decreased in cell migration and its invasion ability (Ding *et al.*, 2011). Under pathological conditions like tumor, inflammation or stroke, the AQP4-related polarity is reduced, which indicates the presence of potassium and water channels in physiologically unsuitable membrane domains.

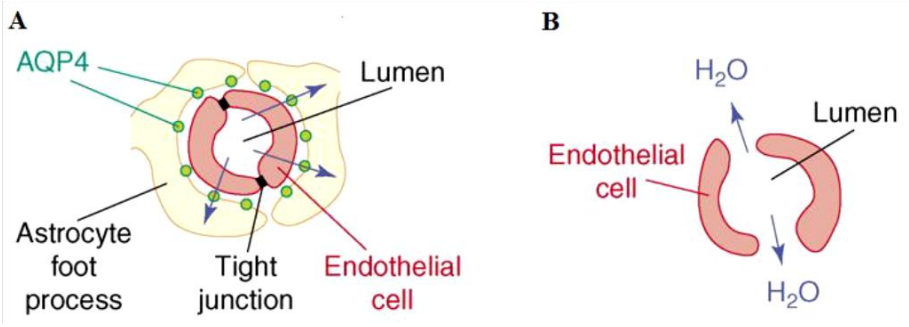

Figure 7 - Cytotoxic and Vasogenic Edema. A) Cytotoxic edema, where the increase in water brain content is dependent of astrocytic AQP activity. B) Vasogenic edema, in which water accumulation is independent of AQP (adapted from Tait *et* al, 2008).

1.8. Methamphetamine

Methamphetamine (METH) is a drug that belongs to the amphetamine-type stimulants (ATS) family, along with amphetamine and ecstasy (or 3,4-methylenedioxymethamphetamine, MDMA), which has great similarity with the neurotransmitter dopamine (Figure 9). METH has two isomers, *d*-methamphetamine

which is a common drug of abuse and *l*-methamphetamine, which has about 25-30% of CNS activity of its *d*-enantiomer (Logan, 2002). Methamphetamine is highly addictive and toxic to the brain (Sulzer *et al.*, 2005), and can be consumed in various ways such as smoked, injected, snorted and taken orally (Winslow *et al*., 2007; Nakama *et al*., 2008).

According to the United Nations World Drug Report 2012 the worldwide amphetamines consumption is between 14 and 52.5 million among people between 15 and 64 years old, which represents 0.3% to 1.2% of annual prevalence, making METH one of the most widely used drug globally (United Nations Office on Drugs and Crime, 2012) (Figure 8). Concerning Portugal, according to the report from the "Instituto da Droga e da Toxicodependência, IDT 2009", between 2001 and 2007, there was an increase in prevalence of amphetamines use from 0.5% to 0.9% in the total population. More recently, data from the IDT report "Estudo sobre o Consumo de Álcool, Tabaco e Drogas (ECATD)/ European School Survey on Alcohol and other Drugs (ESPAD) - Portugal/2011", showed that between 2007 and 2011, there was an increase in prevalence of amphetamines from 0.3% to 2% in students between 13 and 18 years old. However, these reports include all the amphetamines, with the exception of ecstasy, and due to that there are no specific data on METH use.

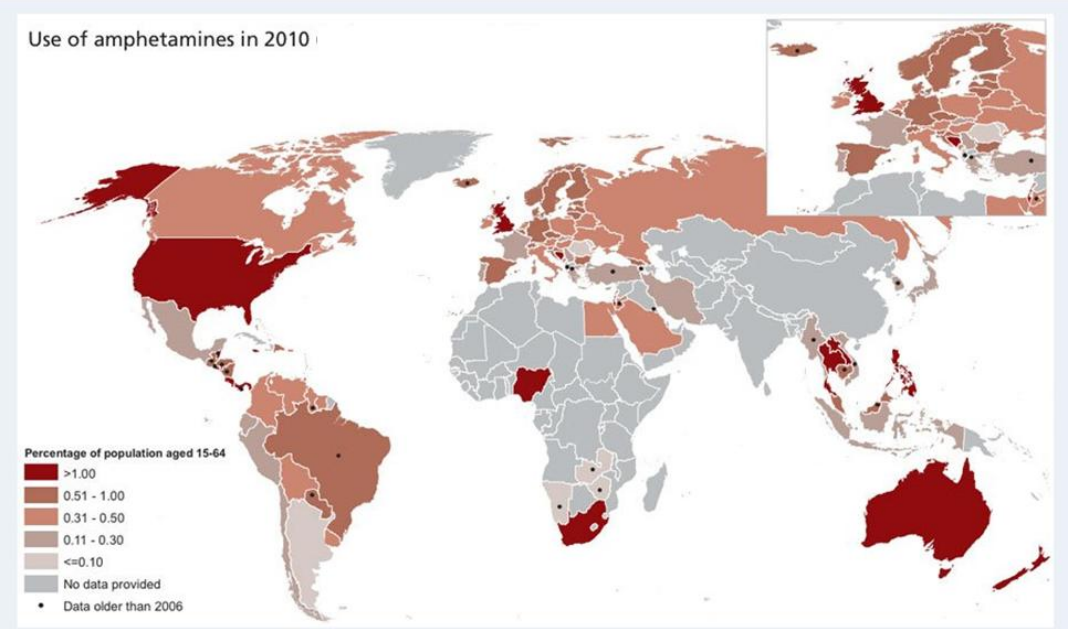

Figure 8 - Worldwide distribution of amphetamines use in 2010 (adapted from United Nations World Drug Report, 2012).

METH has different effects depending if it is taken chronically or acutely. Immediately after consumption, METH leads to alertness, wakefulness, euphoria, increased activity (Quinton et al., 2006; Kish et al., 2008), hyperthermia, and decrease in appetite (Yamamoto *et al.*, 2010). Moreover, at long-term use originates mood disturbances, confusion, anxiety, weight loss, insomnias, among others (Buchanan *et al.*, 2010). In fact, chronic METH abusers can experience psychotic and violent behavior, verbal learning and memory impairment, hallucinations, and even seizures (Quinton *et al.*, 2006; Ramirez *et al.*, 2009, Yamamoto *et al.*, 2010; Buttner, 2011).

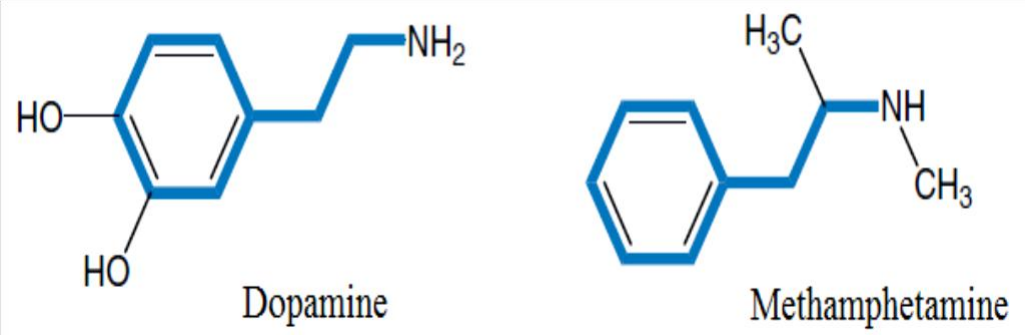

Figure 9 - Chemical structure of methamphetamine and dopamine showing their similarities in blue (adapted from Fleckenstein *et al.*, 2007).

1.8.1. Neurotoxicity

METH neurotoxicity has been characterized by the disruption of synaptic integrity of the dopaminergic system. This drug has the ability to increase neuronal release of monoamines, particularly the catecholamine dopamine (DA), which is the major neurotransmitter impacted by METH use. This occurs due to alterations in both the membrane dopamine transporter (DAT) and the vesicular monoamine transporter-2 (VMAT-2) (Riddle *et al.*, 2006). The function of VMAT-2 in normal cells is to sequester cytoplasmic DA into vesicles for storage and subsequent release. METH interferes with the function of VMAT-2, impairing its ability to store DA into vesicles (Fumagalli *et al.*, 1999; Fleckenstein *et al.*, 2003). Additionally, under normal conditions, DAT clears extracellular DA from the synaptic cleft back into the nerve terminal. This reuptake of DA will further allow the storage of this neurotransmitter into synaptic vesicles via VMAT-2 for later release (Riddle *et al.*, 2006).

When METH reaches the brain there is an increase in extracellular DA that leads to the formation of DA quinones and hydroxyl radicals which results in the production of reactive oxygen species (ROS) (Graham *et al.,* 1978; Chiueh *et al.,* 1993). Moreover, METH also increases the release of glutamate that in turn will increase intracellular calcium concentrations causing activation of nitric oxide synthase (NOS) and the consequent generation of reactive nitrogen species (RNS). In turn, ROS and RNS can alter proteins, lipids, and DNA, as well as inhibit mitochondrial function to produce energy deficits in the nerve terminals. This energy depletion may contribute for neuronal damage or even death (reviewed by Davidson *et al.,* 2001). Alterations in neurons will then trigger a response by surrounding cells, such as microglia and astrocytes (Thomas *et al.,* 2004) (Figure 10).

These reactive glial cells may release several factors (*e.g.* TNF-α, NO) that usually aggravate the brain damage. Indeed, glial cells seem to be activated in response to METH, but its effects on microglial cells are not fully understood (Gonçalves et al., 2010, 2012). In fact, animal studies, demonstrated that a single high dose of METH induces a neuroinflammatory response in mouse hippocampus, and this response was characterized by the activation of microglia and production of proinflammatory cytokines, namely TNF-α (Gonçalves *et al.,* 2010). Nevertheless, it has been shown that cytokines, which are mainly released by activated microglia, may have a dual role in response to brain injury (Coelho-Santos, 2012). *In vitro* studies performed by the same group, showed that METH increased TNF-α and IL-6 levels, as well as their receptors protein levels. However, the endogenous proinflammatory cytokines did not contribute to METH-induced microglial cell death. On the other hand, exogenous low concentrations of TNF-α or IL-6 demonstrated a protective effect. In the same study, it was also verified that the anti-apoptotic role of TNF-α was mediated by activation of IL-6 signaling, specifically the janus kinase (JAK)-STAT3 pathway, which in turn induced down-regulation of pro- and anti-apoptotic proteins (Coelho-Santos *et al.,* 2012).

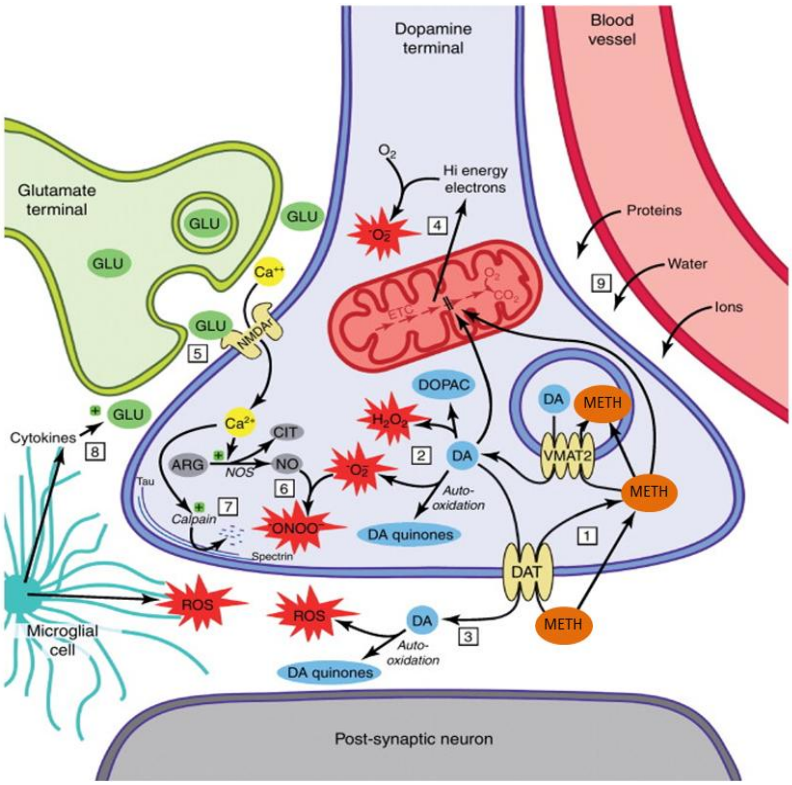

Figure 10- Mechanisms of METH neurotoxicity. METH enters in dopaminergic terminals (1), causing efflux of DA from intraneuronal vesicles. This DA is oxidized intracellularly, producing reactive species (2) such as hydrogen peroxide (H_2O_2) and superoxide ($^{\cdot}O_2^-$), and is transported to extracellular spaces (3) where it is also oxidized producing reactive oxygen species (ROS). High intracellular concentrations of DA and METH can inhibit the electron transport chain (ETC) in mitochondria (4), causing leakage of high-energy electrons which trigger the formation of superoxide. METH-induced increases in GLU release (5)**,** stimulate NMDA receptors (NMDAR) on dopaminergic terminals to cause increases in intracellular Ca^{2+}. These Ca^{2+} increases stimulate nitric oxide synthase (NOS) activity, increasing the production of nitric oxide (NO), which can combine with superoxide to form peroxynitrite ($^{\cdot}ONOO^-$) (6)**.** METH also stimulates microglia to release ROS and cytokines (8) which increase extracellular GLU levels. Finally, METH causes leakage of the BBB, allowing plasma proteins to enter the brain (9) (adapted from Marshall and O'Dell, 2012).

Studies with human subjects have shown that METH chronic users demonstrate structural abnormalities in the brain, namely loss of grey matter, white matter hypertrophy and altered glucose metabolism in specific regions such as hippocampus, prefrontal cortex, cingulated gyrus and amygdale (Thompson *et al.*, 2004). Additionally, data from animals show that a high dose of METH damages striatal dopamine nerve terminals (McCann *et al.*, 2004). However, there is no evidence for dopamine nerve terminal damage in humans who take therapeutic doses of amphetamines (Ricaurte *et al.*, 2005). Although METH can be abused, it can be also used for therapeutic purposes. Indeed, Desoxyn (Methamphetamine Hydrochloride) is a FDA approved drug marketed in USA

to treat attention-deficit hyperactivity disorder (ADHD). Furthermore, METH has been used in other human conditions beyond ADHD, such as mood disturbance (Hart *et al.*, 2005), eating disorders like bulimia nervosa (Drimmer, 2003), and in Parkinson's disease (Devos *et al.*, 2007; Moreau *et al.*, 2012).

1.9. Cell cycle and drugs

Cell division is mainly characterized by two consecutive processes, DNA replication and segregation of replicated chromosomes into two separated cells. Cell division can be divided into two stages: mitosis (M), the process of nuclear division; and interphase, the interval between two M phases. Mitosis stages include prophase, metaphase, anaphase and telophase. At interphase, cells grow in size and pass through G_1, S and G_2 phases. In S phase occurs the replication of DNA. Importantly, this phase is preceded by a gap, G_1, during which the cell is preparing for DNA synthesis, and is followed by a second gap, G_2, when the cell prepares for mitosis. G_1, S, G_2 and M phases are the traditional subdivisions of the standard cell cycle. Cells in G_1 can, before start DNA replication, enter in a resting state called G_0 (Figure 11). Cells in G_0 account for the major part of the non-proliferating, non-growing cells in the human body (Reviewed by Vermeulen *et al.*, 2003).

