LEBANESE AMERICAN UNIVERSITY

Potential Mechanisms of Human Recombinant Arginase I (Co)- PEG5000 [HuArgI (Co)-PEG5000]-Induced Cytotoxicity in Glioblastoma Cells

By

YASSMINE EL JAWHARI

A thesis

submitted in partial fulfillment of the requirements

for the degree of Master of Science in Molecular Biology

School of Arts and Sciences

June 2018

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS APPROVAL FORM

Student Name: Yassmine El-Jawhari I.D. #: 201206169

Thesis Title: Potential mechanisms of human recombinant arginase induced cytotoxicity in GB

Program: Master of Science in Molecular Biology

Department: Natural Sciences

School: Arts and Sciences

The undersigned certify that they have examined the final electronic copy of this thesis and approved it in Partial Fulfillment c the requirements for the degree of:

Masters in the major of Molecular Biology

Thesis Advisor's Name	Signatu	[redacted]	DATE: 23 / 6 / 18 (Day / Month / Year)
Committee Member's Name	Signatu		DATE: 23 / 6 / 18 (Day / Month / Year)
Committee Member's Name	Signatu		DATE: 23 / 6 / 18 (Day / Month / Year)

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS COPYRIGHT RELEASE FORM

LEBANESE AMERICAN UNIVERSITY NON-EXCLUSIVE DISTRIBUTION LICENSE

By signing and submitting this license, you (the author(s) or copyright owner) grants the Lebanese American University (LAU) th non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwid in print and electronic formats and in any medium, including but not limited to audio or video. You agree that LAU may, withou changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree tha LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that th submission is your original work, and that you have the right to grant the rights contained in this license. You also represent tha your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the submission contains material fo which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to gran LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within th text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED B AN AGENCY OR ORGANIZATION OTHER THAN LAU, YOU REPRESENT THAT YOU HAVE FULFILLED ANY RIGHT OF REVIEW OR OTHER OBLIGATIONS REQUIRED BY SUCH CONTRACT OR AGREEMENT. LAU will clearly identify your name(s) as the author(s) or owner(s of the submission, and will not make any alteration, other than as allowed by this license, to your submission.

Name: Yassmine El-Jawhari

Signature: [redacted]

Date: 20-6-2018

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

PLAGIARISM POLICY COMPLIANCE STATEMENT

I certify that:

1. I have read and understood LAU's Plagiarism Policy.
2. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me.
3. This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: Yassmine El-Jawhari

Signature: [redacted]

Date: 20-6-2018

Acknowledgment

First, I would like to express my deepest gratitude to Dr. Ralph Abi Habib for his continuous support. I am grateful for his help and encouragement that helped me to carry on with my thesis work. Working with such a talented professor has been delightful since the very first day.

Also, I would like to extend my appreciation to my committee members, Dr. Roy Khalaf and Dr. Sima Tokajian and thank them for their contribution to my thesis.

I am also thankful to all my lab colleagues who helped make the lab such a friendly and pleasant place to work in. I would like to extend my thankfulness especially to Amani Ezzeddine, Noura Ghazale, Oula Atat, and Ghenwa Nasreddine for guiding me through this process offering their love and support.

Finally, my deepest and most sincere appreciation goes to my family and my loving husband. I couldn't have done this without your endless love and support.

Potential Mechanisms of Human Recombinant Arginase I (Co)- PEG5000 [HuArgI (Co)-PEG5000]-Induced Cytotoxicity in Glioblastoma Cells

Yassmine El Jawhari

Abstract

Glioblastoma multiforme (GBM) is a highly aggressive glioma arising in the central nervous system accounting for more than 70% of all brain tumors. Due to the resistance and complexity of the tumor itself, it is characterized by a low survival rate and decreased prognosis. Therefore, more selective and efficient therapeutic methods for targeting GBM are needed. In this study, we attempted to investigate the potency of arginine depletion to target GBM cells through the use of human recombinant Arginase I cobalt [HuArgI(Co)] coupled with polyethylene glycol 5000 [HuArgI (Co)-PEG5000]. Cytotoxicity of [HuArgI (Co)-PEG5000] was tested on two GBM cell lines, namely A172 and U251. Both cell lines showed increased sensitivity at longer incubation periods of [HuArgI (Co)-PEG5000] treatment with IC_{50} values in the pM range, a result attributable to the arginine auxotrophy expressed in those two cell lines. The addition of chloroquine, an autophagy inhibitor, increased the sensitivity of cells to [HuArgI (Co)-PEG5000]-mediated arginine depletion at earlier time points. We also concluded that autophagy was activated in response to arginine depletion and plays a protective role at early time points. However, the excessive accumulation of intracellular autophagosomes upon treatment with chloroquine at later time points illustrates the role of autophagy in response to

[HuArgI (Co)-PEG5000]-mediated arginine depletion resulting in ultimate cell death by autophagy. Overall, in this study we showed how arginine auxotrophy in GBM could be used to target such cells through HuArgI (Co)-PEG5000. Hence, HuArgI (Co)-PEG5000 is an efficient and selective therapeutic method for targeting GBM.

Keywords: Glioblastoma multiforme, Cytotoxicity, Autophagy, Arginine Deprivation, HuArgI (Co)-PEG5000, Arginine Auxotrophy

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADI: arginine deiminase.

AED: antiepileptic drugs.

AET: active efflux transporters.

AKT: protein kinase B.

ALA: 5-aminolevulinic acid.

AML: acute myeloid leukemia.

ASL: argininosuccinate lyase.

ASS1: arginosuccinate synthetase.

ATP: adenosine triphosphate.

BAD: Bcl-2-associated death protein.

BBB: blood brain barrier.

Cdk2: cyclin-dependent kinase-2.

CNS: central nervous system.

CQ: chloroquine.

CSCs: cancer stem cells.

CT: computed tomography.

DIC: differential interference contrast.

DMEM: Dulbecco's modified Eagle’s medium.

DNA: deoxyribonucleic acid.

ECM: extracellular matrix.

EGFR: epidermal growth factor receptor.

ERK: extracellular-regulated kinase.

FBS: fetal bovine serum.

FDA: Food and Drug Administration.

GBM: Glioblastoma Multiforme.

GDP: guanosine diphosphate.

GTP: guanosine triphosphate.

GTR: gross total resection.

HCC: hepatocellular carcinoma.

HuArgI (Co)-PEG5000: pegylated cobalt-substituted recombinant human arginase.

IC50: inhibitory concentration.

ICP: increased intracranial pressure.

LOH: loss of heterozygosity.

MAPK : mitogen activated-protein kinase.

MDM2: mouse double minute 2 homolog.

MGMT: O-6-methylguanine-DNA methyltransferase.

MRI: magnetic resonance imaging.

mTOR: mammalian target of rapamycin.

ODC: ornithine decarboxylase.

OS: overall survival.

OTC: ornithine transcarbamylase.

PI3K: phosphatidylinositol-3-OH kinase.

PIP2: phosphatidylinositol 4,5-bisphosphate.

PIP3: phosphatidylinositol (3,4,5)-trisphosphate.

PTEN: phosphatase and tensin homolog.

RTK: receptor tyrosine kinase.

STR: subtotal resection.

TFs: transcription factors.

TMZ: Temozolomide.

VEGF: vascular endothelial growth factor.

VEGFR: vascular endothelial growth factor receptor.

WHO: World Health organization.

CHAPTER ONE
INTRODUCTION

1.1- Glioblastoma: Characteristics

Glioblastoma multiforme (GBM) is the most common prevalent primary tumor of the CNS in adults, arising from normal glial cells through multistep oncogenesis and accounts for more than 70% of all brain tumors (Ohgaki & Kleihues, 2005). With a grim prognosis and a median survival rate of approximately 15 months, GBM is recognized as a critical public health issue in the world of cancer. This specific type of cancer is mainly diagnosed at an older age with an average age of 64 at the time of diagnosis and an increased occurrence with age peaking at 75-84 years (Tamimi & Juweid, 2017). Recent studies revealed that the recorded cases in Europe and North America are 3 per 100 000 individuals per year, with a lower occurrence rate among Blacks compared to Caucasian populations, and males having a higher incidence rate at a ratio of 1.26 to 1 compared to females (Jovcevska, Kocevar, & Komel, 2013; Urbańska, Sokołowska, Szmidt, & Sysa, 2014). In comparison to the western world, under developed countries usually have a lower GBM occurrence, mostly due to under reporting of glioma cases, different diagnostic methods, and inadequate access to proper health care (Fisher, Schwartzbaum, Wrensch, & Wiemels, 2007).

More than 100 cases of GBM resulting from exposure to high doses of ionizing radiation have been reported making it a highly potential risk factor (Salvati et al., 2003). Recently, life span studies analyzing the aftermath of ionizing radiation on atomic bomb

survivors in Nagasaki and Hiroshima demonstrated a trend of increasing incidence of gliomas with increasing age. Furthermore, it has been suggested that frequent CT scans cause prominent cancer risks. According to BEIR (Biological effects of ionizing radiation) methodology, patients who had more than five CT scans have a 2.7% higher risk of developing cancer (Prasad & Haas-Kogan, 2009). Moreover, the HHP and HAAS (Honolulu Heart Program and Honolulu-Asia Aging Study) studies revealed an increased glioma incidence rate among patients with previous blood transfusions and jobs that expose them to elevated levels of carbon tetrachloride. No direct association was concluded between glioma and physical or environmental factors including, age, BMI, smoking, coffee and tea consumption, physical trauma, surgery, dietary food intake, infectious agents, medications, and pesticide exposure (Nelson, Burchfiel, Fekedulegn, & Andrew, 2012). In addition, germ line genetic polymorphisms, such as *p53* mutations, that pre dispose to glioma formation appear to run in families with a history of multiple cancers, but only account for 1% of all cases (Kyritsis, Bondy, Rao, & Sioka, 2010; Urbańska et al., 2014).

Unlike other dense tumors, GBM largely invades the brain, infrequently metastasizing to other organs in the body and is commonly characterized by a high level of angiogenesis and chemo- resistance (Lathia, Mack, Mulkearns-Hubert, Valentim, & Rich, 2015; Mao, LeBrun, Yang, Zhu, & Li, 2012). It has been postulated that the decreased metastatic ability of GBM is due to the presence of barriers formed by the cerebral meninges and fast tumor progression, thus hindering its paracellular diffusion (El-Habashy et al., 2014; Urbańska et al., 2014). In 95% of cases, GBM mainly develops in the supratentorial region of the brain, specifically in the cerebral hemispheres, rarely

arising in the cerebellum, brainstem, and spinal cord (Hanif, Muzaffar, Perveen, Malhi, & Simjee, 2017; Tanowitz, Machado, Avantaggiati, & Albanese, 2013). Microscopically, glioblastoma has a variable gross appearance, but a key characteristic in diagnosis is the presence of necrotic tissue surrounding the tumor, thus making it appear soft and yellow in color (Dong et al., 2005). On a macroscopic level, the topographically diffuse nature of the tumor resembles a single, gelatinous, irregular shaped neoplasm typically arising in the white matter (Hanif et al., 2017).

The present standard nomenclature used for the organization and diagnosis of all gliomas is the WHO (World Health organization) classification. In this grouping method, an immunohistochemical criteria is used to determine the level of malignancy of the tumor, proliferative index, and survival time accordingly classifying it as a grade I, II, III, or IV glioma (Hanif et al., 2017; Urbańska, Sokołowska, Szmidt, & Sysa, 2014). Non-malignant tumors with a low proliferative rate are referred to as Grade I gliomas, whereas highly invasive malignant tumors with median survival of 6 – 12 months are grouped in Grade II to IV (Urbańska et al., 2014). Due to its invasiveness and high degree of undifferentation, glioblastoma multiforme has been designated Grade IV according to the WHO classification (Jovcevska et al., 2013). When referring to glioblastoma multiforme specifically, an additional classification is applied that subdivides the tumors as either primary or secondary glioblastoma. Primary tumors grow rapidly lacking key clinical indications of a less malignant precursor, while secondary glioblastomas which account for less than 10% of cases are derived progressively from pre-existing lower grade astrocytomas. Hence, revealing a heterogeneous cellular structure among the two subgroups of GBMs (Galli et al., 2004; Ohgaki & Kleihues, 2007; Ravikanth, 2017).