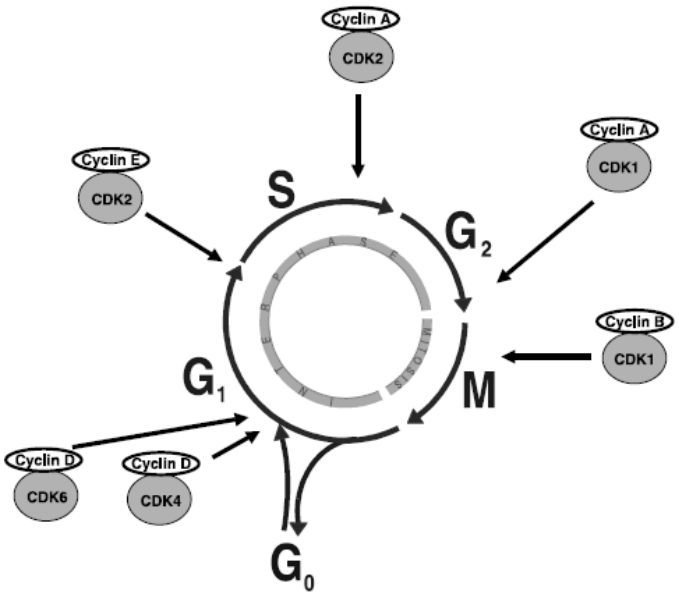

Figure 11 - Cell cycle phases and their regulators: cyclins and cyclin dependent kinases (CDKs) (adapetd from Vermeulen *et al.*, 2003).

The cyclins and their catalytic associates, the cyclin dependent kinases (CDKs), are cell cycle regulators. Cyclins act in concert with their CDKs to drive cells from one phase of the cell cycle to the next (Gautschi *et al.*, 2007), and different cyclins are required at different phases of the cell cycle (Figure 11). The three D type cyclins (cyclin D1, D2 and D3) bind to CDK4 or to CDK6, and CDK-cyclin D complexes are essential for entry in G_1 phase. Unlike the other cyclins, cyclin D is not expressed periodically, but is synthesized as long as growth factor stimulation persists. Another G_1 cyclin is the cyclin E, which associates with CDK2 to regulate progression from G_1 into S phase (reviewed by Vermeulen *et al.,* 2003). Cyclins D1 and E are involved in proliferation, apoptosis, invasion, differentiation and angiogenesis (Wang *et al.,* 2012; Spruck *et al.*, 1999). In fact, these cyclins are considered to be key oncogenes and they are overexpressed in breast, liver, lung and brain cancers (Gillet *et al.,* 1996; Hall *et al*., 1996; Molenaar *et al*., 2008; Akli and Keyomarsi 2003). Regarding cyclin A, it binds to CDK2 and this complex is required during S phase. In late G_2 and early M, the complex cyclin A-CDK1 promotes entry into M phase. In line with this, cyclin A overexpression has been found to be an adverse prognostic factor in non-small-cell lung cancer (Volm *et al.,* 1997), breast cancer (Bukholm *et al.,* 2001) and colorectal cancer (Handa *et al.,* 1999). In addition, expression of cyclin A has been found to be associated with a high malignancy potential in sarcomas (Huuhtanen et al., 1999) and prostate cancer (Aaltomaa *et al.,* 1999). Mitosis is further regulated by cyclin B-CDK1 complex (reviewed by Vermeulen *et al*., 2003).

Cyclins expression could be altered by several drugs (Basso *et al.,* 2002; Li *et al.,* 2000; Hashemolhosseini *et al.,* 1998). DOX, MTX and METH are able to change cyclins expression or/and function and consequently deregulate cell cycle. Concerning cell cycle, METH down-regulates D1 and A1 gene expression in neuroblastoma cells (Bachmann *et al.,* 2009). Recently, our group also demonstrated that METH delayed cell cycle in the G1-to-S phase transition, which was correlated with a decrease in cyclin E and pERK1/2 protein levels in DG neurospheres (unpublished data).

MTX, as an antimetabolite that hind the biosynthesis of purines and thymidines (Boiardi *et al.*, 1999) necessary for DNA and RNA synthesis, has a great influence in cell cycle. Some studies involving oral carcinomas cells, demonstrated that MTX treatment leads to a reduction of cyclin B1 expression (Pavelic *et al.,* 2009) and to a cell cycle arrest at the S and G_2 phases (Duran *et al.,* 2001).The MTX-induced cell cycle arrest at S phase was also observed in primary cultured astrocytes (Bruce-Gregorios *et al*., 1991),

melanoma (Sáez-Ayala *et al.,* 2012), osteosarcoma (Martins-Neves *et al.,* 2012), leukemia (Tsurusawa *et al*., 1990) and breast cancer cells (Costantini *et al*., 2010).

It is known that DOX induces cellular apoptosis by intercalating into the DNA (Zhang *et al.,* 2010) destabilizing the normal cell cycle. Studies involving glioma cells, showed that around 70% of the cells treated with DOX were in G_0/G_1 phase, which proves the DOX ability to inhibit cell cycle progression. This inhibition could also be related with the decrease of cyclin E protein expression that is required for the cell cycle progression into the S phase (Gopinath *et al.,* 2009). DOX has a similar effect in human colon cancer cells, arresting cells in G_0/G_1 phase (Lupertz *et al*., 2010). However, in human breast carcinoma cells (Kuznetsov *et al.,* 2011), osteosarcoma cells (Martins-Neves *et al.,* 2012) and bladder cells (Bilim *et al.,* 2000) treated with DOX showed a significant increase in cells arrested in the G_2/M phase of the cell cycle.

1.10. Methamphetamine and Blood-Brain Barrier

There are several studies showing that METH can lead to BBB disruption. Animal studies demonstrated that mice administered with an acute high dose of METH show an increase in BBB permeability in the medial and ventral amygdala, hippocampus (Martins *et al*., 2011; Bowyer and Ali, 2006) caudate-putamen (Bowyer *et al.*, 2008) and striatum (Urrutia *et al.,* 2013). Moreover, *in vitro* studies published by Mahajan and others (2008) demonstrated that METH alters the BBB function through direct effects on endothelial cells, specifically by modulating the expression of tight junction proteins, and also by enhancing the production of reactive oxygen species (Ramirez *et al.*, 2009). More recently, it was demonstrated that METH transiently increases BBB permeability in the hippocampus due to a downregulation of the TJ proteins, namely ZO-1, claudin-5 and occludin, which may be also correlated with the increase in MMP-9 activity and expression by hippocampal neurons (Martins *et al,* 2011). Additionally, it was demonstrated that the decrease in BBB integrity in the stratum induced by METH was mediated by the JNK pathway, which activates MMP-9 causing degradation of laminin and BBB leakage (Urrutia *et al.,* 2013). Other explanation for METH-induced BBB permeability was raised by Park and collaborators (2011) by demonstrating the activation of a NOX complex and caveolae-associated pathways that resulted in the generation of ROS and alterations of occludin protein levels. Recently, our group showed that the increased in endothelial cells permeability triggered by METH involves eNOS/NO-

mediated transcytosis (Martins *et al.*, 2013). Additionally, METH also seems to interfere with the glucose uptake by endothelium cells compromising the BBB normal function. Studies conducted by Abdul Muneer *et al.* (2011) demonstrated that an impairment of GLUT1 at the brain endothelium by METH may contribute to energy-associated disruption of tight junction assembly and loss of BBB integrity.

It is worthwhile to point out that METH-induced BBB permeability could be considered an useful approach to allow the passage across BBB of drugs that could have a potential therapeutic effect in brain diseases, such as chronic myelogenous leukaemia and glioblastoma (Kast, 2009; Kast and Focosi, 2010). In clinics, METH is successfully used to treat attention-deficit hyperactivity disorder, mood disturbance (Hart *et al.*, 2005) and eating disorders (Drimmer, 2003), as it was mentioned previously. This drug is, in general, well tolerated with low risk of abuse in this patient's and could be easily administered to normal or medically ill and frail patients. Methamphetamine's high doses and long term use should be avoided due to its addictive and neurotoxic potential. Nevertheless, it has been discussed that risks of low doses and short-term use of METH are low and well known by the physicians. So, under conditions of brain tumors the severity of CNS malignancy is such that the small risks of METH use could be justifiable (Kast, 2007). Importantly, METH ability to generate reversible disruption of BBB in rodents, adding to no evidence of brain damage in humans who take therapeutic doses of amphetamines and the pharmaceutical circulating half-life of 9 to15 hours, C_{max} of 1 hour (Kast and Focosi, 2010), can support the use of METH to open the BBB during intravenous or oral administration of MTX or DOX to allow the entrance of these chemotherapeutic drugs into the brain.

CHAPTER 2

MATERIAL AND METHODS

CHAPTER 2

Material and Methods

2.1. Cell culture

The human glioblastoma cell line U-118MG (American Type Culture Collection, Manasas, VA, USA) was cultured in Dulbecco's modified essential medium (DMEM) high glucose (4 g/L; Gibco, Scotland, UK) supplemmented with 10% fetal calf serum (FBS; Gibco), 3.7 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco), at pH 7.4. Cells were maintained in a humidified atmosphere with 5% CO_2, at 37°C, and were discarded after 40 passages.

2.2. MTT assay

The effects of METH and of chemotherapeutic agents (DOX, MTX and TMZ) on U118 cells were analyzed using the [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT) colorimetric assay. This method is based on the enzyme mitochondrial dehidrogenase NADH activity which is active only in viable metabolically active cells. This enzyme reduces the yellow tetrazolicum salt converting it in insoluble purple crystals (formazan crystals). Formazan crystals can be quantified by spectrophotometry where the resultant intensity is proportional to the enzyme activity and, consequently, to the viable cells number.

U118 cells at a confluence of 70% were harvested and seeded in 96 multiwell plates at a density of 150 000 cells/well in 100 µL and allowed to attach overnight. Then, cells were treated with METH or/and anti-tumoral drugs as follows (in µM): METH (Methamphetamine hydrochloride, Sigma, USA) - 0.01, 1, 10, 100, 500, 650, 850, 1000 µM; DOX (DOXO-cell®, Portugal) - 0.001, 0.01, 0.25, 0.5, 1, 5, 10, 50, 100; MTX (Teva Pharma, Portugal) - 0.001, 0.01, 0.025, 0.05, 0.075, 0.1, 1, 10, 100, 1000; and TMZ (Sigma Chemicals, St. Louis, MO, USA) - 0.01, 1, 10, 25, 50, 75, 100, 250, 500, 1000. After 48 h of drug exposure, cells were washed with Krebs solution [142 mM NaCl, 4 mM KCl, 1 mM MgCl, 1 mM CaCl, 10 mM Glucose, 10 mM HEPES pH 7.4], and 0.5 mg/mL MTT (Sigma) solution was added to each well and incubated at 37 °C, for 1 h, protected from light. The formazan crystals were vigorously dissolved with 0.04 M isopropanol-HCl (50 µl per well). Absorbance was measured at 570 nm (reference 620 nm) using the SynergyTM HT

multi-detection microplate reader (BioTek, Windoski, USA). The results were expressed as percentage of control and the IC_{50} values (concentration of drug that reduces cell survival by 50%) were calculated using non-linear regression analysis (GraphPad Prism).

2.3. Migration assay

U-118 cells were seeded in 12-well plates at a density of 200 000 cells/ well and allowed to attach overnight. Then, cells were treated for 24 h with DOX (0.25 μM) or MTX (0.01 μM) alone or plus METH (1 μM). When plates were confluent, the cell monolayer was scraped in a straight line with a small tip, culture medium was removed in order to clean debris, and new culture medium was added. The plate was observed under the phase-contrast microscope and an image from each well was acquired at 0 h, 4 h, 8 h, 24 h, 28 h, 32 h and 48 h, after the scratch was done. Migration was evaluated using a homemade MATLAB-based software (Matlab®). Briefly, for each condition, all images were filtered with an adaptive filter to smooth the image while preserving the edges, and then a region of interest was drawn in the first image, which was used as reference (0h) Afterwards, the algorithm analyze the other images obtained at different time points by measuring the signal in the region defined by the user, and consequently the level of cell migration. Each experiment was repeated six times.

2.4. Chemotaxis assay

Chemotaxis assay was carried out using HTS 96 transwell permeable supports with 8 μm pores (Corning, NY, USA). Cells were starved for 24 h, resuspended in Krebs solution and plated in the transwell (upper chamber). CXCL12 (Peprotech, Rocky Hill, NJ, USA) (100 ng/mL) was added to the medium in the lower chamber. Cells were treated with DOX (0.25 μM) or MTX (0.01 μM) alone or plus METH (1 μM) at 37ºC in a 5% CO_2 incubator for 12h. At least one well per experience was used as blank (with no cells) and as a control without CXCL12. After this period, calcein-AM (Invitrogen) (0.002 μg/μL) was added to the lower chamber, at 37ºC for 1 h, followed by fluorescence measurement at 485 nm and 528 nm (excitation and emission, respectively) using the SynergyTM HT multi-detection microplate reader (BioTek, Windoski, USA) (Figure 12).

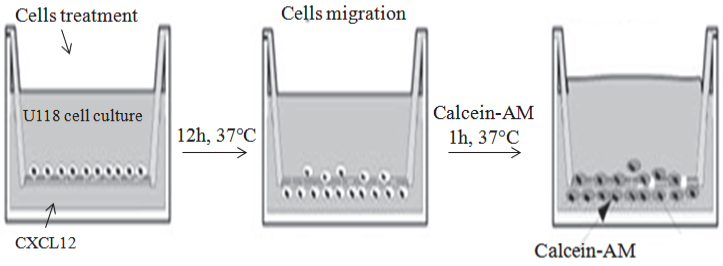

Figure 12 - Schematic representation of the chemotaxis assay using HTS 96 transwell permeable support. U-118 cells were seeded into the upper chamber, while CXCL12 was added into the lower chamber. The chamber was incubated at 37° C, 5% CO_2. After 12 hours of incubation, invasive cells that passed through the membrane were labeled with calcein-AM. After 1h of incubation, the fluorescence was measured. The percentage of invaded cells was calculated using a standard curve performed before the assay.

A standard curve was performed for each assay (cells/well: 50000, 25000, 12500, 6250, 3125, 1562.5, 781.25, 0). A volume of 50 µl from dilution was added in triplicate to a well from a 96-well solid black microplate. Additionally, 50 µl of calcein AM (0.002 µg/µL) was added to each well of the standard curve. The plate was incubated for 1 h in the dark at room temperature then read using the fluorescence top reader option in the SynergyTM HT multi-detection microplate reader (485 nm and 528 nm, excitation and emission, respectively). The obtained relative fluorescence units (RFU) values for each concentration were averaged and then background (no cells) was subtracted.

The total number of cells that passed through the membrane of each well was calculated as shown in the equation:

$$\% \text{ Invasion} = \frac{\text{Cell concentration}}{\text{Inicial cell seeded}} \times 100$$

2.5. Flow cytometry

Cells were plated in 12-well plates at a density of 300 000 cells/well and allowed to attach overnight. Then, cells were treated with DOX (0.25 µM) or MTX (0.01 µM) alone or plus METH (1 µM) for 24h or 48h, Afterwards, cells were centrifuged at 132 x*g* for 5 min, the culture medium was discarded and the pellet was resuspended in a solution of 75% ethanol and maintained overnight at 4°C. Fixed cells were then centrifuged at 206 x*g* for 20 min, the pellet was resuspended and incubated for 1 h 15 min in the dark at RT in a solution of 1x PBS containing 10 µl/ml propidium iodide (PI, Invitrogen, Paisley, UK) and 500 µg/ml RNAse (Sigma). The PI fluorescence was measured on a FACScan flow cytometer (BD FACSCalibur™) and the data were gated to exclude cell debris and aggregates by using the software ModFit LT™ 3.0. For each sample were acquired

$2x10^4$ events.