This inter/intra tumor heterogeneity is one of the most important hallmarks of GBM, challenging the design and effectiveness of targeted therapies (Friedmann-Morvinski, 2014; Inda, Bonavia, & Seoane, 2014). The first evidence of heterogeneity in gliomas was confirmed in isolated cells from clinical samples that showed variable expression of surface antigenic markers (CD133, CD44). One of the many reasons for such extensive heterogeneity is the fluctuations in the tumor's micro- atmosphere, such as variations in growth factors, oxygen pressure, and constitution of ECM. Such changes affect tumor cells and could lead to genomic and phenotypic alterations permitting proliferative growth. Moreover, the co-existence of various interacting clones in the tumor bulk that provide a protective environment against determined therapies has been observed in various tumors, further demonstrating tumor heterogeneity (Inda et al., 2014).

1.2- Clinical Presentation, Diagnosis, and Imaging

With a median survival of nearly 4 months without treatment, or 12 months after surgery and radiation therapy, GBM continues to rank among the most commonly aggressive brain tumors (Faraz, Pannullo, Rosenblum, Smith, & Wernicke, 2016). Clinical presentation of newly diagnosed GBM greatly depends on the size and localization of the tumor, and as a result of the clinical progression of the disease, most of the common signs include headache, vision disturbances, dizziness, nausea, vomiting, increased intracranial pressure and seizures (Davis, 2016; Pan & Prados, 2003; Urbańska et al., 2014). Seizures are the preliminary indications of a brain tumor in 15% of glioma patients co-occurring with slower-developing neoplasms and are usually treated with antiepileptic drugs (AEDs) as the present standard of care (Glantz et al., 2000; Pan & Prados, 2003). Children with tumor- related seizures that are hard to control account for

1% of all cases and should be analyzed by MRI (Pan & Prados, 2003). Because of such undefined and common occurring symptoms, GBM is frequently misdiagnosed for infections, viral diseases, or immunological responses and even strokes if symptoms progress rapidly (Lakhan & Harle, 2009; Omuro & DeAngelis, 2013).

The various presented symptoms of GBM are produced by two distinct processes, the first being through direct effect in which brain tissue is damaged due to necrosis leading to cognitive impairment (Hanif, Muzaffar, Perveen, Malhi, & Simjee, 2017). Such cognitive abnormalities are frequently observed in frontal lobe tumors (25%) causing variations in mental status such as lower attention span, personality changes, or even depression in rare cases (Davis, 2016; Pan & Prados, 2003). Patients with GBM in the frontal and temporal lobes may have a moderately longer survival time due to the ease of accessibility to tumor excision (Nakada et al., 2011). Increased intracranial pressure (ICP) resulting from the progressive growth of the tumor and increased surrounding edema constitute symptoms brought on through secondary effects (Hanif et al., 2017). Such elevated pressures result in the movement of intracranial contents and consequently headaches which are a common feature in 30-50% of patients bearing GBM, yet indistinguishable from tension headaches with no exact pain pattern (Hanif et al., 2017; Omuro & DeAngelis, 2013).

Conclusive GBM diagnosis is established according to the histopathological examination of the removed tumor (Urbańska, Sokołowska, Szmidt, & Sysa, 2014). In an attempt of avoiding such invasive surgical resections, recent technological advances have resorted to the use of magnetic resonance imaging (MRI) scans and computed tomography (CT) which demonstrated efficacy in non-invasive detection, grading and diagnosis of

tumors (Kalpathy-Cramer, Gerstner, Emblem, Andronesi, & Rosen, 2014). Magnetic fields radiating from heart pace makers interfere with MRI magnetic resonance leading to false results, thus such patients bearing body-inserted metals are advised to undergo CT scans (Omuro & DeAngelis, 2013). However, MRI is the imaging model of choice regarding tumor visualization and precise identification of high-grade gliomas allowing the quantification of changes in tumor vasculature and enhancement (Kalpathy-Cramer et al., 2014; Ravikanth, 2017). Moreover, the excellent soft tissue contrast provided by MRI scans allows the heterogeneity of tumor abrasions to be perfectly visualized appearing as a necrotic deformed mass surrounded by white matter edema in comparison to contiguous brain tissue (Davis, 2016; Hanif et al., 2017; Kalpathy-Cramer et al., 2014).

1.3- Pathogenesis and Pathways involved

The highly complicated pathogenesis of glioblastoma multiforme is due to an extremely uncontrolled tumor genome involving genetic modifications such as removal of tumor suppressor genes and amplification or excessive activation of tumorigenic oncogenes (Nakada et al., 2011). Various high dimensional profiling studies have been carried out in an attempt to study the mechanisms that initiate tumorigenesis in GBM (Seymour, Nowak, & Kakulas, 2015). It was found that a population of self-regenerating, tumorigenic CSCs in GBM greatly contribute to tumor origination and medical resistance. As normal stem cells undergo regular tissue development, such developmental mechanisms re-emerge in glioma initiating CSCs to support the continuous proliferation of tumors instead of normal constant growth (Lathia et al., 2015). These rapidly dividing tumorigenic cells eventually lead to the formation of a tumor bulk following the accumulation of additional deleterious mutations and genetic aberrations that allow them

to escape processes which usually control their cell-cycle checkpoint, proliferation and apoptosis (Davis, 2016; Fürthauer & González-Gaitán, 2009; Haque, Banik, & Ray, 2011). The signaling cascades associated with such developmental mechanisms are described in three steps: (1) an extracellular afferent signal is mediated by membrane surface receptors and their ligands; (2) a central step in which the signal is transferred to the nucleus by protein kinases; and (3) an intra-nuclear step where TFs control genes affecting cellular processes (Nakada et al., 2011).

Molecular analyses of glioblastoma cell samples showed a variety of altered pathways involved in apoptosis, invasion, cell cycle, and DNA repair that aid in the pathogenesis of glioblastoma, making it one of the most aggressive and fatal types of primary brain cancers (Haque et al., 2011; Seymour, Nowak, & Kakulas, 2015). One mechanism regulating proliferative growth and apoptosis among other functions is the PI3K/AKT/mTOR pathway, found to be deregulated in 80% of all GBM (Valdés-Rives, Casique-Aguirre, Germán-Castelán, Velasco-Velázquez, & González-Arenas, 2017). Mostly, the PI3K/Akt signaling cascade is over activated through deletions or mutations in PTEN (a tumor suppressor gene), or through excessive stimulation of PI3K (Paw, Carpenter, Watabe, Debinski, & Lo, 2015). Excess EGFR signaling on the cell surface results in recruitment of PI3K to the cell membrane, which in turn phosphorylates and activates Akt through downstream effector molecules such as PIP3 and PIP2 (Haque et al., 2011; Valdés-Rives et al., 2017). Activated phospho-Akt leads to the inhibition of apoptosis through the phosphorylation and inactivation of pro-apoptotic proteins such as BAD (Seymour et al., 2015). Phospho-Akt also activates mTOR (mammalian target of rapamycin) which acts as both a downstream effector and an upstream regulator of the

PI3K integrating signals from multiple pathways to promote angiogenesis, inhibition of autophagy, and cellular proliferation (X. Li et al., 2016; Mao, LeBrun, Yang, Zhu, & Li, 2012; Watanabe, Wei, & Huang, 2011). Although EGFR amplification is rarely observed in secondary and pediatric glioblastoma, it is detected in 40% of primary glioblastoma cases (Haque, Banik, & Ray, 2011).

Another important signal transduction cascade de-regulated in glioblastoma is the RAS/MAPK pathway. Constitutive ligand binding to RTKs on cell surface activates RAS through exchange of GDP with GTP, which recruits RAF kinase to the membrane and activates it through direct binding (Mao et al., 2012; Seymour et al., 2015). After a series of downstream phosphorylation events, the activated MAPK, also known as ERK, translocates into the nucleus triggering various TFs involved in cell cycle progression, resistance to apoptosis, and tumorigenesis (Nakada et al., 2011). It was shown that neither activated Ras nor Akt alone induced tumor initiation in mouse models, however this was reversed when a combination of both activators was used at the same time (Seymour et al., 2015).

Genetic modifications of specific genes, such as deletions and/or mutations of tumor suppressor genes, also play an important role in the events that lead to the pathogenesis of gliomas (Y. Yang et al., 2010). One such gene is PTEN, which is located on chromosome 10 and acts as a tumor suppressor (Mao et al., 2012). PTEN is mutated or deleted (<2%) in almost 44% of GBM cases and mostly observed in primary GBMs (Kanu et al., 2009; Pearson & Regad, 2017). The role of PTEN is to block Akt signaling by reducing intracellular PIP3 levels through dephosphorylation and thus antagonizing the PI3K/Akt signal transduction pathway (Haque et al., 2011). Mutations or deletions

through loss of heterozygosity in chromosome 10 disrupt the regulation of developmental process involved in the PI3K pathway such as proliferation and invasion, hence leading to continuously growing tumors (Endersby & Baker, 2008). PTEN LOH- mediated malignancies have been implicated in various cancers, including breast, pancreatic, prostate, and GBM (Joshi, Lucic, & Zuniga, 2015).

Another widely studied tumor suppressor in human gliomas is the *p53* gene. Recently, the TCGA reported that in 87% of newly diagnosed GBM cases, almost 35% of patients had *p53* mutations and/or deletions (Endersby & Baker, 2008; Pearson & Regad, 2017). Intracellular and extracellular stress signals such as hypoxia, radiation exposure, DNA damage, and temperature shock all induce a *p53* response and lead to the activation of MDM2, a p53 degrading molecule (Harris & Levine, 2005; Kanu et al., 2009; Pearson & Regad, 2017). Under normal conditions, the p53 protein binds to DNA and complexes with a cell-division stimulating protein (cdk2), inhibiting the passage of cells through the stages of cell division and eventually halting the cell cycle at G1 phase. Mutations in *p53* allow an inefficient DNA binding and dismiss the impeded signal for cell division, thus forming continuously dividing tumors (Information (US), 1998). MDM2 hyper activation leading to decreased p53 levels is observed in approximately 10% of GBM cases (Nakada et al., 2011). In an aim to restore p53 expression in GBM, a nanocomplex was used to deliver wild-type p53 into Temozolomide resistant GBM cells. It was shown to reduce MGMT protein levels leading to improved sensitivity to TMZ, thus demonstrating the importance of p53 in GBM pathogenesis (Pearson & Regad, 2017).

In addition to the direct glioma- inducing events (amplifications/deletions/mutations), other processes such as DNA instability and DNA

repair contribute to gliomagenesis indirectly. For example, methylation of the gene encoding MGMT (O_6-methylguanine-DNA methyltransferase), a DNA repair enzyme, has been demonstrated in 36% of primary GBMs and 75% of secondary GBMs and confers resistance to alkylating chemotherapeutic agents. (Kanu et al., 2009).