2.6. Western blot

Glioblastoma cells were seeded in six-well plates at a density of 350 000 cells/mL, and when 70% confluence was reached, cells were treated with DOX (0.25 μM) or MTX (0.01 μM) alone or plus METH (1 μM) for 48h. Cytosolic fraction was obtain by lysing the cells using a buffer solution composed by 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA (Ethylenediamine tetraacetic acid), 0.4% NP-40 and 1 mM dithiothreitol (DTT) (Bioron, Porto, Portugal), supplemented with protease inhibitor cocktail tablets (Roche, Indianapolis, IN, USA). Lysates were placed on ice for 30 min and centrifuged at 16000 x*g*, at 4°C for 7 min. The supernatant (cytosolic fraction) was collected and the pellet was washed with 1 ml 1x PBS (Phosphate Buffered Saline) and centrifuged at top speed for 1 min. Nuclear fraction was obtain by resuspending the pellet using a buffer solution composed by 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 10% glycerol and 1 mM dithiothreitol (DTT) supplemented with inhibitor cocktail tablets. The resuspension was placed on ice for 1h, sonicated and centrifuged at 16000 x*g*, at 4°C, for 7 min. The supernatant was saved and pellet was discarded. Protein quantification was performed using the bicinchoninic acid (BCA) method (Pierce, Rockford, USA) with BSA as a standard. Cytosolic fraction was stored at -20°C and nuclear fraction at -80°C until further use. Protein samples were prepared under reduced conditions by adding sample buffer (0.5 M Tris-HCl, 4% SDS, 30% glycerol, 10% SDS, 0.6 M DTT, bromophenol blue; pH 6.8) and heating at 95°C for 5 min. Proteins (Table 1) were separated by electrophoresis on 12% polyacrylamide gels at 160 V and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Madrid, Spain) that were blocked with 5% (w/v) low fat milk in PBS-T [PBS containing 0.1% (v/v) Tween-20 (Sigma)]. Then, membranes were incubated overnight at 4°C with primary antibodies as described in Table 3. After washing with PBS-T during 30 min, membranes were incubated with the respective alkaline phosphatase-conjugated secondary antibody (anti-mouse 1:10000 or anti-rabbit 1:20000) (Amersham GE Healthcare Life Science, USA), for 1 h, at RT. The membranes were again washed with PBS-T, and bands were visualized, using the enhanced chemiofluorescence (ECF) reagent (Amersham), on the Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden). To ensure equal sample loading, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used in cytosolic fraction samples and lamin B antibody was used in nuclear fraction samples (Table 2). Quantification of band density was performed using ImageQuant 5.0 software.

Table 2 – List of proteins identified by western blot analysis.

Protein	Molecular weight (kDa)	Amount of protein (µg)
Cyclin D1	36	25
Cyclin A	60	25
Lamin B	66	25
GAPDH	37	25

Table 3 - List of primary and secondary antibodies used in western blot studies.

Primary antibody	Dilution	Source	Secondary antibody	Dilution	Source
Mouse anti-cyclin D1	1:100	Santa Cruz Biotechnology	Anti-mouse IgG alkaline phosphatase conjugated	1:10000	Amersham GE Healthcare Life Science
Mouse anti-cyclin A	1:200	Abcam	Anti-mouse IgG alkaline phosphatase conjugated	1:10000	Amersham GE Healthcare Life Science
Rabbit anti-lamin B	1:1000	Abcam	Anti-rabbit IgG alkaline phosphatase conjugated	1:20000	Amersham GE Healthcare Life Science
Mouse anti-GAPDH	1:1000	Abcam	Anti-mouse IgG alkaline phosphatase conjugated	1:10000	Amersham GE Healthcare Life Science

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Data was analyzed using the one-way ANOVA analysis of variance followed by Dunnett's post hoc to compare experimental conditions with control. Data were considered to be statistical different at values of $P<0.05$ and were presented as means + SEM (standard error of the mean).

In cell cycle and migration studies, data were analyzed using an unpaired one tailed Mann-Whitney test or one-way ANOVA analysis of variance followed Bonferroni's post hoc to compare differences between experimental conditions.

CHAPTER 3

RESULTS

CHAPTER 3

Results

Effect of methamphetamine and chemotherapeutic drugs on:

3.1. Glioblastoma cell viability

In the present study our main goal was to evaluate the possible effect of METH as a co-adjuvant for chemotherapeutic drugs, specifically as a toll to transiently open the BBB. So, we started by analyzing the effect of METH on cell viability, and for that, we used the MTT assay. METH induced a significant decrease in U-118 cell viability for a drug concentration equal and above 650 µM (Figure 13; control: 100.0+5.3%; METH: 0.01 µM, 97.9+5.6%; 1 µM, 101.9+9.1%; 10 µM, 97.5+9.8%; 100 µM, 95.2+6.7%; 500 µM, 82.0+4.2%; 650 µM, 74.8+6.6%; 850 µM, 51.1+6.4%; 1000 µM, 0.9+0.2%).

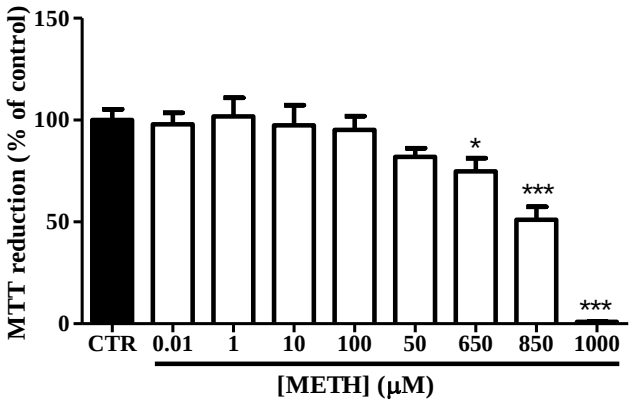

Figure 13- Effect of METH on glioblastoma cell viability. U-118 cells were exposed to increasing METH concentrations (0.01–1000 µM) for 48 h. Cell viability significantly decreased at a concentration equal or above 650 µM METH. The results are expressed as mean % of control + SEM, n=4 performed in triplicate. **P* <0.05,****P* <0.001, significantly different when compared to the control using one way ANOVA followed by Dunnett's Multiple comparison test.

For this work we were particularly interested in identify a non-toxic concentration of METH, *e.g.* that could increase endothelial cells permeability without leading to cell death. In fact, a previous work by our group showed that 1 µM METH increases endothelial cell permeability, without affecting cell viability (Martins *et al*., 2011). Additionally, we also know that METH at low

concentrations can increase cell proliferation (Coelho-Santos *et al.*, 2012). So, based on our previous studies and in our results (Figure 13), we decided to use 1 μM METH in the following studies.

MTX is a chemotherapeutic agent that is widely used in pediatric neuro-oncology and function as a folate antagonist with a potential effect against malignant gliomas (Wolff *et al.*, 1999, 2011). DOX has also been demonstrated to have a cytotoxic activity against highly invasive human glioblastoma cell lines (Nabissi *et al.*, 2013). However, unlike TMZ that crosses the BBB, these chemotherapeutic drugs have limited penetration into the brain. Thus, the transient opening of BBB induced by METH (Martins *et al.*, 2011, 2013) could be considered an useful therapeutic approach to allow the entrance of these drugs. To explore this idea, we further evaluated U-118 cell viability in the presence of DOX or MTX alone or in the presence of METH. Cells were treated with different concentrations of DOX (0.001, 0.01, 0.25, 0.5, 1, 5, 10, 50, 100 μM) for 48h and it was observed a concentration-dependent decrease in cell viability (Figure 14). Moreover, METH did not significantly interfere with the effect of DOX alone (Table 4, Figure 14) and, indeed, the IC_{50} values were very similar (DOX: 0.23±0.05 μM; DOX+METH: 0.21±0.07 μM) (Table 6).

Table 4 - Effect of DOX and DOX plus METH (1μM) on glioblastoma cell viability.

	Cell viability (% of control)	
[DOX] (μM)	**DOX**	**DOX+METH**
0	100±7.8	100±2.9
0.001	99.2± 9.5	110.0±8.7
0.01	97.8±9.6	109.6±8.7
0.1	77.5±14.1	86.8±8.2
0.25	52.3±13.4	42.1±15.2
0.5	13.2±2.5	13.6±3.5
1	7.8±3.4	6.8±2.6
5	0.6±0.4	0.5±0.2
10	0.6±0.3	0.4±0.2
50	0.8±0.5	0.5±0.2
100	1.1±0.7	0.7±0.2

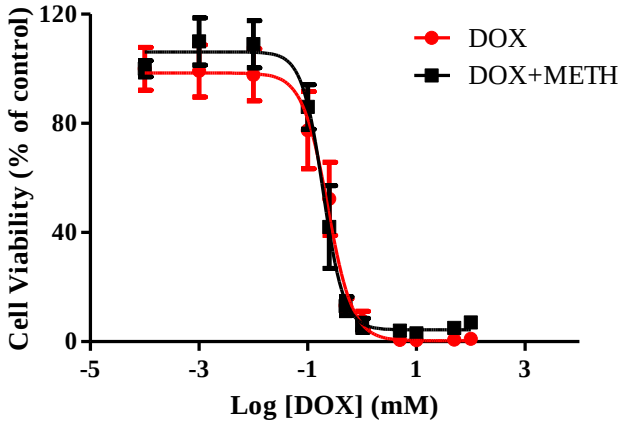

Figure 14 - Effect of DOX and DOX plus METH (1 µM) on glioblastoma cell viability. U-118 cells were treated for 48 h with increasing concentrations of DOX (0.001, 0.01, 0.25, 0.5, 1, 5, 10, 50, 100 µM) or in combination with 1 µM METH. Cell viability was determined by MTT assay. Data are expressed as mean ± SEM and were fitted to a sigmoid function for IC_{50} calculation; n=6, performed in triplicate.

As above mentioned for DOX, U-118 cells were also treated with MTX (0.001, 0.01, 0.025, 0.05, 0.075, 0.1, 1, 10, 100, 1000 µM), which induced a decrease in cell viability in a concentration-dependent manner (Table 5, Figure 15). No significant differences between MTX alone and MTX+METH were observed (Table 6: IC_{50} values – MTX: 0.01+0.005 µM; MTX+METH: 0.01+0.001 µM).

Table 5- Effect of MTX and MTX plus METH (1µM) on glioblastoma cell viability.

	Cell viability (% of control)	
[MTX] (µM)	**MTX**	**MTX+METH**
0	100±5.5	100±4
0.001	100.3±7.4	103.1±7.2
0.01	54.7±15.1	53.2±5.5
0.025	26.8±6.5	27.7±3.4
0.05	17.1±3.6	17.5±2.9
0.075	11.8±4.5	11.2±2.7
0.1	10.2±4.3	9.4±2.4
1	8.7±2.2	7.6±2.1
10	6.8±2.1	5.1±1.3
100	5.8±2	4.5±1.7
1000	4.8±2	4.1±1.5

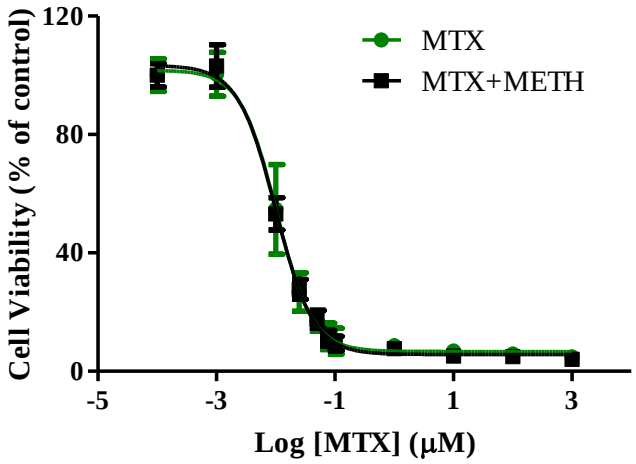

Figure 15 - Effect of MTX and MTX plus METH (1µM) on glioblastoma cell viability. U-118 cells were treated for 48 h with different concentrations of MTX alone (0.001, 0.01, 0.025, 0.05, 0.075, 0.1, 1, 10, 100, 1000 µM) or in combination with 1 µM METH. Cell viability was determined by MTT assay. Data are expressed as mean ± SEM and were fitted to a sigmoid function for IC_{50} calculation; n=5, performed in triplicate.

Table 6 - IC_{50} values obtained by MTT assay after 48h of drugs exposure.

Drug(s)	IC_{50} (µM)
DOX	0.23 ± 0.02 (n=6)
DOX+METH	0.21 ± 0.04 (n=4)
MTX	0.01 ± 0.002 (n=5)
MTX+METH	0.01 ± 0.0004 (n=4)

TMZ is widely used in standard chemotherapy in clinics but still has some limitations (reviewed by Chamberlain, 2010). Here, we also evaluated its effect on U-118 cell viability (Figure 16) and a significant decrease was observed only for concentrations above 500 µM, indicating that U-118 cells are highly resistant to TMZ. Additionally, METH, as previously observed with DOX and MTX, did not significantly interfere with the cytotoxicity of TMZ in U-118 cells (Table 7, Figure 16).

Table 7 - Effect of TMZ and TMZ plus METH (1µM) on glioblastoma cell viability.

	Cell viability (% of control)	
[TMZ] (µM)	**TMZ**	**TMZ+METH**
0	100.0+3.7	100.0+1.4
0.01	103.7+3.2	96.8+5
1	109.3+2.1	108.0+3.1
10	102.6+3.6	101.7+4.3
25	100.1+2.2	100.5+4.6
50	102.1+2	102.6+4.6
75	101.4+2.4	101.8+6.2
100	91.45+4.1	99.7+5.6
250	92.37+3.2	93.6+5.2
500	59.14+2.9***	73.8+7**
1000	25.94+2.2***	41.1+6.5***

Data shown are expressed as mean + SEM, n=3 performed in triplicate. ***P*< 0.01,****P*<0.001, significantly different when compared to the respective control (untreated cells) using one way ANOVA followed by Dunnett's Multiple comparison test.

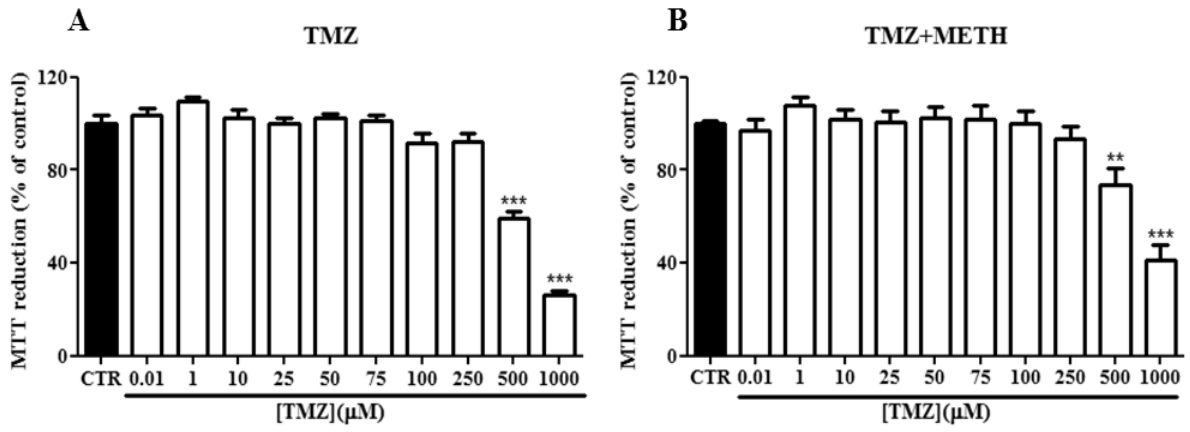

Figure 16 - Effect of TMZ and TMZ plus METH (1µM) on glioblastoma cell viability. U-118 cells were treated for 48 h with different concentrations of (A) TMZ (0.01, 1, 10, 25, 50, 75, 100, 250, 500, 1000 µM) or in combination with (B) METH (1 µM). Cell viability was determined by MTT assay. Data shown are expressed as mean + SEM, n=3 performed in triplicate.***P*< 0.01,****P*<0.001, significantly different when compared to the control using one way ANOVA followed by Dunnett's Multiple comparison test.

3.2. Glioblastoma cell migration

Glioblastoma is characterized by its aggressive behavior and high recurrence rate mainly because of its ability to migrate and invade other brain tissues (reviewed by Furnari *et al*., 2007). Taking this into consideration, we evaluated the effects of 1 µM METH alone and in combination with 0.25 µM DOX or 0.01 µM MTX in the migration of U-118 cells by using a scratch assay. The tested concentrations of DOX and MTX correspond to the respective IC_{50}, obtained from previous studies. The results indicate that DOX or MTX impairs cell migration, whereas METH did not have any effect by itself and also did not interfere with the effect induced by DOX or MTX (Figure 17).

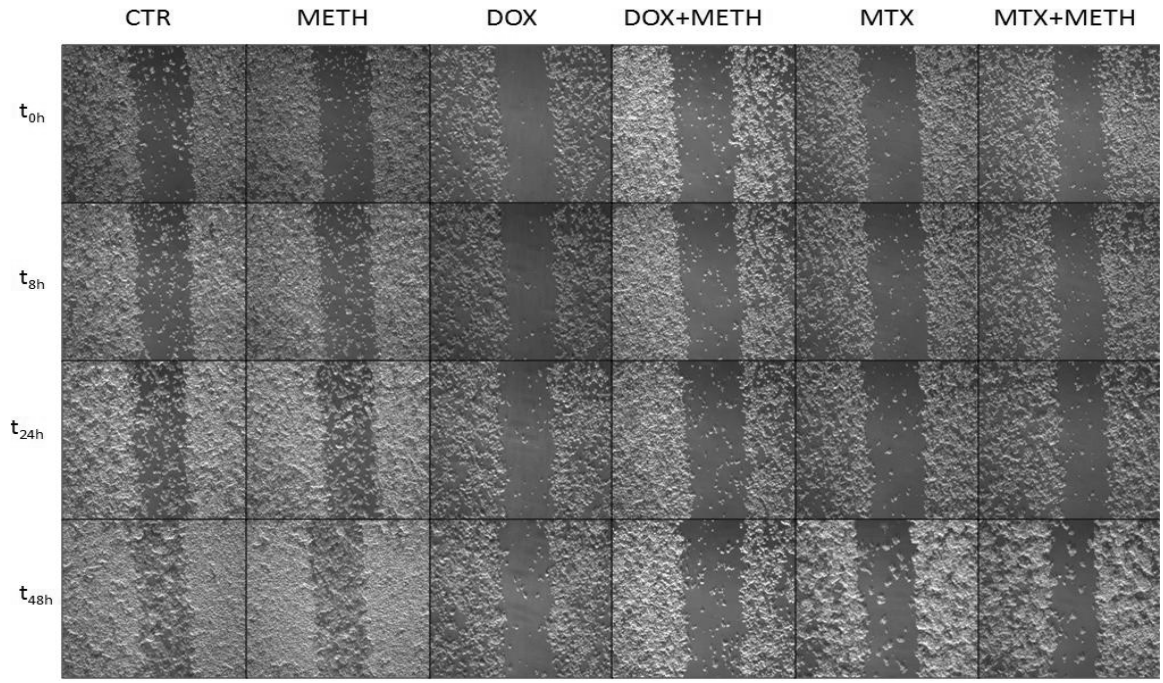

Figure 17- Effect of 24 h treatment with 1 µM METH, 0.25 µM DOX, DOX plus METH, 0.01 µM MTX or MTX plus METH in cell migration, using a scratch assay. Representative images were taken at different time points as follows: starting point, t_{0h}; after 8h, t_{8h}; 24h, t_{24h}; and 48h, t_{48h}. Migration of cells resulted in an increased signal intensity into the scratch. Magnification x10.

Glioblastoma cell migration was quantified by using homemade based-Matlab® software. It was possible to observe that 1 µM METH did not significantly interfere with cell migration at all the time points analyzed (Figure 18, Table 8). However, a significant alteration in glioblastoma cell migration ability was observed in the presence of chemotherapeutic drugs, as demonstrated by the significant reduction in the signal intensity into the scratch (Figure 18, Table 8). This effect was not modified by METH.

Table 8 - Quantification of signal intensity in the scratch under different drug(s) treatment(s).

Time	CTR	METH	DOX	DOX+METH	MTX	MTX+METH
0 h	0+0	0+0	0+0	0+0	0+0	0+0
4 h	1753+573	1945+388	1323+483	1773+527	1298+386	1500+419
8 h	3850+922	3886+767	2608+853	2880+735	2130+820	1864+414
24 h	5873+1196	5695+1109	3130+957	2826+597	2391+690*	2277+488*
28 h	7282+1842	6970+1655	3579+1041*	3287+443*	3177+771*	2822+548*
32 h	7686+1998	6942+1679	3628+1094*	3104+495*	3179+924*	2794+655*
48 h	9386+2166	8928+2043	3375+1316*	3919+888	5667+1295	4800+1472

Data shown are expressed as mean + SEM, n=12. *P<0.05, significantly different when compared to the control at the same time-point using one way ANOVA followed by Dunnett's multiple comparison test.

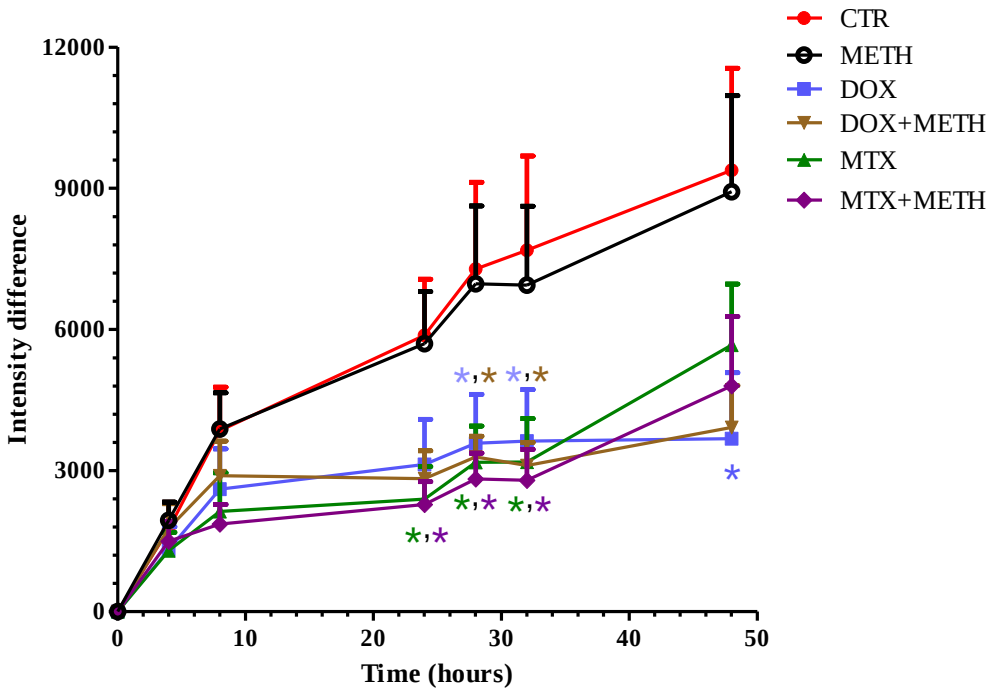

Figure 18 - Quantification of cell intensity in the scratch field using the home-made based-Matlab® software. Intensity difference is directly proportional to the number of cells that migrated into the scratch. Data shown are expressed as mean + SEM, n=12. *P<0.05, significantly different when compared to the control at the same time-point using one way ANOVA followed by Dunnett's multiple comparison test.

3.3. Glioblastoma cell chemotaxis

Previous studies showed that glioblastoma robustly express CXCR4, a chemokine stromal-derived factor-1 (SDF-1 or CXCL12) receptor, and demonstrated a relationship between expression levels of the CXCR4 receptor and the infiltrative extension and growth of the tumor (Maru *et al.*,

2008; Liu *et al.*, 2010, Bian *et al.*, 2007). Here, we also aimed to evaluate the chemotactic activity of U-118 cells treated with 1 µM METH, 0.25 µM DOX, DOX plus METH, 0.01 µM MTX or MTX plus METH. The results indicate that U-118 glioblastoma cells chemotactic ability was significantly increased in the presence of CXCL12 (control:89.8+8.9%; - CXCL12: 3.7+0.55%, ***P <0.001) (Figure 19). Furthermore, the chemotactic ability of glioblastoma cells was significantly decreased in the presence of METH, DOX or MTX when compared to the control (control: 89.8+8.9%; METH: 66.3+3.2%, **P <0.01; DOX: 58.8+4.3%, ***P <0.001; MTX: 62.2+4 %, ***P <0.001) (Figure 7). Additionally, METH did not interfere with the chemotactic ability of DOX or MTX (DOX+METH: 56.8+3.7%; MTX+METH: 64.7+4 %).

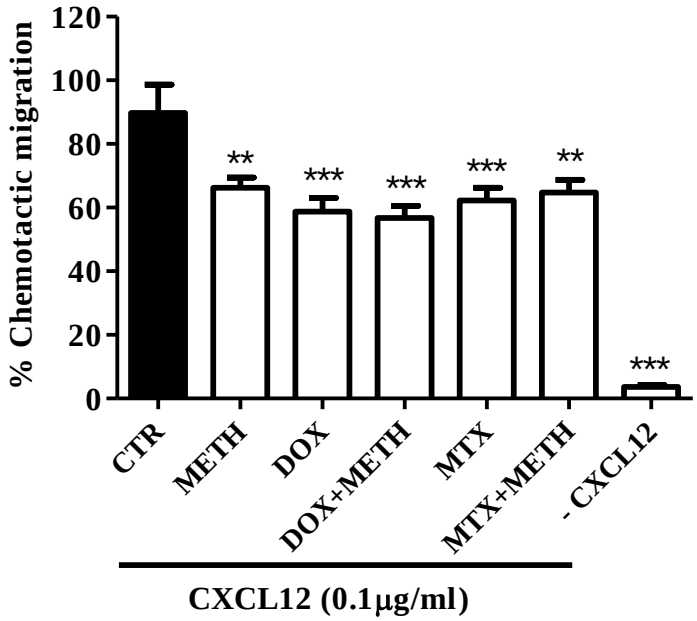

Figure 19 - Effect of METH (1 µM), DOX (0.25 µM), DOX plus METH, MTX (0.01 µM) or MTX plus METH on the chemotactic migration of U-118 glioblastoma cells. The results are expressed as mean % of control + SEM, n=4 performed in quadruplicate. **P<0.01, ***P<0.001 significantly different when compared to the control using one way ANOVA followed by Dunnett's Multiple comparison test.

3.4. Glioblastoma cell cycle

In previous studies it was observed that DOX alter the cell cycle of some types of malignant cells such as glioblastoma, breast carcinoma and colon cancer cells (Gopinath *et al.*, 2009, Kuznetsov *et al.*, 2011, Lupertz *et al*., 2010). MTX also induce cell cycle alterations in melanoma, osteosarcoma and leukemia cells (Sáez-Ayala *et al.*, 2012, Martins-Neves *et al*. 2012, Costantini *et al*., 2010). Additionally, we recently showed that METH interferes with the cell cycle in

hippocampal stem cells (unpublished data). So, in the present study we evaluated the effect of the different drugs and combinations on U-118 cell cycle.

The cell cycle of untreated cells was characterized by a long and well-defined G_0/G_1 peak, with a slightly prominent G_2/M peak (Figure 20). METH by itself did not significantly interfere with cell cycle, neither at 24 h nor at 48 h (Figure 20 and 21; Table 9). Treatment with 0.25 µM DOX for 24 h induced a decrease in the percentage of U-118 cells into the S phase (25.6+3%) and a significant increase in the number of cells in the G_2/M phase (19.2+3.3%,**P* <0.05) relatively to untreated cells (S: 36.8+2.1%; G2/M: 6.1+0.6%). The cell cycle arrest at G2/M phase (18.6+4.7%, ***P* <0.01) together with a lower percentage of cells in S phase (24.5+2%) was maintained after 48h exposure to DOX. Co-incubation with METH did not induce significant alterations in the cell cycle progression neither at 24 h nor at 48 h, comparatively to cells treated with DOX alone.

Regarding MTX, 24 h exposure to this drug induced an arrest of U-118 cells in S phase (control: 36.8+2.1%; MTX: 67.7+12%, **P* <0.05) and a decrease in G_0/G_1 phase (control: 57.3+2.5%; MTX: 30.3+11%) when compared to the control. At 48 h, even though there is a small increase in percentage of cells in G_2/M phase the difference was not significant (control: 4.9+1.4%; MTX:3.3+0.1%; Table 9). Once again, METH treatment did not induce significant alterations in the cell cycle progression neither at 24 h nor at 48 h, comparatively to cells treated with MTX alone (Figure 20 and 21; Table 9).

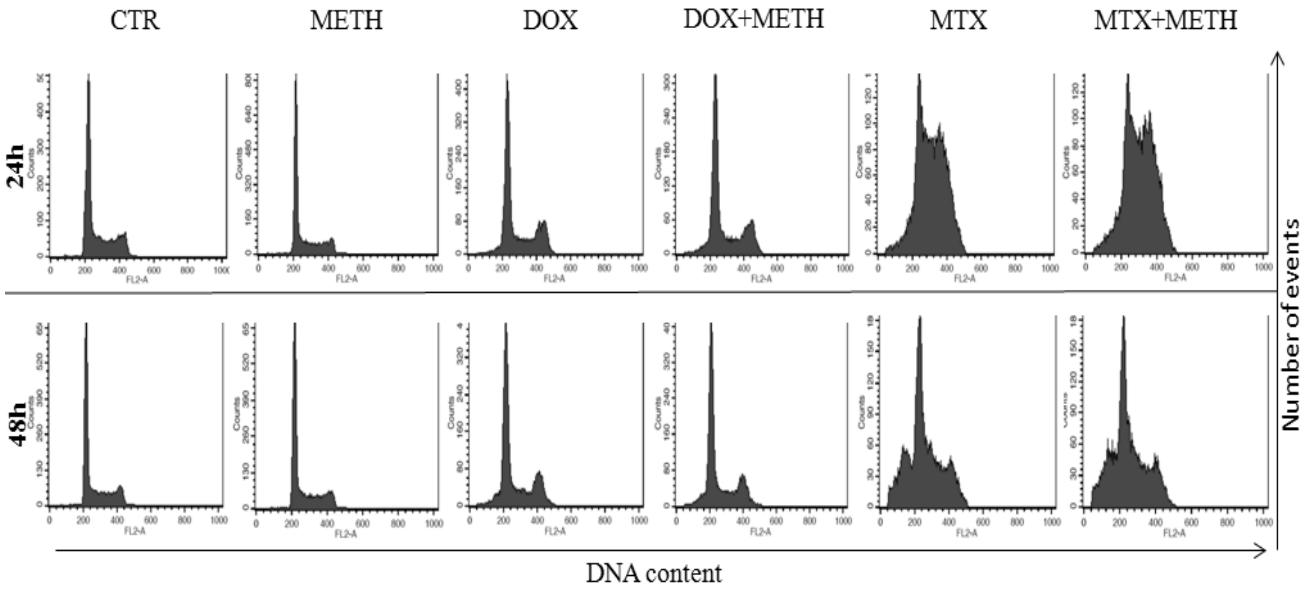

Figure 20 - Representative histograms of U-118 cell cycle after 24 h and 48 h of incubation with METH (1 µM), DOX (0.25 µM), DOX plus METH, MTX (0.01 µM) or MTX plus METH. Cell cycle analysis was performed by flow cytometry using propidium iodide (PI). A total of 20 000 events were analyzed for each experiment, n=3.

Table 9 - Quantitative analysis of the cell cycle distribution after 24 h and 48 h of U-118 cells incubation with METH (1 µM), DOX (0.25 µM), DOX plus METH, MTX (0.01 µM) or MTX plus METH.

	G_0/G_1 phase		S phase		G_2/M phase	
	24 h	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
CTR	57.3+2.5%	59+4.3%	36.8+2.1%	36+3.3%	6.1+0.6%	4.9+1.4%
METH	56.1+2.9%	59.41+2.9%	39.1+3%	35.9+2.4%	4.6+0.2%	4.7+1.3%
DOX	55.2+3.8%	56.9+3.9%	25.6+3%	24.5+2%	19.16+3.3%*	18.6+4.7%**
DOX+METH	55.5+3.6%	59+3.2%	25+1.4%	23.8+2.5%	19.6+2.8%*	17.2+3.8%**
MTX	30.3+11%	31.5+2.6%**	67.7+12%*	65.2+2.5%***	2+1%	3.3+0.1%
MTX+METH	29.8+13.3%	25.7+6.3%**	68.9+14%*	70.6+6.7%***	1.4+0.7%	3.8+0.5%

The results are expressed as mean % of total cells + SEM, n=3. **P*<0.05, ***P*<0.01, ****P*<0.001, significantly different when compared to the control using one way ANOVA followed by Dunnett's Multiple comparison test.