1.4- GBM Therapies

While mechanisms describing GBM initiation and development remain mostly unknown, modern advances in the interpretation of the signaling cascades that trigger GBM pathogenesis have led to the development of new therapeutic methods targeting several oncogenic signaling alterations associated with GBM (Mao et al., 2012). Since 2005, the suggested conventional treatment of GBM has been mainly focused on achieving greatest surgical resection followed by radiation and concurrent and subsequent adjuvant TMZ chemotherapeutic administration (Lathia et al., 2015; W. Li et al., 2012; Patel, Kim, Ruzevick, Li, & Lim, 2014). These treatment options present patients with an increased survival time to almost 22 months and an average quality of life (Lathia et al., 2015; W. Li et al., 2012). Surgical resection is categorized as either subtotal resection (STR) or gross total resection (GTR), with the latter providing an additional one-year survival in patients with more than 90% tumor removal (Orringer et al., 2012). ALA (5-aminolevulinic acid), a tumor- specific fluorescent method that allows tumor cells to fluoresce under blue light, is being utilized for better resolution in order to achieve maximal resection. Moreover, only 36% of resections reached GTR through conventional therapy, whereas 65% achieved GTR using ALA. In the case when GTR is unachievable, radiation therapy inducing apoptosis through extreme DNA damage could be used in

combination with surgery in a dose-dependent manner. Nonetheless, resistance to radiotherapy has been observed in GBM through the up-regulation of DNA repair mechanisms (Carlsson, Brothers, & Wahlestedt, 2014). Due to the tumor's heterogeneity and nature, GBMs have been reported to recur within 2 years following conventional therapies, thus the degree of tumor resection including the remaining bulk of tumor left greatly affects OS and relapse (W. Li et al., 2012; Nakada et al., 2011; Patel et al., 2014). Because of the low survival rate of GBM patients undergoing conventional therapy, novel therapeutic methods are urgently needed to improve patient prognosis and further develop existing therapeutic strategies to manage this fatal malignancy. (X. Li et al., 2016; Pandey, 2011; Pearson & Regad, 2017).

The present chemotherapeutic standard of care for glioblastoma multiforme is Temozolomide (TMZ), an orally administered DNA alkylating agent with remarkable tissue absorption, stability, and accumulation potential in the brain. TMZ induces nucleotide mispairing leading to cell cycle arrest at the G2/M phase eventually inducing apoptosis and autophagy (Carlsson et al., 2014; Patel et al., 2014; Ramirez, Weatherbee, Wheelhouse, & Ross, 2013). First introduced in 2005, TMZ administration along with radiation therapy has shown an increase in OS rates to approximately 27% at 2 years compared to the 10% observed with radiation therapy only (Carlsson et al., 2014). Recent studies have established an inverse relation correlating TMZ sensitivity to MGMT expression and promoter methylation, both *in vitro* and *in vivo* (Ohka, Natsume, & Wakabayashi, 2012; Ramirez et al., 2013). It was found that patients with methylated MGMT promoters due to gene silencing experience markedly increased survival (21 months versus 15 months) and sensitivity to TMZ treatment following radiotherapy, since

methylation decreases MGMT enzyme expression rendering the tumor cells prone to TMZ toxicity (Ramirez et al., 2013; Weathers & Gilbert, 2014). On the other hand, individuals expressing elevated MGMT levels show an increase in TMZ chemo-resistance, since MGMT strongly diminishes the effects of alkylating drugs. Therefore, MGMT methylation could be utilized as a potential biomarker and prognostic tool to indicate the degree of TMZ responsiveness (Polivka, Polivka, Rohan, Topolcan, & Ferda, 2012; Ramirez et al., 2013). However, a drawback for TMZ usage is the ability of inducing TMZ-related DNA damage in normal cells, thus other chemotherapeutic alternatives are needed to improve selectivity (Carlsson et al., 2014).

Glioblastoma multiforme is described as having elevated VEGF expression in which the ligand, VEGFA, binds to its receptor, VEGFR, promoting migration, proliferation, and most importantly angiogenesis by activating the PI3K pathway (Ramirez et al., 2013). Bevacizumab, an FDA approved monoclonal antibody against VEGF, was recently found to be an effective second-line cure for individuals with recurrent GBM (Mao et al., 2012). This antibody acts to inhibit the binding between VEGF and its cell surface receptors, VEGF-1 and VEGF-2, leading to decreased tumor cell survival and proliferation (Joshi et al., 2015). However, a meta-analysis studying cases of recurrent GBM treated with Bevacizumab showed a 2% increased incidence of ischemic stroke and intracranial hemorrhage resulting from sustained anti- angiogenic therapy (Roy, Lahiri, Maji, & Biswas, 2015). Recent clinical trials proposed that GBMs become resistant to bevacizumab through the activation of VEGF-independent pathways, thus patients experience tumor relapse and re-progression. (Ramirez et al., 2013; Roy et al., 2015).

The absence of remarkable progress in GBM therapy is due to multiple restrictions including the lack of oncogenomic understanding, GBM tumor heterogeneity, multi-drug resistance, and the activation of other signaling pathways leading to immune escape (Kanu et al., 2009; X. Li et al., 2016). One significant factor hindering the ability of selective drugs from reaching their target is the low permeability of the blood brain barrier (BBB), which prevents drugs from spreading to their targeted destination through drug metabolizing enzymes and AETs that carry drugs back to the blood stream (X. Li et al., 2016). Moreover, it has been observed that claudins, proteins that comprise tight junctions in the BBB, are important for prevention of small molecular drugs crossing across the barrier (claudin 5) such that the loss of claudin 3 is linked to elevated BBB penetrability in tumor bulks (Glaser, Han, Wu, & Zeng, 2017). Due to their excellent drug-loading capability and ease of use, liposomes and nanoparticles have been widely studied in an attempt to increase drug trafficking across the BBB, however this approach is mainly suitable for small molecule transport instead of large ones (Glaser et al., 2017).

1.5- Arginine Deprivation as Targeted Treatment

Enzymotherapies targeting cancer metabolic pathways through amino acid and essential nutrient deprivation have become greatly acknowledged in oncological research for the treatment of several tumors (Fiedler et al., 2015; Panosyan, Lin, Koster, & Lasky, 2017; Pavlyk et al., 2015). Such metabolic approaches are based on variations in amino acid requirements between tumor cells versus normal cells such as use of the enzyme arginase in depleting intracellular L- arginine levels, a semi-essential amino acid (Agarwal et al., 2017; Fiedler et al., 2015; Panosyan et al., 2017).

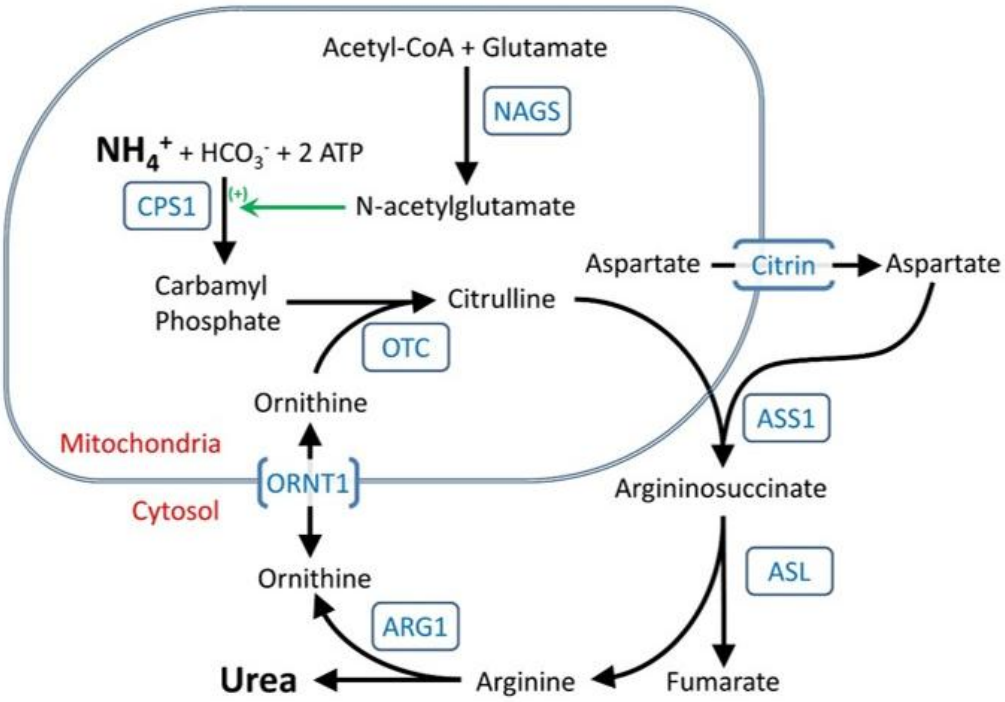


Figure 1. Enzymes and Intermediate compounds involved in the cyclic urea cycle. Arginase catalyzes the conversion of arginine into urea and ornithine.

In humans, citrulline and aspartate are used as substrates by the rate limiting enzyme arginosuccinate synthetase (ASS1) to produce arginosuccinate, which is then converted to arginine and fumarate through arginosuccinate lyase cataylzation (Figure 1) (Ah Mew et al., 2017; Yoon, Frankel, Feun, Ekmekcioglu, & Kim, 2012). Dietary intake and protein breakdown are the major sources of circulating arginine, with only 15% derived through *de novo* endogenous synthesis enough to maintain normal cell requirements (Pavlyk et al., 2015; Phillips, Sheaff, & Szlosarek, 2013). However, as tumors grow beyond a specific size, the surrounding vasculature and endogenous supply of arginine becomes insufficient for such rapidly dividing cells making them dependent

on exogenous nutrient sources for continuous proliferation. Hence, they become 'auxotrophic' for arginine as observed in many tumor types including GBM, both in *vitro* and in *vivo* (Fiedler et al., 2015; Khoury et al., 2015; Patil, Bhaumik, Babykutty, Banerjee, & Fukumura, 2016; Pavlyk et al., 2015; Yoon et al., 2012). Partial arginine auxotrophy has been evident in various tumors types in which such a dependency is reversed upon the addition of exogenous citrulline, however still exhibiting arginine reliance (Khoury et al., 2015; Ohshima et al., 2017). Multiple tumors including GBM, melanoma, hepatocellular carcinoma (HCC) and AML have been identified as deficient in arginine synthesizing enzymes namely ASS due to epigenetic methylation and down-regulation of the ASS1 gene, thus displaying complete arginine auxotrophy and the inability to produce arginine from citrulline (Langer et al., 2014; McAlpine, Lu, Wu, Knowles, & Thomson, 2014; Patil et al., 2016; Yoon et al., 2012). Accordingly, this semi-essential amino acid is needed for synthesis of polyamines, nucleotides, and glutamate in normal cells and for proliferation, invasion, and adhesion in malignant tumors such as GBM (Fiedler et al., 2015; Pavlyk et al., 2015; Phillips et al., 2013). This auxotrophy could be exploited as an anticancer treatment through arginine depravation of tissue surrounding the tumor, specifically in ASS- negative cell lines that critically depend on extracellular arginine, consequently inducing autophagy to sustain survival (Kremer et al., 2017; Yoon et al., 2012). Arginine depletion in regular cells promotes quiescence through cell cycle arrest at the G_o/G_1 phase which is abolished following administration of extracellular arginine reverting cells back to regular growth, unlike tumor cells that do not exhibit cell cycle arrest upon arginine starvation, thus leading to deregulated development and apoptosis (Fiedler et al., 2015; Patil et al., 2016). Accordingly, limiting extracellular

arginine through arginine-depleting enzyme supplementation could potentially target metabolic vulnerability in multiple cancer types, especially in tumors exhibiting arginine auxotrophy (Bean et al., 2016; Phillips et al., 2013).