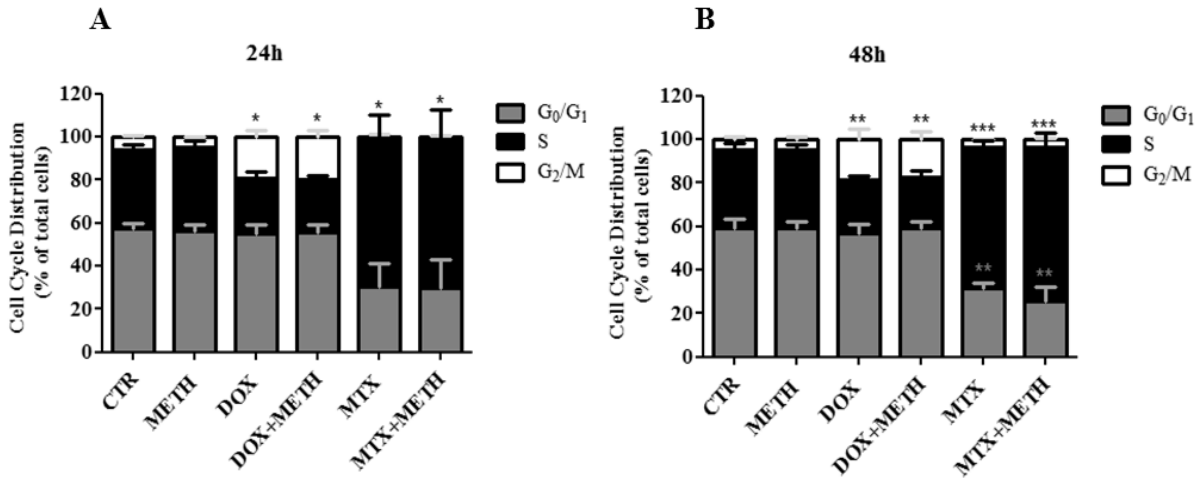

Figure 21 - Quantitative analysis of the cell cycle distribution, after (A) 24 h and (B) 48 h of U-118 cells incubation with METH (1 µM), DOX (0.25 µM), DOX plus METH, MTX (0.01 µM) or MTX plus METH. The results are expressed as mean % of total cells + SEM, n=3. **P* <0.05, ***P* <0.01, ****P* <0.001, significantly different when compared to the control using one way ANOVA followed by Dunnett's Multiple comparison test.

Cell cycle is regulated by several cyclins that act in concert with their cyclin dependent kinases to drive cells through the different phases of the cell cycle (Gautschi *et al.*, 2007). Here, we

evaluated the expression of cyclin A, a critical regulator of the S/G_2 transition and G_2/M phase checkpoint (Figure 22 A). METH (1 µM) did not alter cyclin A protein levels (90.9+3.6%), but DOX (0.25 µM) significantly decrease its protein levels (71.1+4.9% ***P* <0.01; Figure 22 A) compared to the control (100.0+5.5%), an effect that was not altered by METH (METH+DOX, 76.3+6.7%). Moreover, 0.01 µM MTX (83.7+10.4%) or MTX+METH (108.0+3.7%) had no significant effect. These results are in agreement with cell cycle arrest in G_2/M phase that was observed in cells treated with 0.25 µM DOX or DOX plus METH. Additionally, the expression of cyclin D1, a protein responsible for the G_0/G_1 phase progression, was also evaluated (Figure 22 B). However, no significant alterations were observed with all the drugs studied (Figure 22 B - control: 100.0+10.3%; 1 µM METH: 89.6+9.2%; 0.25 µM DOX: 83.3+6.8%; DOX+METH: 81.9+7.2%; 0.01 µM MTX: 79.7+3.7%; MTX+METH: 93.2+8.4%).

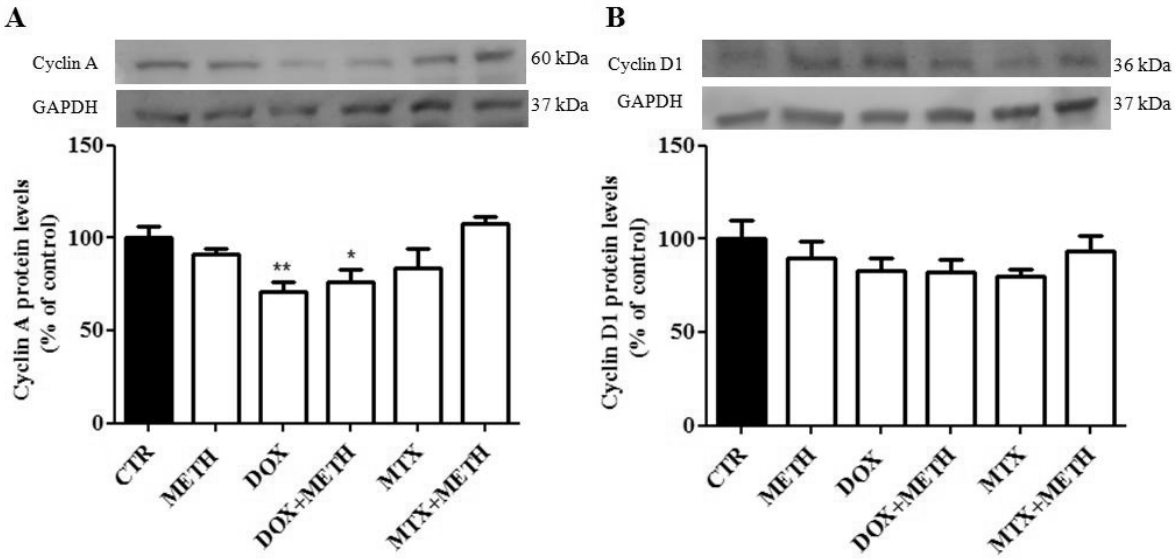

Figure 22- Effect of 48 h treatment with METH (1 µM), DOX (0.25 µM), DOX plus METH, MTX (0.01 µM) and MTX plus METH on the expression of (A) cyclin A and (B) cyclin D1 in U-118 glioblastoma cells. Above the bars, representative western blot images of cyclin A (60 kDa), cyclin D1 (36 kDa) and GAPDH (37 kDa) are shown. The results are expressed as mean % of control+SEM, n=8 (cyclin A) and n=5 (cyclin D1). **P*< 0.05, ***P*< 0.01 significantly different when compared to the control using one way ANOVA followed by Dunnett's Multiple comparison test.

CHAPTER 4

DISCUSSION

CHAPTER 4

Discussion

Glioblastoma multiforme is the most common and aggressive type of astrocytoma. Despite recent therapeutic advances, the median survival time for glioblastoma patients remains short. Among several factors that contribute to the reduced efficacy of treatment in glioblastoma, the blood-brain barrier is one of the most relevant since it limits the delivery of standard chemotherapeutic agents. Taking this into consideration, it was hypothesized if the transient opening of BBB induced by METH (Martins *et al.*, 2011, 2013) could be considered an useful therapeutic approach to allow the entrance of chemotherapeutic drugs, such as MTX (Wolff *et al*., 1999, 2011) and DOX (Nabissi *et al*., 2013). Indeed, these two drugs are successfully used in other type of cancers.

4.1. Effects of methamphetamine and chemotherapeutic drugs on glioblastoma cell viability

In attempt to clarify our hypothesis, we started to evaluate the direct effect of METH on glioblastoma cell viability. Our results showed that METH reduced U-118 glioblastoma cell viability only for higher concentrations above 650 μM. Previous studies performed in our lab with primary cultures of astrocytes (unpublished data) demonstrated that METH is also toxic to astrocytes at similar concentrations ≥500 μM, inducing cells death by apoptosis. Additionally, several other works have shown that METH is toxic to neuronal stem cell (Bento *et al.*, 2011; Baptista *et al.*, 2012), and also to cortical and striatal cells (Deng *et al*., 2001, 2002). Nevertheless, low concentrations of METH were proved to be non-toxic to endothelial cells (Martins et al., 2013) and microglial cells (Coelho-Santos, 2012). Accordingly, based on our results and since our goal was to explore the use of METH as a co-adjuvant of chemotherapeutic drugs, in the present study we used 1 μM METH, a concentration that did not induced U-118 cell death. This METH concentration is also known to be capable of increase endothelial cell permeability, without affecting cell death as above mentioned (Martins *et al*., 2011).

Besides METH, the effect of DOX in cell viability was also evaluated and it was shown that DOX causes a concentration-dependent cytotoxicity in glioblastoma cells, which is in agreement with previous studies (Gopinath *et al*., 2009; Patil *et al*., 2012;

Nabissi *et al.,* 2013). Moreover, METH did not interfere with the cytotoxic effects induced by DOX in glioblastoma cells. The mechanisms by which DOX exerts its cytotoxic effects are related to its ability to disturb DNA function and induce DNA damage. DOX intercalates into DNA double helices, inhibits topoisomerase II and cross-link DNA strands (Gewirtz, 1999). Some studies showed that DOX has the ability to downregulate Bcl-2 mRNA levels (Leung and Wang, 1999). Bcl-2 is an anti-apoptotic protein, whereas Bax is pro-apoptotic, and the balance between both proteins is critical to determine the death or survival of the cell. Pro-apoptotic protein Bax is activated and is translocated to the mitochondrial membranes, where it causes the loss of mitochondrial membrane potential and subsequent cytochrome c release, caspases activation and consequently apoptosis (Narita *et al.*, 1998). Here, we only showed a decrease of cell viability induced by DOX and to clarify the signaling mechanism(s) involved in such effect, further studies are required.

Besides DOX, MTX also induced a concentration-dependent cytotoxicity in glioblastoma cells. MTX inhibits both deoxythymidine monophosphate (dTMP) synthesis and *de novo* purine synthesis, a result of dihydrofolate reductase (DHFR) inhibition that leads to DNA synthesis inhibition and subsequent cell death (Schweitzer *et al.,* 1990). Similarly to DOX, METH at low concentration did not interfere in the cell death induce by MTX.

Regarding TMZ, the current first-line chemotherapeutic agent used in the treatment of glioblastoma, it was observed a significant decrease in cell viability only for higher concentrations above 500 μM, indicating that U-118 cells are more resistant to TMZ. TMZ is an alkylating agent mainly known to act in the formation of O^6-methylguanine in DNA, which mispairs with thymine during DNA replication, leading to activation of apoptotic pathways. Besides this mechanism of action, it was reported that malignant glioma cells can respond to TMZ by arresting cell cycle at G_2/M phase and escape to apoptosis (Hirose *et al.,* 2001). Additionally, Kanzawa and collaborators (2004) reported that TMZ can induce autophagy in glioma cells. The reduced efficacy of TMZ in gliomas was initially attributed to the activity of methylated-DNA-protein-cysteine methyltransferase (MGMT) which removes the DNA adducts, but recently other mechanisms of action and pathways by which glioma cells escape from death are being investigated. Carmo and others (2011) suggested that glioma cells escape from TMZ-induced cell death due to the maintenance of the phosphorylation status of PI3K/Akt and

ERK1/2 MAPK kinase. In this present study, no significant effects in cell viability were observed between TMZ alone or TMZ plus METH, suggesting that 1 μM METH does not interfere in the TMZ cell death mechanisms.

4.2. Effects of methamphetamine and chemotherapeutic drugs on glioblastoma cell migration

Glioblastoma are characterized by their invasive and migratory properties that prevent the complete tumor resection and cause neurological morbidity and mortality (Giese *et al.*, 2003). Since the aim of this work was to explore the possibility of using METH to allow the entrance of chemotherapeutic drugs, it is highly important to evaluate if 1 μM METH, alone or together with DOX or MTX, affects glioblastoma migration. Firstly, we concluded that METH by itself does not interfere in cells migration.

Contrary to METH, it was observed that DOX has the ability to decrease glioblastoma cells migration, and to maintain this trend over time. The ability to decrease the glioblastoma migration is in agreement with previous studies involving DOX and human glioblastoma cells (Gopinath *et al.*, 2009) but also in studies conducted in other type of cancers, such as ovarian cancer cells (Brum *et al*., 2013). This effect, can be justified at least in part by the impairment on cell viability since we used the IC_{50} value. Furhermore, Brum and collaborators showed that DOX-induced cell death and inhibition of cell migration in ovarian cancer cells are associated with cytoskeletal protein reorganization, ataxia telangiectasia mutated (ATM)/p53 activation, and chromatin remodeling (Bum *et al*., 2013). The co-administration of METH did not altered DOX-induced migratory ability.

Regarding MTX, it has also the capacity to decrease glioblastoma migration, and once again METH did not alter this effect. The mechanism responsible for the decreased of glioblastoma migration induced by MTX is still unknown. However, based on the general MTX mechanism of action, it can be hypothesized that the inhibition of nucleotide synthesis, which leads to DNA synthesis inhibition (Schweitzer *et al.*, 1990), will strongly affect the protein expression in all cells and so affecting migration. Specifically, since cell migration is a process that involves a continue expression of cytoskeletal proteins, such as actin and tubulin, monomers of microfilaments and microtubules responsible for the cellular and intercellular movement (Copper and

Hausmann, 2006), the inhibition of DNA synthesis induced by MTX will compromise the entire mechanism involved in cell migration. Other studies also showed that MTX modifies neutrophils migration which may interfere with cytoskeleton elements and metabolic processes (Kraan *et al*., 2000). However, further studies must be done, in order to understand the real mechanism involved in MTX and glioblastoma cells migration.

4.3. Effects of methamphetamine and chemotherapeutic drugs on glioblastoma cell chemotaxis

Chemotaxis is the phenomenon whereby cells direct their movements according to certain chemicals in the environment (reviewed by Wang *et al*., 2011). Chemokines are a family of molecules that regulate the chemotaxis of leucocytes in tissues (Gerard *et al.*, 2001) and more recently recognized as intervenients in the regulation of other cellular processes such as survival, tumor growth and angiogenesis (Vandercappellen *et al.*, 2008). The CXCL12 chemokine and its receptor CXCR4 have been associated with the tumorigenesis in brain cancer (Fulton, 2009). When CXCL12 binds to its receptor, CXCR4 dimerization happens and the activated complex CXCR4/CXCL12 participates in survival, cell proliferation and motility regulation (Barbero *et al.*, 2003). To clarify if METH affects the chemotactic ability of glioblastoma cells, a chemotaxis assay was performed using the chemokine CXCL12 as chemotaxin. In this present work it was observed that glioblastoma cells have a small basal chemotactic ability that significantly increases in the presence of CXCL12, which was been demonstrated already in other study with U-118 glioblastoma cells (Carmo *et al.*, 2010). Here, we observed a significant decreased in glioblastoma cells chemotactic ability in the presence of METH. No specific information is available regarding the effect of METH in cells chemotaxis. However, it was shown that cocaine inhibits the migratory response of neural precursor cells towards CXCL12 stimulus, and this finding was associated with the cocaine ability to induce down-regulation of CXCR4 receptor (Hu *et al*., 2006). Although, additional work is required for a comprehensive understanding of how METH alters chemotaxis in glioblastoma cells.