In metabolism, arginine depletion is mediated by two enzymes, arginase which is expressed in mammalian cells, and arginine deiminase (ADI) derived from bacterial cells (Dillon, Holtsberg, Ensor, Bomalaski, & Clark, 2002). Arginase, recognized as a significant enzyme in the cyclic nature of the urea cycle, catalyzes the conversion of L-arginine to urea and L-ornithine. L-ornithine is either metabolized by ornithine decarboxylase (ODC) to produce polyamines, or alternatively converted to L-citrulline via ornithine transcarbamylase (OTC) in the mitochondria. L-arginine could be regenerated from L-citrulline by the enzymes ASS and ASL to be re-utilized in the urea cycle (Agarwal et al., 2017; Ah Mew et al., 2017; Munder, 2009; Patil et al., 2016; Yoon et al., 2012). Since various tumor types lack ASS1 expression compared to normal cells, arginine depletion by arginase could be employed to selectively target tumor tissue (Pavlyk et al., 2015). Nevertheless, restricted success was accomplished in *vivo* due to the biochemical characteristics of arginase, including short plasma half-life and alkaline pH requirements (9.6) (Patil et al., 2016). In order to overcome such limitations, a new bioengineered form of arginase was developed displaying significantly enhanced features in tumor cells. The endogenous human enzyme L-arginase I (HuArgI) containing two divalent manganese (Mn^{2+}) cations as essential cofactors, exhibits minimal catalytic activity and short circulating half-life at physiological conditions due to the rapid release of Mn^{2+} in serum. In order to extend its 4.5 hour half-life, the two (Mn^{2+}) ions were replaced by cobalt (Co^{2+}) ions [HuArgI (Co)], thus preventing enzyme inactivation

(Glazer et al., 2011). This Co^{2+}-modified arginase lead to five-fold greater serum stability and 10-fold higher catalytic activity with a lowered dissociation constant at physiological pH. Moreover, this ionic substitution resulted in a 15-fold lower IC_{50} value in treatment of HCC and melanoma tumors (Stone et al., 2010). Recently, modifications in Co^{2+}-arginase I were implemented by covalently linking it to polyethylene glycol (5-kDa PEG), thus generating a cobalt-substituted pegylated recombinant human arginase (Co-ArgI-PEG) (Yoon et al., 2012). It was shown that the resultant product induces non-apoptotic cell death in GBM and increased arginine deprivation sensitivity with low IC_{50} values in multiple cancers including HCC, melanoma, and AML (Khoury et al., 2015; Munder, 2009; Phillips et al., 2013).

1.6- The Role of Autophagy in Cancer Therapeutics

The term 'autophagy' meaning 'to eat oneself' refers to the mechanism of cell self-devouring through a lysosomal bulk-degradation route (Kaza, Kohli, & Roth, 2012; Levine & Yuan, 2005). Autophagy, a homeostatic and evolutionary conserved process, involves the degradation of mis-folded proteins and dysfunctional organelles through lysosomal catabolism (Jawhari, Ratinaud, & Verdier, 2016; Levine & Yuan, 2005). This highly regulated mechanism is crucial during nutritional deprivation for sustaining basal turnover of cell components, cellular homeostasis, development, and cell differentiation (Levy, Towers, & Thorburn, 2017; Z. J. Yang, Chee, Huang, & Sinicrope, 2011; Zhuang et al., 2012). Autophagy begins with the dynamic rearrangement of isolated membranes that fuse to form the autophagosome, which engulfs and sequesters cytoplasmic constituents designated for destruction (Kaza et al., 2012; Z. J. Yang et al., 2011). Then, the autophagosome fuses with a lysosome forming an autophagolysosome, which

enzymatically digests and recycles isolated components to maintain cellular biosynthesis and ATP production (Kaza et al., 2012; Levine & Yuan, 2005; Z. J. Yang et al., 2011). Such a process is mostly triggered in response to overcoming nutrient deprivation and oxidative stress such as hypoxia (Jawhari et al., 2016). Autophagy has also demonstrated multiple dynamic roles depending on the tumor type such as eradication of microorganisms, antigen presentation, and tumor suppression or alternatively tumor initiation (Mizushima, 2007; Santana-Codina, Mancias, & Kimmelman, 2017).

Autophagy has context-dependent dual roles in cancer acting as either an apparatus for cell growth by promoting development, or as a tumor suppressor inhibiting the buildup of dysfunctional cytoplasmic components (Kaza et al., 2012; Levy et al., 2017). The process by which autophagy could accomplish such contrasting actions has continued to be ambiguous until recently (Yan et al., 2016). In glioblastoma, it has been proposed that such a dynamic mechanism is firmly tangled with GBM tumorigenesis (Noonan, Zarrer, & Murphy, 2016). *Beclin 1*, an essential autophagy gene, was found to be monoallelically deleted in a high percentage of cancers including prostate, ovarian, and breast, hence providing a link between autophagy and tumor suppression (Mizushima, 2007). Moreover, lower cytoplasmic concentrations of beclin 1 protein were observed in GBM compared to healthy brain tissue which was found to correlate with increased tumor proliferation (Kaza et al., 2012). Recent evidence in mammalian cells has showed autophagy to be positively regulated by PTEN and negatively through the PI3K signaling cascade (Levine & Yuan, 2005). Furthermore, treatment with rapamycin, an autophagy inducer and mTOR inhibitor, showed a 90% decrease in lung tumors in mouse models, further displaying tumor suppression characteristics (Z. J. Yang et al., 2011). Although

increased autophagic flux has been linked to cancer regression, it may alternatively contribute to cancer initiation as a survival strategy to cope with adverse conditions such as nutrient deprivation, hypoxia, and high metabolic demands (Jawhari et al., 2016). For example, knockdown of vital autophagy genes in cancers has been shown to induce tumor cell death and reduce GBM development. Kanzawa *et al.* reported that autophagy is a necessary precursor for KRAS- driven gliomagenesis in GBM that results in chemotherapeutic resistance to TMZ (Gammoh et al., 2016). Continuous stress and prolonged autophagy eventually result in cell death when cytoplasmic component turnover overwhelms the cell's potential (Z. J. Yang et al., 2011). These opposing aspects of autophagy are as well observed in GBM. For example, upon autophagy inhibition, Palumbo *et al.* showed a decrease in GBM migration, whereas Catalano *et al*. indicated such a change following autophagy stimulation. Moreover, some anticancer treatments were shown to inhibit GBM progression by increasing autophagic influx, yet others lead to tumor survival and protection following induced autophagy (Jawhari et al., 2016). Recent evidence indicated that amino acid deprivation, namely arginine, promotes autophagy and could be used as a potential new strategy for drug development (Khoury et al., 2015; Szlosarek, 2014). The exact role of autophagy in tumorigenesis in response to arginine starvation has yet to be elucidated. A better understanding of such a process is needed for designing enhanced therapeutic approaches (Kaza et al., 2012).

In this study, we attempted to determine the long term effects of arginine deprivation on GBM cells and investigated the mechanism by which arginine depletion leads to cell death particularly the contribution of autophagy. We also studied the effect of autophagy inhibition on the sensitivity of GBM to HuArgI (Co)-PEG5000 treatment.

CHAPTER TWO
MATERIALS AND METHODS

2.1- Cell Lines and culture

The sensitivity of GBM to HuArgI (Co)-PEG5000 was tested on two human adult cell lines: U251 and A172. Both cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin at 37°C and 5% CO2 in a humidified incubator.

2.2- Expression and purification of [HuArgI (Co)-PEG5000]

Pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] was expressed and purified by Georgiou and Everett Stone at the University of Texas in Austin, TX, USA, as described previously (Zakalskiy et al., 2012).

2.3- Proliferation Inhibition Assay (cytotoxicity)

The effect of HuArgI (Co)-PEG5000 on GBM cell sensitivity, with or without the addition of chloroquine, was determined using a proliferation inhibition assay, as previously described (Abi-Habib et al., 2005). Briefly, aliquots of 10^3 cells/well in 100 ul DMEM were plated in a Costar flat-bottomed 96-well plate (Corning Inc. Corning, NY). Chloroquine was added to the cells at a concentration of 50 µM. Following 2 hours incubation, 50 µl HuArgI (Co)-PEG5000 in media was added to each well to obtain concentrations ranging from 10^{-7} to 10^{-13} M. Plates were incubated at 37°C/5% CO2 for

24, 48, 72, 96, and 120 hours to establish a time course. Following incubation, 50 µl of XTT cell proliferation reagent (Roche, Basel, Switzerland) was added to each well and further incubated for 4 hours. Absorbance at 450 nm was then determined using a microplate reader (Thermo Fisher Scientific, Waltham, MA). Nominal and percent average absorbance versus the log of the HuArgI (Co)-PEG5000 concentration was plotted. GraphPad Prism 5 software was used to generate a non-linear regression with a variable slope sigmoidal dose-response curve along with IC_{50} (GraphPad Software, San Diego, CA).

2.3- Autophagy Assay

To examine the potential role played by autophagy in arginine-deprivation mediated cytotoxicity of GBM, cells were incubated with HuArgI (Co)-PEG5000 alone and in combination with chloroquine, an autophagy inhibitor, and rapamycin, an autophagy inducer. Aliquots of 10^3 cells/ well were grown on coverslips in Dulbecco's Modified Eagle's Medium (DMEM) in a 6-well plate (Corning Inc. Corning, NY). After 2 hours incubation, HuArgI (Co)-PEG5000 was added to a subset of wells at a concentration of 10^{-7} M, followed by the addition of chloroquine at a concentration of 50 µM and rapamycin as a positive control at a concentration of 500 nM. This was followed by incubation of the plates for 24, 48, 72, 96, and 120 hours at 37°C/ 5% CO_2. After incubation, cells were washed twice with 1X Assay Buffer and 50 µl of Microscopy Dual Detection reagent obtained from CYTO-ID Autophagy Detection Kit (Enzo Life Sciences, Switzerland) were dispensed onto each coverslip and incubated for 30 minutes in the dark at 37°C/5% CO_2. Then, cells were washed with 100 µl of 1X Assay buffer and

fixed using 4% formaldehyde for 10 minutes at room temperature. Following fixation, cells were washed and placed on microscopic slides using mounting buffer. The stained cells were analyzed by fluorescence microscopy using a standard 488-Alexa Flour filter set for imaging the autophagic signal at 488 nm.

CHAPTER THREE

RESULTS

3.1-Long Term Effect of Arginine Deprivation on GBM Cell Lines

We had previously demonstrated that GBM cell lines were auxotrophic for arginase (partially and completely auxotrophic) and sensitive to the HuArgI (Co)-PEG5000-induced arginine deprivation. We also demonstrated that the degree of arginine auxotrophy in GBM cells (partially versus completely auxotrophic) was dependent on expression levels of argininosuccinate synthetase 1 (ASS1). Since the sensitivity of GBM cell lines to arginine deprivation may vary in a time-dependent manner, and as we have previously shown that GBM cells are sensitive to arginine deprivation at early time points, we attempted to investigate the long term effects of HuArgI (Co)-PEG5000-induced arginine deprivation. Both cell lines showed an increased sensitivity to HuArgI (Co)-PEG5000 and lower IC_{50} values with increasing incubation times, compared to the 24-hour time point, and culminating in IC_{50} values ranging between 150 and 348 pM at the longest incubation time tested of 120 hours (Figure 2, Table 1).

Table 1. Sensitivity of GBM cell lines to HuArgI (Co)-PEG5000 at 24, 48, 72, 96, and 120 hrs.

Cell line	HuArgI (Co)-PEG5000 (24hrs)	HuArgI (Co)-PEG5000 (48hrs)	HuArgI (Co)-PEG5000 (72hrs)	HuArgI (Co)-PEG5000 (96hrs)	HuArgI (Co)-PEG5000 (120hrs)
U251	>10,000 pM	354 pM	383 pM	361 pM	150 pM
A172	3157 pM	1016 pM	406 pM	392 pM	348 pM

Furthermore, both cell lines showed an increase in the percent cell death at the highest HuArgI (Co)-PEG5000 concentration when subjected to arginine deprivation for longer periods (from 83% at 72 hours to 86% at 96 hours and to 88% at 120 hours for A172; and from 88% at 72 hours to 91% at 96 hours and to 89% at 120 hours for U251) compared to shorter incubation times (52% and 55% at 24 hours for A172 and U251, respectively) (Figure 2). Such an increase in cell death further shows the extent of the cytotoxic effects of HuArgI (Co)-PEG5000 on GBM cells denoting the arginine auxotrophy displayed by such cell lines.

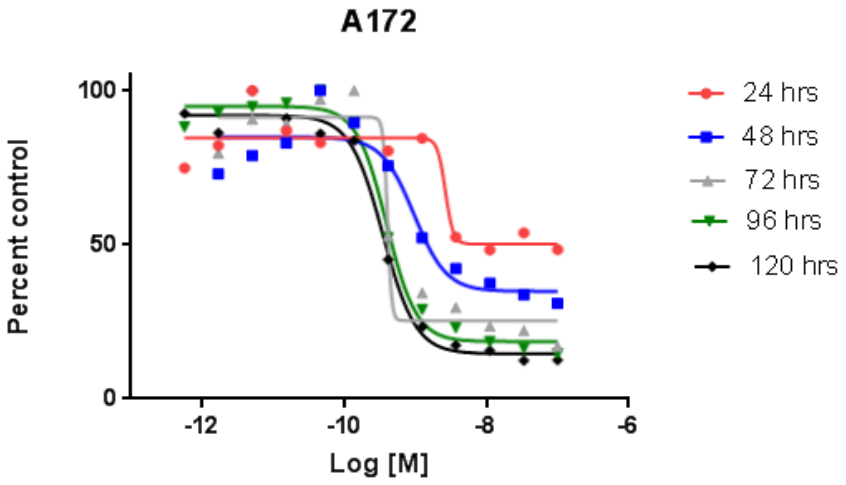


Figure 2. Sensitivity of A172 cells to HuArgI (Co)-PEG5000 at different time points. Non-linear regression curves are shown with HuArgI (Co)-PEG5000 at 24 hours (red), 48 hours (blue), 72 hours (grey), 96 hours (green), and 120 hours (black).

These results demonstrate the long term increased sensitivity of GBM cells to HuArgI (Co)-PEG5000- mediated arginine depletion compared to the early effects described in previous studies, thus displaying the characteristics of arginine auxotrophy.

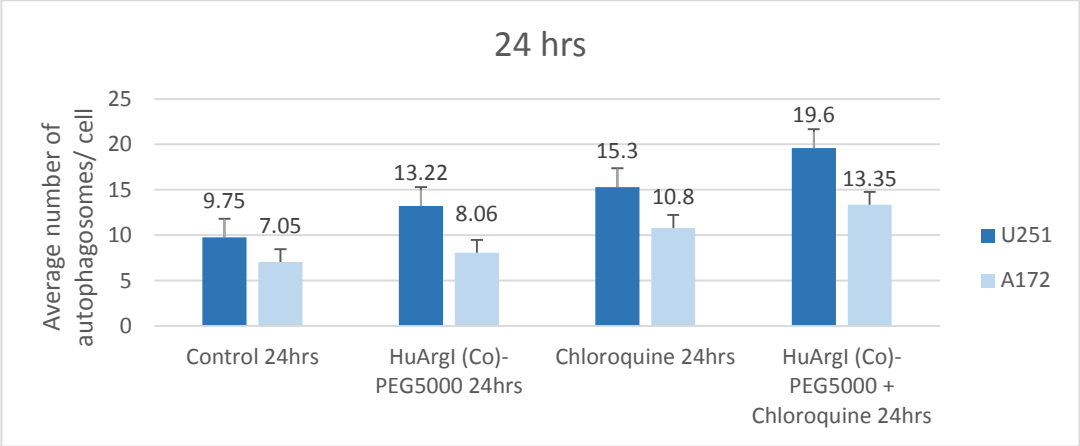
3.2 Activation of Autophagy

In order to investigate the mechanisms of sensitivity of GBM cells to HuArgI (Co)-PEG5000-induced arginine deprivation, particularly the contribution of autophagy, GBM cells were incubated with HuArgI (Co)-PEG5000 alone or in combination with 50 μM of the autophagy inhibitor, chloroquine (CQ). Chloroquine alone was used as a control. We were able to show a time-dependent increase in the formation of autophagosomes, hence an increase in the flux through autophagy, in both cell lines following HuArgI (Co)-PEG5000-induced arginine deprivation. This was evident by the increase in autophagosome formation in arginine depleted cells compared to controls (from an average of 9.75 autophagosomes/cell in controls to 13.22 autophagosomes/cell with HuArgI (Co)-PEG5000 and from an average of 7.05 autophagosomes/cell in controls to 8.06 autophagosomes/cell with HuArgI (Co)-PEG5000 in U251 and A172, respectively) (Figure 3 A, Figure 4 A). Moreover, HuArgI (Co)-PEG5000 treatment at 24 hours lead to a slight increase in autophagosome size compared to non-treated cells (from 78 au in controls to 90 au with HuArgI (Co)-PEG5000 and from 77.6 au in controls to 85.5 au with HuArgI (Co)-PEG5000 in U251 and A172, respectively) (Figure 3 B).

Since the impact of autophagy in response to arginine depletion should be time dependent, similarly to cytotoxicity, longer incubation periods were tested on both cell lines. A172 and U251 cells showed increased activation of autophagy, hence an increased autophagic flux, with time as evidenced by an increase in the number of autophagosomes formed at 72 and 120 hours post-treatment compared to early time points in cells incubated with HuArgI (Co)-PEG5000 (from 13.2 at 24 hours to 40 at 72 hours to 57.5 at

120 hours and from 8.06 at 24 hours to 33.4 at 72 hours to 44.1 at 120 hours in U251 and A172, respectively) (Figure 4 A, C, and D). Moreover, the average area of autophagosomes also increased at longer incubation periods compared to early time points in cells incubated with HuArgI (Co)-PEG5000 alone (from 90 au at 24 hours to 123.4 au at 72 hours and from 85.5 au at 24 hours to 130.5 at 72 hours for U251 and A172, respectively) (Figure 4 A, C). However, the formation of autophagic vesicles in U251 occurs at a faster rate producing more autophagosomes with a greater area compared to A172 cells at both early and late time points following HuArgI (Co)-PEG5000 treatment.

A



B

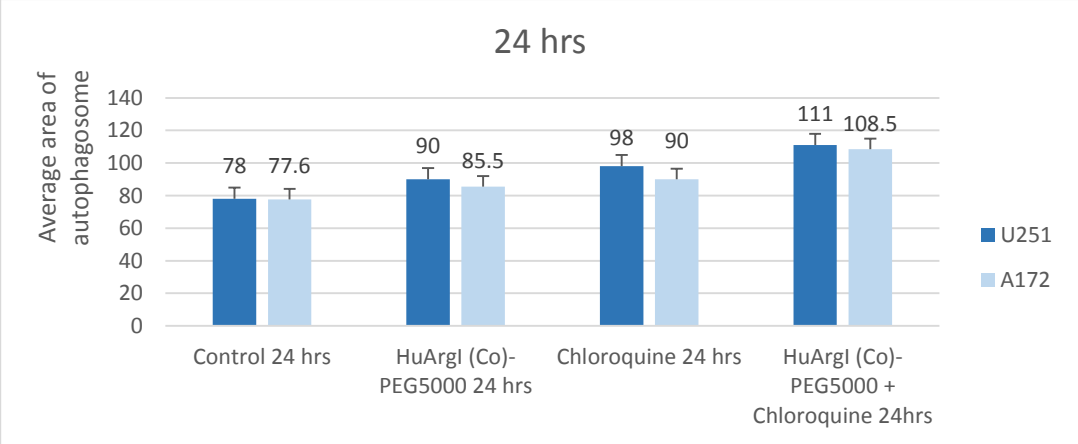
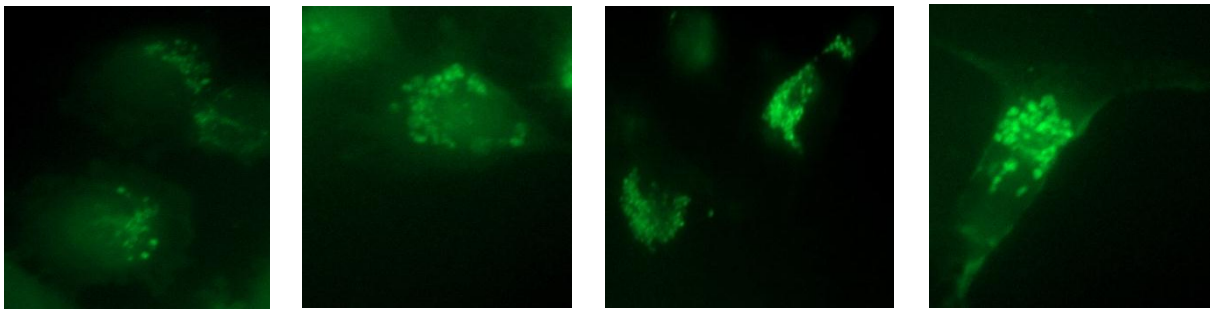


Figure 3. Graphs showing the effect of arginine depletion on the number of autophagosomes/ cell (A) and area of autophagosomes (B) following a 24 hour incubation period with HuArgI (Co)-PEG5000 alone, with 50 µM chloroquine alone, and with HuArgI (Co)-PEG5000 in combination

with 50 µM chloroquine. U251 cell line is depicted in dark blue and A172 cell line is depicted in light blue.

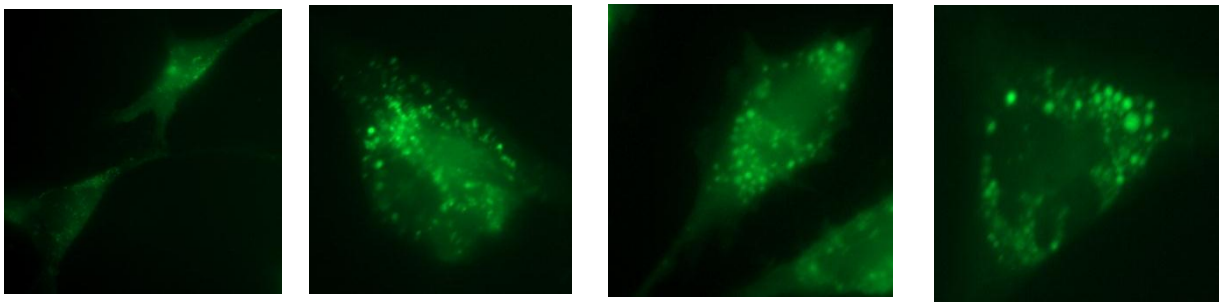
A) 24 hours

U251



Control | HuArgI (Co)-PEG5000 | Chloroquine | HuArgI (Co)-PEG5000 + chloroquine

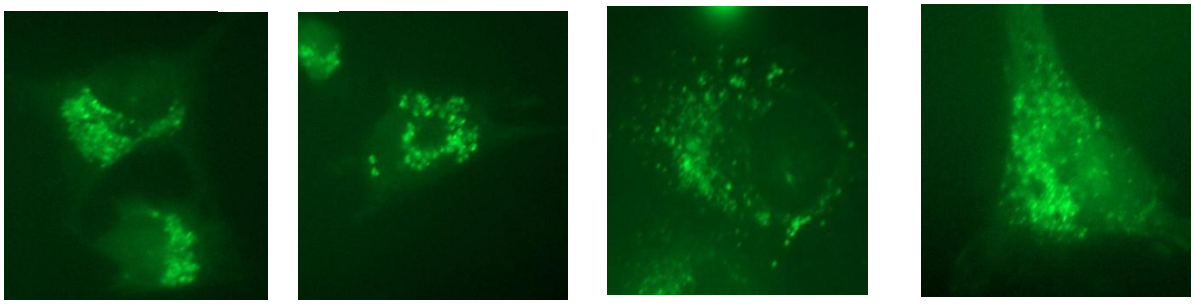
A172



Control | HuArgI (Co)-PEG5000 | Chloroquine | HuArgI (Co)-PEG5000 + chloroquine

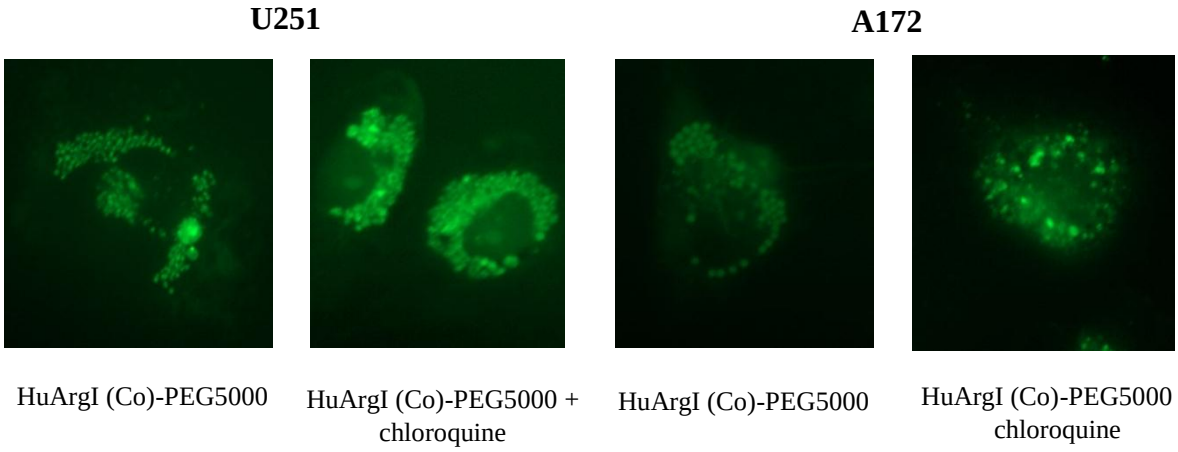
B) 48 hours

U251 **A172**



HuArgI (Co)-PEG500 | HuArgI (Co)-PEG5000 + chloroquine | HuArgI (Co)-PEG5000 | HuArgI (Co)-PEG5000 + chloroquine