Regarding DOX it was observed that it had the ability to decrease glioblastoma cells chemotactic migration. This effect was maintained when cells were incubated with DOX plus METH, with no significant differences between both treatments. Based on a

previous study by Carmo and collaborators (2010), the effect of DOX on glioblastoma chemotactic migration could be related with its impact on cell survival, motility or proliferation pathways induced by CXCR4/CXCL12 complex, the PI3K/Akt pathway (Carmo *et al*., 2010). Moreover, it was demonstrated that topoisomerase II inhibitors lead to an increase in ceramide in glioma cells (Sawada *et al*., 2000), which in turn can induce the association between Akt and Protein Phosphatase 1 (PP1) that dephosphorylates Akt (Yao *et al.,* 2012) leading to its inactivation. Taking this into consideration, the ceramide mediated Akt inactivation will contribute to the anti-chemotactic effect exerted by DOX. To clarify whether ceramide and the PI3K/Akt pathway are responsible for this DOX anti-chemotactic effect in glioblastoma cells, more studies must be performed.

The effect of MTX on glioblastoma chemotactic ability was also explored, and the results obtained demonstrated that this antimetabolite has the capacity to decrease the chemotactic migration of glioblastoma cells. This capacity was maintained when cells were incubated with MTX and METH, once again showing that METH does not potentiate MTX-induced impairment of chemotactic migration. Little is known about the role of MTX in chemotaxis. However, a recent study performed by Georgiou and collaborators (2012) showed a deregulation of CXCL12/CXCR4 axis in bone marrow cells treated with MTX, which may be associated with the reduced CXCR4 gene and protein expression following MTX chemotherapy. Alterations in gene expression induced by MTX could be due to the inhibition of DHFR that results in a depletion of the intracellular reduced folate pools required for the biosynthesis of purines and thymidine that leads to DNA synthesis inhibition (Schweitzer *et al.,* 1990). This issue would be of particular interest to clarify how MTX affects glioblastoma cells chemotaxis.

4.4. Effects of methamphetamine and chemotherapeutic drugs on glioblastoma cell cycle

Previous studies conducted in our lab demonstrated that METH can interfere with the cell cycle in hippocampal stem cells (unpublished data). It is also known that chemotherapeutic drugs that target DNA, such as DOX and MTX, alter the normal cell cycle. So, in the present study our goal was to evaluate the effect of METH alone or in combination with DOX or MTX on U-118 cell cycle.

Although in previous studies METH had demonstrated an ability to interfere with the hippocampal stem cells cycle by inducing a delay in the G1-to-S phase transition and

a down-regulation of cyclin E (unpublished data), in the present work METH did not significantly altered the cell cycle of glioblastoma cells. Also, no alterations were observed in the protein levels of cyclin A and cyclin D1. These results suggest that U-118 cells are more resistant to METH-induced cell cycle alterations than the hippocampal stem cells or neuroblastoma cells (Bachmann *et al*., 2009). Actually, studies using microarrays analysis revealed a down-regulation of cyclin D1 and cyclin A1 genes in neuroblastoma cells induced by METH (Bachmann *et al*., 2009). Regarding this issue, it would be interesting to further clarify if there is any alteration in cyclin D1 or cyclin A gene expression in glioblastoma cells treated with METH.

Concerning the effects of DOX in cell cycle, it was observed a significant increase in percentage of cells in the G_2/M phase demonstrating a cell cycle arrest at this point. Checkpoint protein levels were also analyzed and it was possible to conclude that the G_2/M phase arrest was accompanied by a decreased in cyclin A protein levels, the protein responsible for the G_2/M checkpoint. Our results are in agreement with other published works involving osteosarcoma cells (Martins-Neves *et al*., 2012) and breast carcinoma cells (Kuznetsov *et al.,* 2011). Additionally, studies performed in cervical cancer and melanoma cells showed that cyclin A is activated during normal G_2/M progression and that this activation is blocked in G2 arrested cells induced by etoposide, a topoisomerase inhibitor, which suggests that the G_2 phase cyclin A is a target of the G_2 phase DNA damage response. To reinforce the role of cyclin A in G_2/M phase arrest, it was seen that the blockade activation of the G_2 pool of cyclin A during G2 arrest maintained the cycle arrest (Goldstone *et al*., 2001).

This study is in accordance with our showing that DOX is also able to induce a G_2/M checkpoint arrest by compromising the expression of cyclin A, which limits the normal cell cycle progression. Cyclin D1 protein levels were also analyzed, but no differences were observed, which is in agreement with cell cycle analyses that did not show significant alterations in the percentage of cells in G_0/G_1 phase. Moreover, METH did not significantly interfere with the effect of DOX alone in cell cycle and checkpoint regulator proteins.

Here, we also explored the effect of MTX in cell cycle. Previous studies have shown that MTX exerts its toxicity in melanoma and osteosarcoma cells due to a S phase arrest in cell cycle (Sáez-Ayala *et al.,* 2012, Martins-Neves *et al.,* 2012). In line with these studies, MTX also induced a S phase arrest in glioblastoma cells. This arrest was accompanied by a significant decreased in the percentage of cells in the G_0/G_1 phase.

Usually, alterations in G_0/G_1 phase lead to alterations in G_0 checkpoint protein expression, cyclin D1. Based on this, cyclin D1 and cyclin A protein levels were also analyzed but no alterations were observed. These results suggest that the decrease in the percentage of cells in G_0/G_1 and the S phase arrest were not dependent of cyclin D1 nor cyclin A protein expression. Thus, one possible explanation for the S phase arrest seen in glioblastoma cells could be the depletion of reduced folates required for the production of purine deoxyribonucleotides and thymidylate necessary for DNA synthesis and repair (Schweitzer *et al.,* 1990). Once again, MTX effects in cell cycle and in checkpoint regulator proteins were not altered by METH.

Since there were no significant alterations among the cell cycle phases between 24h and 48 h, it could be suggested that the effects of DOX and MTX last for at least 48h. Additionally, our results emphasize that the DOX and MTX effects in glioblastoma cell viability, migration, chemotaxis and cell cycle, are related to their ability to interfere with DNA synthesis and repair.

CHAPTER 5

CONCLUSIONS

CHAPTER 5

Conclusions

With this work it was demonstrated that METH by itself did not interfere with U-118 glioblastoma cell viability, migration and cell cycle, but impaired cell chemotaxis. Moreover, the effects of DOX or MTX on cell viability, migration, chemotaxis and cell cycle were not altered by METH.

In conclusion, since METH is able to transiently open the BBB and did not interfered with the effects of both chemotherapeutic drugs, it is plausible that METH could be considered a co-adjuvant of DOX or MTX for the treatment of glioblastoma. Nevertheless, since METH can be an addictive drug, further studies are needed to consider this a safe approach.

CHAPTER 6

REFERENCES

CHAPTER 6

References

Aaltomaa S., Eskelinen M., Lipponen P., 1999. **Expression of cyclin A and D proteins and their relation to clinicopathological variables and patient survival.** Prostate 38, 175-182.

Abbott N. J., 2004. **Evidence for bulk flow of brain interstitial fluid: significance for physiology and pathology.** Neurochem Int 45, 545-552.

Abbott N. J., Patabendige A. A. K., Dolman D. E. M., Yusof S. R., Begley D. J., 2010. **Structure and function of the blood-brain barrier**. Neurobiol Dis 37, 13-25.

Abbott N.J., Rönnbäck, L., Hansson, E., 2006. **Astrocyte-endothelial interactions at the blood-brain barrier.** Nature Rev Neurosci 7, 41-53.

Akli S., Keyomarsi K. 2003**. Cyclin E and its low molecular weight forms in human cancer and as targets for cancer therapy.** Cancer Biol Ther 2, 38-47.

Arifin D. Y., Lee K. Y. T., Wang C., Smith K. A., 2009. **Role of convective flow in Carmustine delivery to a brain tumor**. Pharmaceut Res 26, 2289-2302.

Arko L., Katsyv I., Park G. E., Luan W, P., Park J. K., 2010. **Experimental approaches for the treatment of malignant gliomas**. Pharmacol Therapeut 128, 1-36.

Bachmann R. F., Wang Y., Yuan P., Zhou R., Li X., Alesci S., Du J., Manji H. K., 2009. **Common effects of lithium and valproate on mitochondrial functions: protection against methamphetamine-induced mitochondrial damage**. Int J Neuropsychopharmacol 12, 805-822.

Baptista S., Bento A. R., Gonçalves J., Bernardino L., Summavielle T., Lobo A., Fontes-Ribeiro C., Malva J. O., Agasse F., Silva A. P., 2012. **Neuropeptide Y promotes neurogenesis and protection against methamphetamine-induced toxicity in mouse dentate gyrus-derived neurosphere cultures**. Neuropharmacol 62, 2413-2423.

Barbero S., Bonavia R., Bajetto A., Porcile C., Pirani P., Ravetti J.L., *et al*., 2003. **Stromal Cell-derived Factor-1α stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt.** Cancer Res 63,1969-1974.

Basso A. D., Solit D. B., Munster P. N., Rosen N., 2002. **Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2**. Oncogene 21(8), 1159-1166.

Behin A., Hoang-Xuan K., Carpentier A.F., Delattre J.Y., 2003. **Primary brain tumours in adults**. Lancet 361,323-331.

Bellavance M. A., Blanchette M., Fortin D., 2008. **Recent advances in blood-brain barrier disruption as a CNS delivery strategy**. AAPS J 10, 166-177.

Bento A. R., Baptista S., Malva O. P., Silva A. P., Agasse F. R., 2011**. Methamphetamine exerts toxic effects on subventricular zone stem/progenitor cells and inhibits neuronal differentiation**. Rejuven Res 14, 205-214.

Bertossi M., Virgintino D., Maiorano E., Occhiogrosso M., Roncali L., 1997. **Ultrastructural and morphometric investigation of human brain capillaries in normal and peritumoral tissues.** Ultrastruct Pathol 21, 41-49.

Bian X.W., Yang S.X., Chen J.H., Ping Y.F., Zhou X.D., 2007**. Preferential expression of chemokine receptor CXCR4 by highly malignant human gliomas and its association with poor patient survival.** Neurosurgery 61, 570-579.

Bilim V., Kawasaki T., Takahashi K., Tomita Y., 2000. **Adriamycin induced G2/M cell cycle arrest in transitional cell cancer cells with wt p53 and p21(WAF1/CIP1) genes**. J Exp Clin Cancer Res 19(4), 483-488.

Boiardi A., Silvani A., Pozzi A., Fariselli L., Broggi G., Salmaggi., 1999. **Chemotherapy is effective as early treatment for primary central nervous system lymphoma**. J Neurol 246, 31-37.

Bonavia R., Bajetto A., BarberoS., Pirani P., Florio T., Schettini G., 2003. **Chemokines and their receptors in the CNS: expression of CXCL12/SDF-1 and CXCR4 and their role in astrocyte proliferation**. Short communication, Toxicology Letters 139, 181-189.

Bota D. A., Desjardins A., Quinn J. A., Affronti M. L., Friedman H. S., 2007. **Interstitial chemotherapy with biodegradable BCNU (Gliadel®) wafers in the treatment of malignant gliomas**. Ther Clin Risk Manag 3, 707-715.

Bowyer J.F., Ali S., 2006. **High doses of methamphetamine that cause disruption of the blood–brain barrier in limbic regions produce extensive neuronal degeneration in mouse hippocampus**. Synapse 60, 521-532.

Bowyer J.F., Robinson B., Ali S., Schmued L.C., 2008. **Neurotoxic-related changes in tyrosine hydroxylase, microglia, myelin, and the blood–brain barrier in the caudate-putamen from acute methamphetamine exposure**. Synapse 62, 193-204.

Bredel M., 2001. **Anticancer drug resistance in primary human brain tumors.** Brain Res Rev 35, 161-204.

Brem H., Gabikian P., 2001. **Biodegradable polymer implants to treat brain tumors**. Journal of Controlled Release 74, 63-67.

Brem H., Piantadosi S., Burger P. C., Walker M., Selker R., *et al*., 1995. **Placebo- controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas**. Lancet 345, 1008-1012.

Bruce-Gregorios J.H., Soucy D., Chen M.G., 1991. **Effect of methotrexate on cell cycle and DNA synthesis of astrocytes in primary culture: flow cytometric studies**. J Neuropathol Exp Neurol 50(63), 63-72.

Brugnoletti F., Morris E. B., Laningham F. H., Patay Z., Pauley J. L., Pui C., Jeha S., 2009. **Recurrent intrathecal methotrexate induced neurotoxicity in an adolescent with acute lymphoblastic leukemia: serial clinical and radiologic findings**. Pediatr Blood Cancer 52, 293-295.

Brum G., Carbone1 T., Still E., Correia V., Szulak K., Calianese D., Wan Y., 2013. ***N*-acetylcysteine potentiates doxorubicin-induced ATM and p53 activation in ovarian cancer cells,** Int J Oncol 42, 211-218.

Bukholm I.R., Bukholm G., Nesland J.M., 2001. **Over-expression of cyclin A is highly associated with early relapse and reduced survival in patients with primary breast cancers.**Int J Cancer 93, 283-287.

Buttner A., 2011. **The neuropathology of drug abuse.** Neuropathology and Applied Neurobiology 37, 118-134.

Cardoso F.L., Brites D., Brito M.A., 2010**. Looking at the blood–brain barrier: Molecular anatomy and possible investigation approaches**. Brain Research Reviews 64, 328-363.

Carmo A., Carvalheiro H., Crespo I., Nunes I., Lopes M. C., 2011. **Effect of temozolomide on the U-118 glioma cell line**. Oncology Letters 2, 1165-1170.

Carmo A., Patricio I., Cruz M. T., Carvalheiro H., Oliveira C. R., Lopes M. C., 2010. **CXCL12/CXCR4 promotes motility and proliferation of glioma cells.** Cancer Biol Ther 9, 56-65.

Chamberlain M.C., 2010. **Temozolomide: therapeutic limitations in the treatment of adult high-grade gliomas**. Expert Rev Neurother 10, 1537-1544.

Chen J., McKay R. M., Parada L. F., 2012. **Malignant Glioma: Lessons from Genomics, Mouse Models, and Stem Cells.** Cell 149, 36-47.

Chetty C., Vanamala S. K., Gondi C. S., Dinh D. H., *et al.*, 2012. **MMP-9 induces CD44 cleavage and CD44 mediated cell migration in glioblastoma xenografts cells.** Cel Signal 24, 549-559.

Chiueh C.C., Miyake J., Peng M.T., 1993. **Role of dopamine autoxidation, hydroxyl radical generation, and calcium overload in underlying mechanisms involved in MPTP-induced parkinsonism**. Adv Neurol 60, 251-258.

Coelho-Santos V., Gonçalves J., Fontes-Ribeiro C., Silva A.P., 2012. **Prevention of methamphetamine-induced microglial cell death by TNF-α and IL-6 through activation of the JAK-STAT pathway.** J Neuroinflamm 9, 1-14.

Cokgor I., Friedman A.H., and Friedman H.S., 1998. **Pediatric Update gliomas**. Eur J Cancer 34, 1910-1918.

Collins V.P., 2007. **Mechanisms of disease: genetic predictors of response to treatment in brain tumors**. Nat Clin Pract Oncol 4, 362-374.

Copper G.M and Hausmann R. E., 2006. **The cell: A molecular Approach**. Washington, D.C.: ASM press. p. 473-514.

Costantini D.L., Villani D.F., Vallis K.A., Reilly R.M., 2010. **Methotrexate, paclitaxel, and doxorubicin radiosensitize HER2-amplified human breast cancer cells to the Auger electron-emitting radiotherapeutic agent (111)In-NLS-trastuzumab**. J Nucl Med 51(3), 477-483.

Crocetti E.,Trama A., Stiller C., Caldarella A., Soffietti R., et al., 2012. **Epidemiology of glial and non-glial brain tumours in Europe**. Eur J Cancer 48, 1532-1542.

Dalmarco E. M., Fröde T. S., 2007. **Methotrexate inhibits integrin adhesion molecules in the mouse model of pleurisy induced by carrageenan**. Braz Arch Biol Techn 50, 777-784.

Davidson C., Gow A. J., Lee T. H., Ellinwood E. H., 2001. **Methamphetamine neurotoxicity: necrotic and apoptotic mechanisms and relevance to human abuse and treatment**. Brain Res Rev 36, 1-22.

de Vries N. A., Beijnen J. H., Boogerd W., van Tellingen O., 2006. **Blood-brain barrier and chemotherapeutic treatment of brain tumors.** Expert Neurotherapeutics 6, 1199-1209.