C) 72 hours



D) 120 hours

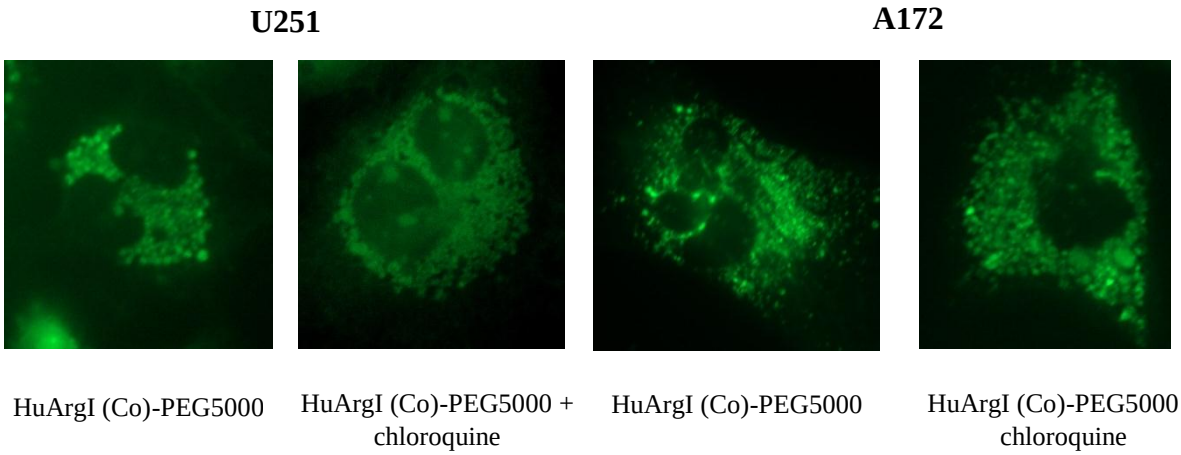


Figure 4. Microscopic images showing autophagosome formation induced by HuArgI- (Co) PEG5000 treatment in U251 and A172 at 24 hours (A), 48 hours (B), 72 hours (C), and 120 hours (D). Cells were incubated with HuArgI (Co)-PEG5000 alone, with 50 µM chloroquine alone, or with HuArgI (Co)-PEG5000 in a combination with 50 µM chloroquine.

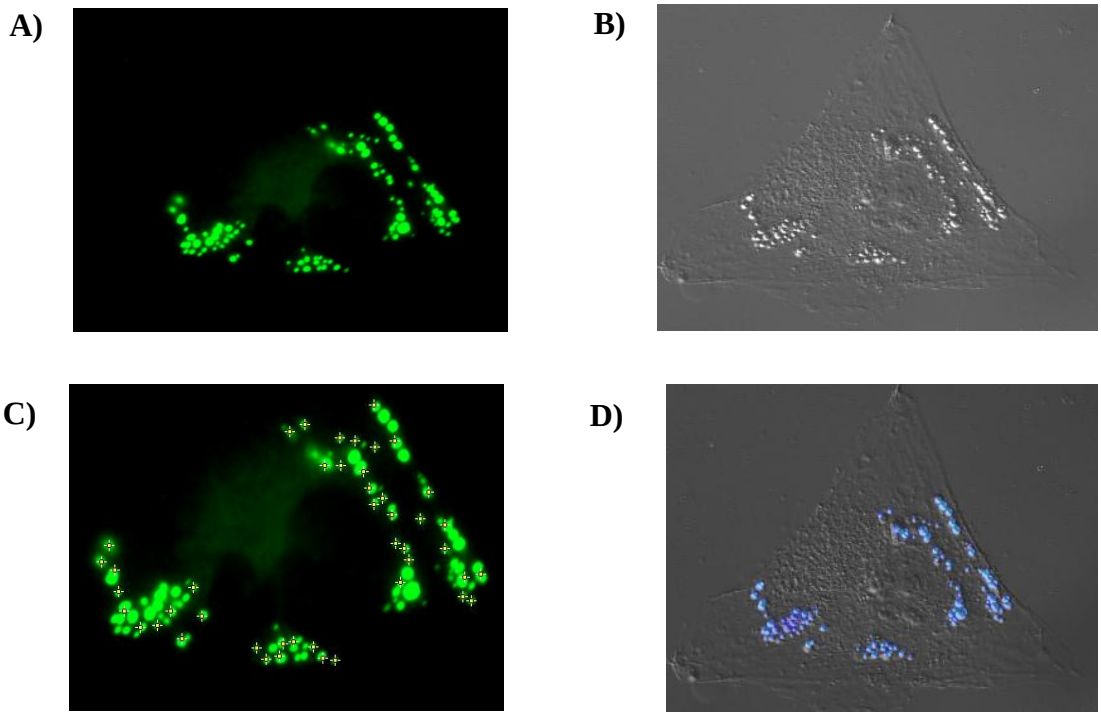


Figure 5. Images illustrating the method used to quantify intracellular autophagosomes using ImageJ software. Fluorescent image (A), differential interference contrast (DIC) (B), quantification of each autophagosome (C), overlay of fluorescent and DIC images (D). The figures are A172 cells treated with HuArgI (Co)-PEG5000 and rapamycin for 72 hours.

The autophagosomes formed following HuArgI (Co)-PEG5000 treatment were analyzed producing a fluorescent image (Figure 5 A) showing individual autophagosomes along with the DIC images showing the entire cell (Figure 5 B). Overlays of both images clearly illustrate the autophagosomes formed at the margins of the cell (Figure 5 D).

In order to test the effect of autophagy inhibition on the formation of autophagosomes, we used chloroquine (CQ), a downstream inhibitor of autophagy. The inhibitory action of chloroquine blocks the processing of autophagosomes, hence leading to their excessive accumulation, which was evident from 24 hours to 120 hours. The co-incubation of GBM cells with HuArgI (Co)-PEG5000 and 50 µM chloroquine led to a significant increase in the number of autophagosomes produced compared to controls and

HuArgI (Co)-PEG5000 alone at an early time point of 24 hours (from 9.75 autophagosomes/cell in controls to 13.22 in HuArgI (Co)-PEG5000 alone to 19.6 in HuArgI (Co)-PEG5000 and chloroquine in U251; and from 7.05 autophagosomes/cell in controls to 8.06 in HuArgI (Co)-PEG5000 alone to 13.35 in HuArgI (Co)-PEG5000 and chloroquine in A172) (Figure 3 A, Figure 4 A). In addition to the increase in number, the average area of the autophagosomes also increased when cells were incubated with HuArgI (Co)-PEG5000 in combination with chloroquine (111 and 108.5) compared to controls (78 and 77.6) and HuArgI (Co)-PEG5000 alone (90 and 85.5) at 24 hours for U251 and A172, respectively (Figure 3 B, Figure 4 A).

To determine the long term effect of autophagy inhibition, we incubated cells with HuArgI (Co)-PEG5000 and chloroquine at 72 and 120 hours. At such late time points, the average number of autophagosomes was significantly higher in cells incubated with HuArgI (Co)-PEG5000 and chloroquine compared to HuArgI (Co)-PEG5000 alone (45.8 autophagosomes/ cell in HuArgI (Co)-PEG5000 and chloroquine compared to 40 autophagosomes/cell in HuArgI (Co)-PEG5000 alone in U251; and 37.5 autophagosomes/cell in HuArgI (Co)-PEG5000 and chloroquine compared to 33.4 autophagosomes/cell in HuArgI (Co)-PEG5000 alone in A172) at 72 hours post treatment (Figure 4 C). Moreover, when comparing the effect of autophagy inhibition in response to arginine deprivation at different time points, it was shown that over time, the average number and area of autophagosomes maintained a constant increase from 24 hours up to 120 hours, hence showing the important role played by autophagy in response to arginine depletion. Therefore, incubation of cells with both HuArgI (Co)-PEG5000 and CQ led to a further accumulation of autophagosomes since CQ inhibits their downstream

processing, while HuArgI (Co)-PEG5000-induced arginine deprivation leads to increased production of autophagosomes, hence demonstrating that HuArgI (Co)-PEG5000-induced arginine deprivation leads to a time-dependent increase in the flux through autophagy in GBM cell lines.

3.3- Effect of Autophagy Inhibition on Sensitivity of GBM Cells to Arginine Deprivation

In order to determine the impact of autophagy inhibition on cell cytotoxicity in response to arginine deprivation following HuArgI (Co)-PEG5000 treatment in GBM cells, we used chloroquine, an autophagy inhibitor, in a proliferation inhibition assay. A172 and U251 were tested for sensitivity to HuArgI (Co)-PEG5000 alone and in combination with 50 µM chloroquine. Co- incubation of U251 with HuArgI (Co)-PEG5000 and 50 µM chloroquine resulted in a decreased IC_{50} value compared to HuArgI (Co)-PEG5000 alone and hence higher sensitivity at an early time point of 24 hours. However, at late time points (96 and 120 hours) the addition of chloroquine to U251 cells led to a decrease in sensitivity to HuArgI (Co)-PEG5000 and was evident in the increased percentage of surviving cells following treatment (from 10% with HuArgI (Co)-PEG5000 alone to 28% in combination with chloroquine at 96 hours and from 11 % with HuArgI (Co)-PEG5000 alone to 29% with chloroquine at 120 hours) (Figure 6 B). The IC_{50} values obtained with the combination of HuArgI (Co)-PEG5000 and chloroquine were 7-fold lower for A172 compared to HuArgI (Co)-PEG5000 alone at 24 hours, hence showing increased sensitivity (Figure 6 A). This indicates that the activation of autophagy plays a protective role at early time points in GBM cells subjected to arginine deprivation.

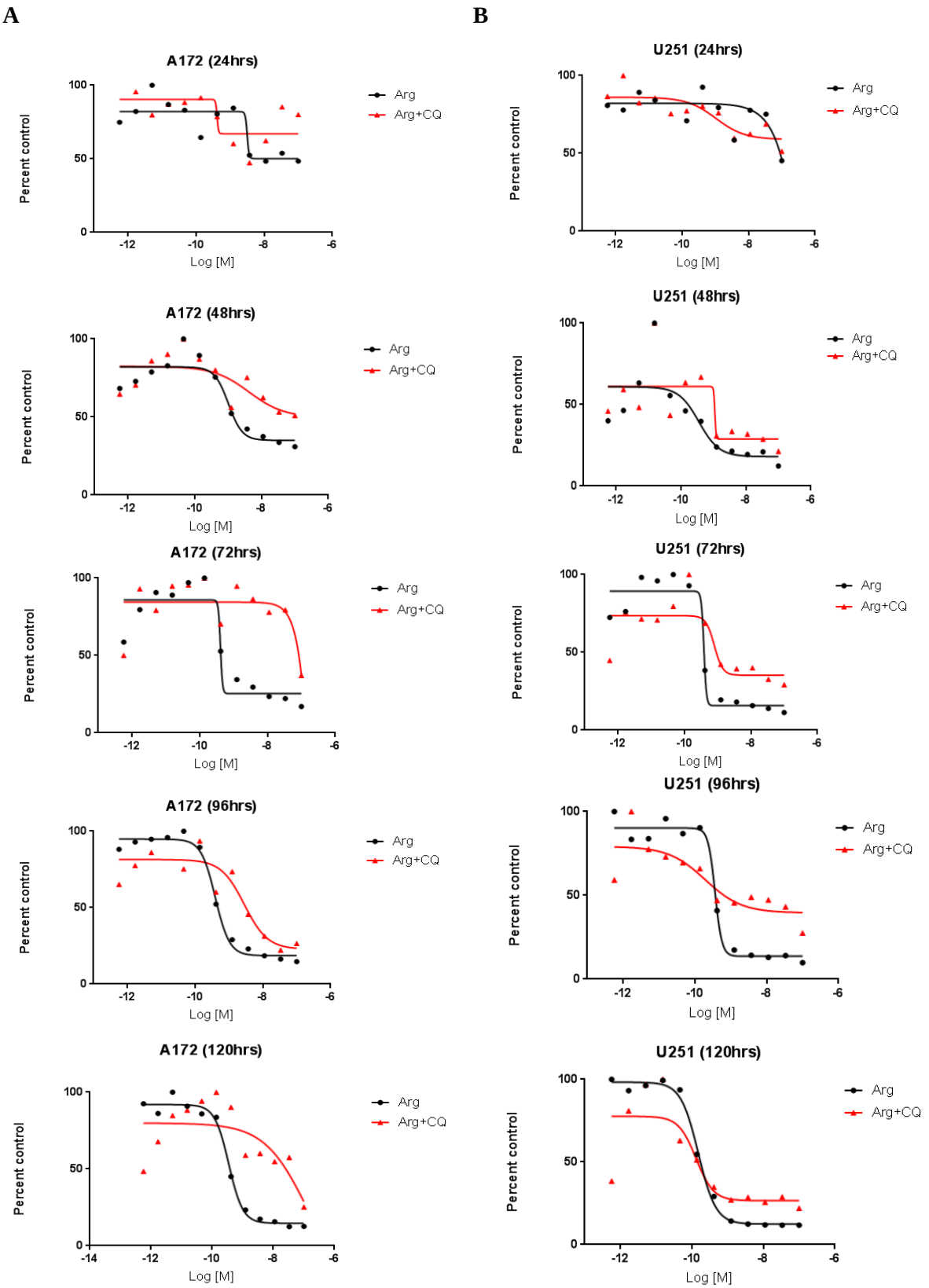


Figure 6. Sensitivity of A172 cells (A) and U251 cells (B) to HuArgI (Co)-PEG5000 in the

presence and absence of 50 μM of the autophagy inhibitor chloroquine at 24, 48, 72, 96, and 120 hrs incubation times. Non-linear regression curves are shown with HuArgI (Co)-PEG5000 alone **(**black circle) and HuArgI (Co)-PEG5000 in combination with chloroquine (red triangle).

However, at later time points, the combination of HuArgI (Co)-PEG5000 and chloroquine resulted in an increase in IC_{50} values for A172 at 72, 96, and 120 hours, hence exhibiting decreased HuArgI (Co)-PEG5000 sensitivity with longer incubation periods. Furthermore, the percent of surviving A172 cells increased from 14% with HuArgI (Co)-PEG5000 alone to 27% in combination with chloroquine at 96 hours and from 12% with HuArgI (Co)-PEG5000 alone to 26% following chloroquine addition at 120 hours (Figure 6 A). These results indicate that the excessive activation of autophagy, at later time points, leads to cell death in GBM cells subjected to arginine deprivation. Hence, these results demonstrate the dual role played by autophagy in response to HuArgI (Co)-PEG5000-induced arginine deprivation in GBM cells, with autophagy playing a protective role at early time points and leading to cell death (death by autophagy) at late time points.

These results demonstrate that arginine depletion through treatment of GBM with HuArgI (Co)-PEG5000 induces the activation of autophagy. Since autophagy inhibition lead to an increase in cell sensitivity to HuArgI (Co)-PEG5000 at early time points, this indicates that autophagy activated in response to arginine deprivation plays a protective role against HuArgI (Co)-PEG5000-mediated cell death of GBM at early time points. On the other hand, the inhibition of autophagy at later time points lead to a decrease in cell sensitivity to HuArgI (Co)-PEG5000, thus showing how HuArgI (Co)-PEG5000-mediated arginine-depletion leads to death by autophagy in GBM cells.

CHAPTER FOUR

DISCUSSION

Glioblastoma multiforme is a central nervous system Grade IV malignant tumor mainly affecting higher age groups (Urbańska et al., 2014). Even with innovative therapeutic advances and the low metastatic rate of such a type of cancer, its prognosis remains poor with less than 5% of patients surviving more than 2.5 years after diagnosis (Tamimi & Juweid, 2017). The conventional applied treatment for GBM is surgical resection followed by radiation therapy and TMZ administration. However, due to the low percentage of patients surviving after undergoing conventional therapy, finding novel therapeutic methods to improve patient survival and prognosis is of significant importance (W. Li et al., 2012).

In a previous study we established that glioblastoma cells are sensitive to HuArgI (Co)-PEG5000-induced arginine depletion with some cell lines being partially auxotrophic for arginine and others being completely auxotrophic and how this phenomenon was linked to arginosuccinate synthetase expression (Khoury et al., 2015). In this study, we examined the long term effects of arginine deprivation on GBM cells and investigated the mechanism by which arginine depletion leads to cell death particularly the contribution of autophagy. We have shown that GBM cells are auxotrophic for arginine due to their increased sensitivity following HuArgI (Co)-PEG5000-induced arginine depletion especially at longer time points. Both cell lines were shown to be sensitive to HuArgI (Co)-PEG5000 with IC_{50} values in the pM range, thus revealing the increased effectiveness of arginine deprivation in targeting GBM cell lines.

Moreover, arginine depletion showed an increase in percent cell death ranging between 69% and 88% at highest toxin concentration 48 hours following HuArgI (Co)-PEG5000 treatment, thus demonstrating the high potency of arginine deprivation in targeting GBM. A significant decrease in IC_{50} values was also observed following longer incubation periods with HuArgI (Co)-PEG5000, hence depicting the time-dependent effectiveness of arginine deprivation in arginine auxotrophic cells. The effect of autophagy inhibition was also studied in an attempt to determine the role of such a process in response to HuArgI (Co)-PEG5000- mediated arginine depletion. In order to define whether autophagy contributes to cell protection against arginine deprivation or whether it is responsible for autophagy-mediated cell death, we inhibited the activation of autophagy at various time points using the autophagy inhibitor, chloroquine. It was shown that the inhibition of autophagy by chloroquine at early time points leads to an increase in cell sensitivity to HuArgI (Co)-PEG5000 with lower IC_{50} values compared to treatment with HuArgI (Co)-PEG5000 alone. However, at later time points (96 and 120 hours) the sensitivity of GBM to arginine depletion is reversed with higher IC_{50} values and an increased percentage of cell survival, hence lower sensitivity to amino acid depletion. Since autophagy inhibition resulted in greater cell sensitivity to arginine deprivation at early stages, this explains the protective role contributed by autophagy against HuArgI (Co)-PEG5000-mediated cell death. However, such an inhibition resulted in decreased sensitivity of GBM cells to arginine depletion at later stages indicating that HuArgI (Co)-PEG5000 leads to death by autophagy at later time points. Previous studies have also illustrated such a variation with respect to the contribution of autophagy to arginine depleting cytotoxicity on GBM cell lines (Khoury et al., 2015), however only at early time points. Hence, these results

describe the dual-time-dependent role played by autophagy in arginine auxotrophic GBM cell lines.

In an attempt to further determine the exact role played by autophagy in response to HuArgI (Co)-PEG5000- induced arginine depletion, we tested GBM cells for autophagosome formation at different time points. Our results show that at early time points, HuArgI (Co)-PEG5000 treated cells exhibit an increase in the number and area of autophagosomes produced compared to control cells, thus showing the activation of autophagy in response to arginine deprivation. Moreover, cells treated with HuArgI (Co)-PEG5000 and chloroquine showed a significant increase in autophagosome formation starting at 24 hours up to 120 hours, hence demonstrating the excessive continuous activation of autophagy. The addition of chloroquine interrupts the fusion of autophagosomes with lysosomes, thus blocking the autophagic flux signals and hindering the downstream processing of autophagosomes. This results in the accumulation of autophagosomes subsequently inhibiting autophagy, which describes the results we obtained. The autophagy data obtained confirms our cytotoxicity results in that autophagy contributes to protection at preliminary stages against drug-mediated cytotoxicity, however due to the increase in autophagosome formation and decrease in cell death at late time points observed during autophagy inhibition, it can be concluded that this process eventually leads to autophagy-mediated cell death upon arginine depletion.

As a conclusion, in this study we have established that GBM cell lines are sensitive to HuArgI (Co)-PEG5000-mediated arginine deprivation as a long term effect, since they display extensive arginine auxotrophy which can be used to target different types of tumors. Furthermore, we have shown that autophagy has time-dependent roles acting as

either a protector against drug-induced cell death at early stages or as a mediator of cell death at late stages. Overall, HuArgI (Co)-PEG5000 exhibits excellent potency and selectivity against glioblastoma multiforme.

BIBLIOGRAPHY

Agarwal, P., Pajor, M. J., Anson, D. M., Guda, M. R., Labak, C. M., Tsung, A. J., & Velpula, K. K. (2017). Elucidating immunometabolic targets in glioblastoma. *American journal of cancer research*, *7*(10), 1990-1995.

Ah Mew, N., Simpson, K. L., Gropman, A. L., Lanpher, B. C., Chapman, K. A., & Summar, M. L. (1993). Urea Cycle Disorders Overview. In M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. Bean, K. Stephens, & A. Amemiya (Eds.), *GeneReviews®*. Seattle (WA): University of Washington, Seattle. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK1217/.

Bean, G. R., Kremer, J. C., Prudner, B. C., Schenone, A. D., Yao, J. C., Schultze, M. B., ... & Rubin, B. P. (2016). A metabolic synthetic lethal strategy with arginine deprivation and chloroquine leads to cell death in ASS1-deficient sarcomas. *Cell death & disease*, *7*(10), e2406.

Carlsson, S. K., Brothers, S. P., & Wahlestedt, C. (2014). Emerging treatment strategies for glioblastoma multiforme. *EMBO Molecular Medicine*, *6*(11), 1359-1370.

Davis, M. E. (2016). Glioblastoma: overview of disease and treatment. *Clinical journal of oncology nursing*, *20*(5), S2-S8.

Dillon, B. J., Holtsberg, F. W., Ensor, C. M., Bomalaski, J. S., & Clark, M. A. (2002). Biochemical characterization of the arginine degrading enzymes arginase and arginine deiminase and their effect on nitric oxide production. *Medical Science Monitor*, *8*(7), BR248-BR253.

Dong, S., Nutt, C. L., Betensky, R. A., Stemmer-Rachamimov, A. O., Denko, N. C., Ligon, K. L., ... & Louis, D. N. (2005). Histology-based expression profiling yields novel prognostic markers in human glioblastoma. *Journal of Neuropathology & Experimental Neurology*, *64*(11), 948-955

El-Habashy, S. E., Nazief, A. M., Adkins, C. E., Wen, M. M., El-Kamel, A. H., Hamdan, A. M., ... & Nounou, M. I. (2014). Novel treatment strategies for brain tumors and metastases. *Pharmaceutical patent analyst*, *3*(3), 279-296.