Deng X., Cai N.-S., McCoy M. T., Chen W., Trush M. A., Cadet J. L., 2002. **Methamphetamine induces apoptosis in an immortalized rat striatal cell line by activating the mitochondrial cell death pathway.** Neuropharmacol 42, 837-845.

Deng X., Wang Y., Chou J., Cadet J. L., 2001. **Methamphetamine causes widespread apoptosis in the mouse brain: evidence from using an improved TUNEL histochemical method.** Mol Brain Res 93, 64-69.

Devos D., Krystkowiak P., Clement F., Dujardin K., Cottencin O., Waucquier N., Ajebbar K., Thielemans B., Kroumova M., Duhamel A., Deste´e A., Bordet R., Defebvre L., 2007.**Improvement of gait by chronic, high doses of methylphenidate in patients with advanced Parkinson's disease.** J Neurol Neurosurg Psychiatry 78, 470-475.

Dinda A.K., Sarkar C., Roy S., Kharbanda K., Mathur M., Khosla A.K., Banerji A.K., 1993. **A transmission and scanning electron microscopic study of tumoral and peritumoral microblood vessels in human gliomas.** J Neurooncol 16, 149-158.

Ding, T., Ma, Y., Li, W., Liu, X., Ying, G., Fu, L., Gu, F., 2011. **Role of aquaporin-4 in the regulation of migration and invasion of human glioma cells**. Int J Oncol 38, 1521-31.

Dixon K. H., Lanpher B. C., Chiu J., Kelley K., Cowan K. H., 1994. **A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells**. J Biol Chem 269, 17-20.

Drimmer E.J., 2003. **Stimulant treatment of bulimia nervosa with and without attention-deficit Disorder: three case reports**. Nutrition 19, 76-77.

Duran N., Allahverdüyev A. M., Eetüner S., 2001. **Flow Cytometric Analysis of the Effects of Methotrexate and Vepesid on the HEp-2 Cell Cycle.** Turk J Med Sci 31 , 187-192.

Engelhard H.H., Groothuis D.G., 1999. **The blood-brain barrier: structure, function, and response to neoplasia.** In: Berger MD, Wilson CB (eds) Gliomas. Saunders, Philadelphia, 115-121.

Ferlay J., Shin H.R., Bray F., *et al.* GLOBOCAN 2008, **Cancer incidence and mortality worldwide: IARC Cancer Base.** No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010. Available from: http://globocan.iarc.fr. accessed on day/month/year.

Fisher J.L., Schwartzbaum J.A., Wrensch M., *et al.*, 2007. **Epidemiology of brain tumors**. Neurol Clin 25, 867-890.

Fleckenstein A.E., Hanson G.R., 2003. **Impact of psychostimulants on vesicular monoamine transporter function**.Eur J Pharmacol 479, 283 - 289.

Fleckenstein A.E., Volz T.J., Riddle E.L., Gibb J.W., Hanson G.R., 2007. **New insights into the mechanism of action of amphetamines.** Annu Rev Pharmacol Toxicol 47, 681-98.

Francesca B., Rezzani R., 2010. **Aquaporin and Blood Brain Barrier**. Curr Neuropharmacol 8, 92-96.

Frei E., Faffe N., Tattersall M. H. N., Pitman S., Parker L., 1975. **New approaches to cancer chemotherapy with methotrexate**.N Engl J Med 202, 846-851.

Fulton A., 2009. **The Chemokine Receptors CXCR4 and CXCR3 in cancer.** Cur Oncol Rep 11, 125-131.

Fumagalli F., Gainetdinov R.R., Wang Y.M., Valenzano K.J., Miller G.W., Caron M.G., 1999**. Increased methamphetamine neurotoxicity in heterozygousvesicular monoamine transporter 2 knock-out mice**.J Neurosci 19, 2424 -2431.

Furnari F.B., Fenton T., Bachoo R.M., *et al.*, 2007. **Malignant astrocytic glioma: genetics, biology, and paths to treatment.**Genes Dev 21, 2683-2710.

Gautschi O., Ratschiller D., Gugger M., Betticher D. C., HeighwayJ., 2007.**Cyclin D1 in non-small cell lung cancer: A keydriver of malignant transformation.**Lung Cancer 55, 1-14.

Gerard C., Rollins B.J., 2001. **Chemokines and disease**. Nat Immunol 2, 108-115.

Gewirtz D. A., 1999. **A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin.** Biochem Pharmacol 57,727-741.

Giese A., Bjerkvig R., Berens M.E., Westphal M., 2003. **Cost of migration: invasion of malignant gliomas and implications for treatment.** J Clin Oncol 21,1624-1636.

Gillett C., Smith P., Gregory W., Richards M., Millis R., 1996.**Cyclin D1 and prognosis in human breast cancer.**Int J Cancer 69, 92-99.

Goldstone S., Pavey S., Forrest A., Sinnamon J., Gabrielli B., 2001. **Cdc25-dependent activation of cyclin A/cdk2 is blocked in G2 phase arrested cells independently of ATM/ATR.** Oncogene 20, 921-932.

Gonçalves J., Baptista S., Martins T., Milhazes N., Borges F., Fontes-Ribeiro C., Malva J. O., Silva A. P., 2010. **Methamphetamine-induced neuroinflammation and neuronal dysfunction in the mice hippocampus: preventive effect of indomethacin**. Eur J Neurosci 31, 313-326.

Gonçalves J., Ribeiro C.F., Malva J.O., Silva A.P., 2012. **Protective role of neuropeptide Y Y_2 receptors in cell death and microglial response following methamphetamine injury.** Eur J Neurosci 36, 3173-3183.

Gong W., Wang Z., Liu N., Lin W., Wang X., Xu D., Liu H., Zeng C., *et al.*, 2011. **Improving efficiency of adriamycin crossing blood-brain barrier by combination of thermosensitive liposomes and hyperthermia**. Biol Pharm Bull 34, 1058-1064.

Gopinath S., Vanamala S. K., Gujrati M., Klopfenstein J. D., Dinh D. H., Rao J. S., 2009**. Doxorubicin-mediated Apoptosis in Glioma Cells Requires NFAT3.** Cell Mol Life Sci 66(24), 3967-3978.

Graham D.G., 1978. **Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones.** Mol Pharmacol 14, 633-643.

Guerin C., Olivi A., Wingart J. D., Lawson H. C., Brem H., 2004. **Recent advances in brain tumor therapy: local intracerebral drug delivery by polymers.** Invest New Drug 22, 27-37.

Hagemann C., Anacker ., Haas S., Riesner D., Schömig B., Ernestus R., Vince G. H., 2010. **Camparative expression pattern of Matrix-Metalloproteinases in human glioblastoma cell-lines and primary cultures.** BMC Research 3: 293.

Hall M., Peters G., 1996. **Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer.** Adv Cancer Res 68, 67-108.

Handa K., Yamakawa M., Takeda H., 1999. **Expression of cell cycle markers in colorectal cancer: Superiority of cyclin A as an indicator of poor prognosis.** Int J Cancer 84, 225-233.

Hart C.L., Margaret Haney M., Nasser J., Richard W. Foltin R.W., 2005. **Combined effects of methamphetamine and zolpidem on performance and mood during simulated night shift work. Pharmacology**, Biochem and Behav 81, 559-568.

Hashemolhosseini S, Nagamine Y., Morley S. J., Desrivières S., Mercep L., Ferrari S., 1998. **Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability.** J Biol Chem 23, 14424-14429.

Hau P., Fabel K., Baumgart U., Rümmele P., Grauer O., *et al.*, 2004. **Pegylated liposomal doxorubicin-efficacy in patients with recurrent high-grade glioma.** Cancer 100, 1199-1207.

Hawkins B. T., Davis T. P., 2005. **The blood-brain barrier/Neurovascular unit in health and disease.** Pharmacol Rev 57, 173-185.

Hazards Substances Data Bank [Internet]. Bethesda (MD): National Library of Medicine (US); 2005-[updated 2005 Nov 14; cited 2012 July]. Methotrexate; 3123: **Therapeutic Uses; Human Health Effects**; [about 4p.]. Available from: http://toxnet.nlm.nih.gov/cgi-bin/sis/search/r?dbs+hsdb:@term+@rn+59-05-2

Hirose Y., Berger M.S., Pieper R.O., 2001. **Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells.** Cancer Res 61, 5843-5849.

Hou L.C., Veeravagu A., Hsu A.R., *et al.*, 2006. **Recurrent glioblastoma multiforme: a review of natural history and management options.** Neurosurg Focus 20:E3.

Hu S., Cheeran M. C.-J., Sheng W. S., Ni H. T., Lokensgard J. R., Peterson P. K.., 2006. **Cocaine alters proliferation, migration, and differentiation of human fetal brain-derived neural precursor cells**. J Pharmacol and Experim Ther 318, 1280-1286.

Hu X., Pandolfi P.P., Li Y., Koutcher J.A., *et al.*, 2005. **mTOR promotes survival and astrocytic characteristics induced by Pten/Akt signaling in glioblastoma**. Neoplasia 7, 356-368.

Hurley L. H., 2002. **DNA and its associated processes as targets for cancer therapy**. Nat Rev Cancer 2, 188-200.

Instituto da Droga e da Toxicodependência (IDT), I.P. (2009), Relatório anual 2008. **A situação do país em matéria de drogas e toxicodependências,** IDT, Lisboa.

Instituto da Droga e da Toxicodependência (IDT), I.P. (2011), Relatório anual 2011. **Estudo sobre o Consumo de Álcool, Tabaco e Drogas (ECATD)/ European School Survey on Alcohol and other Drugs (ESPAD) - Portugal/2011**, IDT, Lisboa.

Ironside A. J., Ironside J.W., 2012. **Pathology of tumours of the central nervous system**. **Pathology of tumours of the central nervous system**.

Kanzawa T., Germano I.M., Komata T., Ito H., Kondo Y., Kondo S., 2004. **Role of autophagy in temozolomide-induced cytotoxi-city for malignant glioma cells.** Cell Death Differ 11, 448-457.

Kast R. E., 2007. **Using blood brain barrier disruption by methamphetamine for drug delivery.** J Neurooncol 8, 109-110.

Kast R. E., 2009. **Use of FDA approved methamphetamine to allow adjunctive use of methylnaltrexone to mediate core anti-growth factor signaling effects in glioblastoma.** J Neurooncol 94, 163-167.

Kast R. E., Focosi D., 2010. **Three paths to better tyrosine kinase inhibition behind the blood-brain barrier in treating chronic myelogenous leukemia and glioblastoma with imatinib**. Transl Oncol 3, 13-15.

Khurana G. V., Teo C., Kundi M., Hardell L., Calberg M., 2009. **Cell phones and brain tumors: a review including the long-term epidemiologic data.** Surg Neurol 72, 205-215.

Kish S.J., 2008. **Pharmacologic mechanisms of crystal meth.** Canadian Medical Association Journal 178, 1679-1682.

Kleihues P., Ohgaki H., 1998. **Primary and secondary glioblastomas: From concept to clinical diagnosis**. J Neuro-Oncol 1, 44-51.

Kraan M. C., De Koster B. M., Elferink J. G. R., Post W. J., Breedveld F. C., Tak P.P., 2000. **Inhibition of neutrophil migration soon after initiation of treatment with leflunomide or methotrexate in patients with rheumatoid arthritis.** Arthritis & Rheumatism 43, 1488-1495.

Kuznetsov A. V., Margreiter R., Amberger A., Saks V., Grimm M., 2011.**Changes in mitochondrial redox state, membrane potential and calcium precede mitochondrial dysfunction in doxorubicin-induced cell death**. Biochim Biophys Acta 1813, 1144-1152.

Lesniak M. S. and Brem H., 2004. **Targeted therapy for brain tumours**. Rev Drug Discov 3, 499-508.

Lesniak M.S., Upadhyay U., Goodwin R., Tyler B., Brem H., 2005. **Local delivery of doxorubicin for the treatment of malignant brain tumors in rats**. Anticancer Res 25, 3825-3831.

Leung K.L., Wang Y.T.., 1999. **Differential effects of chemotherapeutic agents on the Bcl-2/Bax apoptosis pathway in human breast cancer cell line MCF-7.** Basic Cancer Res Treat 55, 73-83.

Li X., Motwani M., Tong W., Bornmann W., Schwartz G. K., 2000. **Huanglian, a chinese herbal extract, inhibits cell growth by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells.** Mol Pharmacol58, 1287-1293.

Liebner S., Fischmann A., Rascher G., Duffner F., Grote E.H., Kalbacher H., Wolburg H., 2000. **Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme.** Acta Neuropathol 100, 323-331.

Linos E., Raine T., Alonso A., Michaud D., 2007. **Atopy and risk of brain tumors: a meta-analysis.** J Natl Cancer Inst 99, 1544-1550.

Liu C., Luo D., Reynolds B. A., Meher G., Katritzky A. R., Lu B., Gerard G. J., Bhadha C. P., Harrison J. K., 2010. **Chemokine receptor CXCR3 promotes growth of glioma**. Carcinogenesis 32, 129-137.

Logan B. K., 2002. **Methamphetamine: effects on human performance and behavior**. Forensic Sci Rev 14, 133-151.

Louis D.N., Ohgaki H., Wiestler O.D., *et al.*, 2007. **The 2007 WHO Classification of Tumors of the Central Nervous System**. Lyon, France: IARC.

Lüpertz R., Wätjen W., Kahl R., Chovolou Y., 2010. **Dose- and time-dependent effects of doxorubicin on cytotoxicity, cell cycle and apoptotic cell death in human colon cancer cells**. Toxicology 271, 115-121.

Mahajan S. D., Aalinkeek R., Sykes D. E., Reynolds J. R., Bindakumar B., Adal A., *et al*., 2008. **Methamphetamine alters blood-brain barrier permeability via the modulation of tight junction expression: Implication for HIV-1 neuropathogenesis in the context of drug abuse**. Brain Res 1203, 133-148.

Marshall J.F. and O'Dell S.J., 2012. **Methamphetamine influences on brain and behavior: unsafe at any speed?.** Trends Neurosci. 35, 536-45.

Martins T., Baptista, S., Gonçalves J., Leal E., Milhazes N., Borges F., Ribeiro C.F., Quintela O., Lendoirof E., López-Rivadullaf M., Ambrósio A.F., Silva A.P., 2011. **Methamphetamine transiently increases the blood-brain barrier permeability in the hippocampus: role of tight junction proteins and matrix metalloproteinase-9**. BrainRes 1411, 28-40.

Martins T., Burgoyne T., Kenny B., Hudson N., Futter C. E., Ambrósio A. F., Silva A. P., Greenwood J., Turowski P., 2013. **Methamphetamine-induced nitric oxide promotes vesicular transport in blood-brain barrier endothelial cells.** Neuropharmacology 65, 74-82.

Martins-Neves S. R., Lopes Á.O., do Carmo A., Paiva A. A.4, Simões P. C., Abrunhosa A. J., Célia MF Gomes C. M. L., 2012. **Therapeutic implications of an enriched cancer stem-like cell population in a human osteosarcoma cell line.** BMC Cancer 12:139.

Maru S. V., Holloway K. A., Flynn G., Lancashire C. L., Jane Loughlin A., Male D. K., Romero I. A., 2008. **Chemokine production and chemokine receptor expression by human glioma cells: Role of CXCL10 in tumour cell proliferation.** J Neuroimmunol 199, 35-45.

Maysinger D., Morinville A., 1997. **Drug delivery to the nervous system**. Tibtech 14, 410-417.

McCann U. D., Ricaurte G. A., 2004. **Amphetamine neurotoxicity: accomplishments and remaining challenges.** Neurosci Biobehav Rev 27, 821-826.

McCoy E.S., Haas B.R., Sontheimer H., 2010. **Water permeability through aquaporin-4 is regulated by Protein Kinase C and becomes rate-limiting for glioma invasion**. Neuroscience 168, 971-981.

McGuire J. J., 2003. **Anticancer Antifolates: Current Status and Future Directions.** Current Pharmaceutical Design 9, 2593-2613

Molenaar J.J., Ebus M.E., Koster J., van S.P., van Noesel C.J., 2008. **Cyclin D1 and CDK4 activity contribute to the undifferentiated phenotype in neuroblastoma.** Cancer Res 68, 2599-2609.

Moreau C., Delval A., Defebvre L., Dujardin K., Duhamel A., Petyt G., Vuillaume I., Corvol J., Devos D., 2012. **Methylphenidate for gait hypokinesia and freezing in patients with Parkinson's disease undergoing subthalamic stimulation: a multicentre, parallel, randomised, placebo-controlled trial**. Lancet Neurol 11, 589-96.

Nabissi M., Morelli M. B., Santoni M., Santoni G., 2013. **Triggering of the TRPV2 channel by cannabidiol sensitizes glioblastoma cells to cytotoxic chemotherapeutic agents**. Carcinogenesis 34, 48-57.

Nakama H., Chang L., Cloak C., Jiang C., Alicata D., Haning W., 2008. **Association between Psychiatric symptoms and craving in methamphetamine users.** Am J Addict 17(5), 441-446.

Narita M., Shimizu S., Ito T, Chittenden T., Lutz R.J., *et al*., 1998**. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria**. Proc Natl Acad Sci U S A 95, 14681-6.

Neuwelt E. A., Pagel M., Barnett P., *et al*., 1981. **Pharmacology and toxicity of intracarotid adriamycin administration following osmotic blood-brain barrier modification.** Cancer Res 41, 4466-4470.

Nieder C., Mehtaz M. P., Jalalix R., 2009. **Combined Radio- and Chemotherapy of Brain Tumours in Adult Patients.** Clin Oncol 21, 515-524.

Nitta T., Hata M., Gotoh S., Seo Y., Sasaki H., Hashimoto N., Furuse M., Tsukita S., 2003. **Size-selective loosening of the bloodbrain barrier in claudin-5-deficient mice**. J Cell Biol 161, 653-660.

Niyazi M., Siefert A., Schwarz B., Ganwindt U., Kreth F., *et al*., 2011. **Therapeutic options for recurrent malignant glioma.** Radioth Oncol 98, 1-14.

Oh J.,Drabik K., Kutsch O., Choi C., Tousson A., Benveniste E. N., 2001. **CXC Chemokine Receptor 4 Expression and Function in Human Astroglioma Cells.** J Immunol 166, 2695-2704.

Ohnishi T., Tamai I., Sakanaka K., Sakata A., Yamashima T., Yamashita J., Tsuji A., 1995**. I*n vivo* and *invitro* evidence for ATP-dependency of P-glycoprotein-mediated efflux of doxorubicin at the blood-brain barrier.** Biochem Pharmacol 49, 1541-1544.

Papadopoulos M.C., Saadoun S., Woodrow C.J., Davies D.C., Costa-Martins P., Moss R.F., Krishna S., Bell B.A., 2001. **Occludin expression in microvessels of neoplastic and non-neoplastic human brain.** Neuropathol Appl Neurobiol 27, 384–395.

Parney I.F., Chang S.M., 2003. **Current chemotherapy for glioblastoma**. Cancer J 9, 149-156.

Patil R., Portilla-Arias J., Ding H., Konda B., Rekechenetskiy A., Inoue S., Black K. L., Holler E., Ljubimova J. Y., 2012. **Cellular delivery of doxorubicin via pH-controlled hydrazone linkage using multifunctional nano vehicle based on poly(β-L-malic acid).** Int J Mol Sci 13, 11681-11693.

Pavelic S. K., Marjanovic M., Poznic M., Kralj M., 2009. **Adenovirally mediated p53 overexpression diversely infuencethe cell cycle of HEp-2 and CAL 27 cell lines upon cisplatinand methotrexate treatment.** J Cancer Res Clin Oncol 135, 1747-1761.

Quinton M. S., Yamamoto B. K., 2006. **Causes and consequences of methamphetamine and MDMA toxicity.** AAPS J 8, E337-347.

Quinton M.S., Yamamoto B,K., 2006. **Causes and consequences of methamphetamine and MDMA toxicity.** The AAPS Journal 8, E337-E347.

Ramirez S. H., Potula R., Fan S., Eidem T., Papugani A., Reichenbach N., DykstraH., Weksler B. B., Romero I. A., Couraud P. O., Persidsky Y., 2009. **Methamphetamine disrupts blood–brain barrier function by induction of oxidative stress in brain endothelial cells.** J Cerebr Blood F Met 29, 1933-1945.

Ramirez S.H., Potula R., Fan S., Couraud P.O., Persidsky Y., 2009. **Methamphetamine disrupts blood-brain barrier function by induction of oxidative stress in brain endothelial cells.** Journal of Cerebral Blood Flow & Metabolism 29(12), 1933-1945.

Ricaurte G.A., Mechan A.O., Yuan J., *et al.*, 2005. **Amphetamine treatment similar to that used in the treatment of adult attention-deficit/hyperactivity disorder damages dopaminergic nerve endings in the striatum of adult nonhuman primates.** J Pharmacol Exp Ther 315, 91-98.

Riddle E. L., Fleckenstein A. E., Hanson G. R., 2006. **Mechanisms of methamphetamine-induced dopaminergic neurotoxicity.** AAPS J 8, 413-418.

Roberts H.C., Roberts T.P., Brasch R.C., Dillon W.P., 2000. **Quantitative measurement of microvascular permeability in human brain tumors achieved using dynamic contrast-enhanced MR imaging: correlation with histologic grade.** Am J Neuroradiol 21, 891-899.

Saadoun S., Papadopoulos M.C., Davies D.C., Krishna S., Bell B.A., 2002. **Aquaporin-4 expression is increased in oedematous human brain tumours.** J Neurol Neurosurg Psychiatry 72, 262–265.

Sáez-Ayala M., Fernández-Pérez M. P., Montenegro M. F., Sánchez-del-Campo L., Chazarra S., Piñero-Madrona A., Cabezas-Herrera J., Rodríguez-López J. N., 2012. **Melanoma coordinates general and cell-specific mechanisms to promote methotrexate resistance**. Exp Cell Res 318, 1146-1159.

Sawada M., Nakashima S., Banno Y., Yamakawa H., Hayashi K., Takenaka K., Nishimura Y., Sakai N., Nozawa Y., 2000. **Ordering of ceramide formation, caspase activation, and Bax/Bcl-2 expression during etoposide-induced apoptosis in C6 glioma cells.** Cell Death and Differ 7, 761-772.

Sawada T., Kato Y., Kobayashi M., Takekekawa Y., 2000. **Immunohistochemical study of tight junction-related protein in neovasculature in astrocytic tumor.** Brain Tumor Pathol 17, 1-6.

Schouten L.J., Rutten J., Huveneers H.A., Twijnstra A., 2002. **Incidence of brain metastases in a cohort of patients with carcinoma of the breast, colon, kidney, and lung and melanoma**. Cancer 94, 2698–705.

Schweitzer B. I., Dicker A. P., Bertino J. R., 1990. **Dihydrofolate reductase as a therapeutic target.** FASEB J 4, 2441-2452.

Sciaccaluga M., Fioretti B., Catacuzzeno L., Pagani F., Bertollini C., Rosito M., Catalano M., D'Alessandro G., Santoro A., Cantore G., Ragozzino D., Castigli E., Franciolini F., Limatola C., 2010. **CXCL12-induced glioblastoma cell migration requires activity intermediate conductance Ca^{2+}-activated K^+ channel.** Am J Physiol Cell Physiol 299, C175-C184., 2010.

Seitz R.J., Wechsler W., 1987. **Immunohistochemical demonstration of serum proteins in human cerebral gliomas.** Acta Neuropathol 73, 145-152.

Silber J. R., Mueller B. A., Ewers T. G., *et al*., 1993. **Comparison of O^6-Methylguanine-DNA Methyltransferase activity in brain tumors and adjacent normal brain**. Cancer Res 53, 3416-3420.

Spruck C. H., Won K., Reed S. I., 1999. **Deregulated cyclin E induces chromosome instability.** Nature 401, 297-300.

Stan A.C., Casares S., Radu D., Walter G.F., Brumeanu T.D., 1999. **Doxorubicin-induced cell death in highly invasive human gliomas**. Anticancer Res 19, 941-50.

Steiniger S.C., Kreuter J., Khalansky A.S., Skidan I.N., Bobruskin A.I., Smirnova Z.S., *et al*., 2004. **Chemotherapy of glioblastoma in rats using doxorubicin-loaded nanoparticles.** Int J Cancer 109, 759-67.

Stupp R., Hegi M. E., Mason W. P., van der Bent M. J., Taphoorn M. J., *et al.*, 2009. **Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial**. Lancet Oncol 10, 459-466.

Stupp R., Mason W.P., van den Bent M.J., *et al.*, 2005. **Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma**. N Engl J Med 352, 987-996.

Sulzer D., Sonders M.S., Poulsen N.W., *et al.*, 2005. **Mechanisms of neurotransmitter release by amphetamines: a review.** Prog Neurobiol 75, 406-433.

Tait M.J., Saadoun S., Bell B.A., Papadopoulos M.C., 2008. **Water movements in the brain: role of aquaporins.** Trends in Neurosci 31, 37-43.

Thomas D.M., Walker P.D., Benjamins J.A., Geddes T.J., Kuhn D.M., 2004. **Methamphetamine neurotoxicity in dopamine nerve endings of the striatum is associated with microglial activation.** J Pharmacol Exp Ther 311, 1 -7.

Thompson P. M., Hayashi K. M., Simon S. L., Geaga J. A., *et al*, 2004. **Structural abnormalities in the brains of human subjects who use methamphetamine.** J Neurosci 24, 6028-6036.

Tosoni A., Ermani M., Brandes A.A., 2004. **The pathogenesis and treatment of brain metastases: a comprehensive review.** Crc Cr Rev Oncol-Hem 52, 199-215.

Tsurusawa M., Niwa M., Katani N., Fijimoto T., 1990. **Methotrexate cytotoxicity as related to irreversible S phase arrest in mouse L1210 leukemia cells.** Jpn J Cancer Res 81(1), 85-90.

Ugarenko M., Nudelman A., Rephaeli A., Kimura K., Philips D. R., Cutts S., M., 2010. **ABT-737 overcomes Bcl-2 mediated resistance to doxorubicin–DNA adducts**. Biochem Pharmacol 79, 339-349.

United Nations Office on Drugs and Crime (UNODC) (2012) **World Drug Report**. UNODC, Vienna.

Urrutia A., Rubio-Araiz A., Gutierrez-Lopez M. D., ElAli A., Hermann D.M., O'Shea E., Colado M. I., 2013. **A study on the effect of JNK inhibitor, SP600125, on the disruption of blood–brain barrier induced by methamphetamine.** Neurobiology of Disease 50, 49–58.

Vandercappellen J., Van Damme J., Struyf S., 2008. **The role of CXC chemokines and their receptors in cancer**. Cancer Lett. 267, 226-244.

Varma M. V. S., Sateesh K., Panchagnula R., 2004. **Functional role of P-glycoprotein in limiting intestinal absorption of drugs: Contribution of passive permeability to P-glycoprotein mediated efflux transport**. Mol Pharmaceut 2, 12-21.

Vermeulen K., Dirk R. Van Bockstaele, Berneman Z. N., 2003. **The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer.** Cell Prolif 36, 131-149.

Villano J. L., Seery T. E., Bressler L. R., 2009. **Temozolomide in malignant gliomas: current use and future targets**. Cancer Chemother Pharmacol 64, 647-655.

Volm M., Koomagi R., Mattern J., Stammler G., 1997. **Cyclin A is associated with an unfavourable outcome in patients with non-small-cell lung carcinomas**. Br J Cancer 75, 1774-1778.

Wang C., Li J., Teo C. S., Lee T., 1999. **The delivery of BCNU to brain tumors.** J Control Release 61, 21-41.

Wang J., Wang Q., Cui Y., Liu Z.Y., Zhao W., 2012. **Knockdown of cyclin D1 inhibits proliferation, induces apoptosis, and attenuates the invasive capacity of human glioblastoma cells.** J Neurooncol 106, 473-484.

Wang M., Wang T., Liu S., Yoshida D., Teramoto A., 2003. **The expression of matrix metalloproteinase-2 and -9 in human gliomas of different pathological grades.** Brain Tumor Pathol 20, 65-72.

Wang Y., Chen C., Lijima M., 2011. **Signaling Mechanisms for Chemotaxis.** Dev Growth Differ 5, 495-502.

Wen P.Y., Kesari S., 2008. **Malignant gliomas in adults**. N Engl J Med 359, 492-507.

Westphal M., Hilt D. C., Bortey E., Delavault P., Olivares R., Warnke P., *et al*., 2003. **A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma.** J Neuro-Oncol 5, 79-88.

Winslow B.T., Voorhees K.I, Pehl K.A, 2001. **Methamphetamine abuse**. Am Fam Physician 76(8), 1169-1174.

Wolburg H., Noell S., Fallier-Becker P., Mack A. F., Wolburg-Buchholz K., 2012. **The disturbed blood–brain barrier in human glioblastoma**. Mol Aspects Med 33, 511-704.

Wolburg H., Wolburg-Buchholz K., Kraus J., Rascher-Eggstein G., Liebner S., Hamm S., Duffner F., Grote E.H., Risau W., Engelhardt B., 2003. **Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme.** Acta Neuropathol 105, 586–592.

Wolff J.E., Kortmann R-D., Wolff B., Pietsch T.,Peters O., Schmid H-J., Rutkowski S., Warmuth-Metz M., Kramm C., 2011. **High dose methotrexate for pediatric high grade glioma - results of the HIT-GBM-D Pilot study**. J Neurooncol 102, 433-442.

Wolff J.E., Trilling T., Molënkamp G. Egeler R. M., Jürgens H., 1999. **Chemosensitivity of glioma cells in vitro: a meta analysis**. J Cancer Res Clin Oncol 125, 481-486.

Wrensch M., Minn Y., Chew T., *et al.*, 2002. **Epidemiology of primary brain tumors: current concepts and review of the literature.** J Neuro-oncol 4, 278-299.

Wrensch M., Wiencke J. K., Wiemels J., *et al.*, 2006. **Serum IgE, tumor epidermal growth factor receptor expressin, and inherited polymorphisms associated with glioma survival**. Cancer Res 66, 4531-4541.

Xu T., Chen J., Lu X., Wolff J. EA, 2010. **Effects of bevacizumab plus irinotecan on response and survival in patients with recurrent malignant glioma: a systematic review and survival-gain analysis**. BMC Cancer 10, 252.

Yamamoto B.K., Moszczynska A., Gudelsky G.A., 2010. **Amphetamine Toxicities, Classical and emerging mechanisms.** Annals of the New York Academy of Sciences 1187, 101-121.

Yang F., Lin Y., Kang K., Chao T., 2011. **Reversible blood-brain barrier disruption by repeated transcranial focused ultrasound allows enhanced extravasation.** J Control Release 150, 111-116.

Yao C., Wu S., Li D., Ding H., Wang Z., Yang Y., Yan S., Zhangping Gu Z., 2012. **Co-administration phenoxodiol with doxorubicin synergistically inhibit the activity of sphingosine kinase-1 (SphK1), a potential oncogene of osteosarcoma, to suppress osteosarcoma cell growth both in vivo and in vitro.** Mol Onc 6, 392-404.

Zhang R., Wu X., Guziec L. J., Guziec F. S., Chee G., *et al*, 2010. **Design, synthesis and biological evaluation of a novel series of anthrapyrazoles linked with netropsin-like oligopyrrole carboxamides as anticancer agents**. Bioorg Medicinal Chem 18, 3974-3984.

Zhou Y., Larsen P. H., Hao C., Yong V. W., 2002. **CXCR4 is a major chemokine receptor on glioma cells and mediates their survival**. J Biol Chem 277, 49481-49487.