Endersby, R., & Baker, S. J. (2008). PTEN signaling in brain: neuropathology and tumorigenesis. *Oncogene*, *27*(41), 5416-5430.

Faraz, S., Pannullo, S., Rosenblum, M., Smith, A., & Wernicke, A. G. (2016). Long-term survival in a patient with glioblastoma on antipsychotic therapy for schizophrenia: a case report and literature review. *Therapeutic advances in medical oncology*, *8*(6), 421-428.

Fiedler, T., Strauss, M., Hering, S., Redanz, U., William, D., Rosche, Y., ... & Maletzki, C. (2015). Arginine deprivation by arginine deiminase of Streptococcus pyogenes controls primary glioblastoma growth in vitro and in vivo. *Cancer biology & therapy*, *16*(7), 1047-1055.

Fisher, J. L., Schwartzbaum, J. A., Wrensch, M., & Wiemels, J. L. (2007). Epidemiology of brain tumors. *Neurologic Clinics*, *25*(4), 867-890.

Friedmann-Morvinski, D. (2014). Glioblastoma heterogeneity and cancer cell plasticity. *Critical Reviews in Oncogenesis*, *19*(5), 327-336.

Fürthauer, M., & González-Gaitán, M. (2009). Endocytosis, asymmetric cell division, stem cells and cancer: unus pro omnibus, omnes pro uno. *Molecular oncology*, *3*(4), 339-353.

Galli, R., Binda, E., Orfanelli, U., Cipelletti, B., Gritti, A., De Vitis, S., ... & Vescovi, A. (2004). Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer research*, *64*(19), 7011-7021.

Gammoh, N., Fraser, J., Puente, C., Syred, H. M., Kang, H., Ozawa, T., ... & Jiang, X. (2016). Suppression of autophagy impedes glioblastoma development and induces senescence. *Autophagy*, *12*(9), 1431-1439.

Glantz, M. J., Cole, B. F., Forsyth, P. A., Recht, L. D., Wen, P. Y., Chamberlain, M. C., ... & Cairncross, J. G. (2000). Practice parameter: Anticonvulsant prophylaxis in patients with newly diagnosed brain tumors. Report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology*, *54*(10), 1886-1893.

Glaser, T., Han, I., Wu, L., & Zeng, X. (2017). Targeted nanotechnology in Glioblastoma Multiforme. *Frontiers in pharmacology*, *8*, 166.

Glazer, E. S., Stone, E. M., Zhu, C., Massey, K. L., Hamir, A. N., & Curley, S. A. (2011). Bioengineered human arginase I with enhanced activity and stability controls hepatocellular and pancreatic carcinoma xenografts. *Translational oncology*, *4*(3), 138-146.

Hanif, F., Muzaffar, K., Perveen, K., Malhi, S. M., & Simjee, S. U. (2017). Glioblastoma multiforme: A review of its epidemiology and pathogenesis through clinical presentation and treatment. *Asian Pacific journal of cancer prevention: APJCP*, *18*(1), 3

Haque, A., Banik, N. L., & Ray, S. K. (2011). Molecular alterations in glioblastoma: potential targets for immunotherapy. *In Progress in molecular biology and translational science*, *98*, 187-234.

Harris, S. L., & Levine, A. J. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene*, *24*(17), 2899-2908.

Inda, M. D. M., Bonavia, R., & Seoane, J. (2014). Glioblastoma multiforme: a look inside its heterogeneous nature. *Cancers*, *6*(1), 226-239.

Information (US), N. C. for B. (1998). The p53 tumor suppressor protein. National Center for Biotechnology Information (US). Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK22268/

Jawhari, S., Ratinaud, M. H., & Verdier, M. (2016). Glioblastoma, hypoxia and autophagy: a survival-prone ‘ménage-à-trois’. *Cell death & Disease*, *7*(10), e2434.

Joshi, S. K., Lucic, N., & Zuniga, R. (2015). Molecular pathogenesis of glioblastoma multiforme: Nuances, obstacles, and implications for treatment. *World Journal of Neurology, 5* (3), 88-101.

Jovčevska, I., Kočevar, N., & Komel, R. (2013). Glioma and glioblastoma‐how much do we (not) know? *Molecular and clinical oncology*, *1*(6), 935-941.

Kalpathy-Cramer, J., Gerstner, E. R., Emblem, K. E., Andronesi, O. C., & Rosen, B. (2014). Advanced magnetic resonance imaging of the physical processes in human glioblastoma. *Cancer research*, *74*(17), 4622-4637.

Kanu, O. O., Hughes, B., Di, C., Lin, N., Fu, J., Bigner, D. D., ... & Adamson, C. (2009). Glioblastoma multiforme oncogenomics and signaling pathways. *Clinical medicine*. *Oncology*, *3*, 39-52.

Kaza, N., Kohli, L., & Roth, K. A. (2012). Autophagy in brain tumors: a new target for therapeutic intervention. *Brain pathology*, *22*(1), 89-98.

Khoury, O., Ghazale, N., Stone, E., El-Sibai, M., Frankel, A. E., & Abi-Habib, R. J. (2015). Human recombinant arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-induced arginine depletion is selectively cytotoxic to human glioblastoma cells. *Journal of neuro-oncology*, *122*(1), 75-85.

Kremer, J. C., Prudner, B. C., Lange, S. E. S., Bean, G. R., Schultze, M. B., Brashears, C. B., ... & Shelton, L. (2017). Arginine deprivation inhibits the warburg effect and upregulates glutamine anaplerosis and serine biosynthesis in ass1-deficient cancers. *Cell reports*, *18*(4), 991-1004.

Kyritsis, A. P., Bondy, M. L., Rao, J. S., & Sioka, C. (2009). Inherited predisposition to glioma. *Neuro-oncology*, *12*(1), 104-113.

Lakhan, S. E., & Harle, L. (2009). Difficult diagnosis of brainstem glioblastoma multiforme in a woman: a case report and review of the literature. *Journal of medical case reports*, *3*(1), 87.

Langer, J., Elustondo, F. A., Chan, E. C. Y., Antti, H., Want, E., ONeill, K., & Syed, N. (2014). TM-11 Metabolomic Analysis Of Glioblastoma Multiforme Upon Arginine Deprivation Treatment. *Neuro-oncology*, *16*(suppl_5), v215-v215.

Lathia, J. D., Mack, S. C., Mulkearns-Hubert, E. E., Valentim, C. L., & Rich, J. N. (2015). Cancer stem cells in glioblastoma. *Genes & development*, *29*(12), 1203-1217.

Levine, B., & Yuan, J. (2005). Autophagy in cell death: an innocent convict? *The Journal of clinical investigation*, *115*(10), 2679-2688.

Levy, J. M. M., Towers, C. G., & Thorburn, A. (2017). Targeting autophagy in cancer. *Nature Reviews Cancer*, *17*(9), 528-542.

Li, W. B., Tang, K., Chen, Q., Li, S., Qiu, X. G., Li, S. W., & Jiang, T. (2012). MRI manifestions correlate with survival of glioblastoma multiforme patients. *Cancer biology & medicine*, *9*(2), 120-123.

Li, X., Wu, C., Chen, N., Gu, H., Yen, A., Cao, L., ... & Wang, L. (2016). PI3K/Akt/mTOR signaling pathway and targeted therapy for glioblastoma. *Oncotarget*, *7*(22), 33440-33450.

Mao, H., LeBrun, D. G., Yang, J., Zhu, V. F., & Li, M. (2012). Deregulated signaling pathways in glioblastoma multiforme: molecular mechanisms and therapeutic targets. *Cancer investigation*, *30*(1), 48-56.

McAlpine, J. A., Lu, H. T., Wu, K. C., Knowles, S. K., & Thomson, J. A. (2014). Down-regulation of argininosuccinate synthetase is associated with cisplatin resistance in hepatocellular carcinoma cell lines: implications for PEGylated arginine deiminase combination therapy. *BMC cancer*, *14*(1), 621.

Mizushima, N. (2007). Autophagy: process and function. *Genes & Development*, *21*(22), 2861-2873.

Munder, M. (2009). Arginase: an emerging key player in the mammalian immune system. *British journal of pharmacology*, *158*(3), 638-651.

Nakada, M., Kita, D., Watanabe, T., Hayashi, Y., Teng, L., Pyko, I. V., & Hamada, J. I. (2011). Aberrant signaling pathways in glioma. *Cancers*, *3*(3), 3242-3278.

Nelson, J. S., Burchfiel, C. M., Fekedulegn, D., & Andrew, M. E. (2012). Potential risk factors for incident glioblastoma multiforme: the Honolulu Heart Program and Honolulu-Asia Aging Study. *Journal of neuro-oncology*, *109*(2), 315-321.

Noonan, J., Zarrer, J., Murphy, B. M. (2016). Targeting autophagy in glioblastoma. *Critical Reviews in Oncogenesis, 21* (3-4), 241-252.

Ohgaki, H., & Kleihues, P. (2005). Epidemiology and etiology of gliomas. *Acta neuropathologica*, *109*(1), 93-108.

Ohgaki, H., & Kleihues, P. (2007). Genetic pathways to primary and secondary glioblastoma. *The American journal of pathology*, *170*(5), 1445-1453.

Ohka, F., Natsume, A., & Wakabayashi, T. (2012). Current trends in targeted therapies for glioblastoma multiforme. *Neurology research international*, 2012.

Ohshima, K., Nojima, S., Tahara, S., Kurashige, M., Hori, Y., Hagiwara, K., ... & Kanai, Y. (2017). Argininosuccinate Synthase 1-Deficiency Enhances the Cell Sensitivity to Arginine through Decreased DEPTOR Expression in Endometrial Cancer. *Scientific Reports, 7*, 45504.

Omuro, A., & DeAngelis, L. M. (2013). Glioblastoma and other malignant gliomas: a clinical review. Jama, 310(17), 1842-1850

Orringer, D., Lau, D., Khatri, S., Zamora-Berridi, G. J., Zhang, K., Wu, C., … Sagher, O. (2012). Extent of resection in patients with glioblastoma: limiting factors, perception of resectability, and effect on survival. *Journal of Neurosurgery*, *117*(5), 851–859.

Pan, E., & Prados, M. D. (2003). Clinical Presentation. *Holland-Frei Cancer Medicine. 6th Edition*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK13708/

Pandey, J. P. (2011). Genetic and viral etiology of glioblastoma—a unifying hypothesis. *Cancer Epidemiology and Prevention Biomarkers*, *20*(6), 1061-1063.

Panosyan, E. H., Lin, H. J., Koster, J., & Lasky, J. L. (2017). In search of druggable targets for GBM amino acid metabolism. *BMC cancer*, *17*(1), 162.

Patel, M. A., Kim, J. E., Ruzevick, J., Li, G., & Lim, M. (2014). The future of glioblastoma therapy: synergism of standard of care and immunotherapy. *Cancers*, *6*(4), 1953-1985.

Patil, M. D., Bhaumik, J., Babykutty, S., Banerjee, U. C., & Fukumura, D. (2016). Arginine dependence of tumor cells: targeting a chink in cancer's armor. *Oncogene*, *35*(38), 4957.

Pavlyk, I., Rzhepetskyy, Y., Jagielski, A. K., Drozak, J., Wasik, A., Pereverzieva, G., ... & Redowicz, M. J. (2015). Arginine deprivation affects glioblastoma cell adhesion, invasiveness and actin cytoskeleton organization by impairment of β-actin arginylation. *Amino acids*, *47*(1), 199-212.

Paw, I., Carpenter, R. C., Watabe, K., Debinski, W., & Lo, H. W. (2015). Mechanisms regulating glioma invasion. *Cancer letters*, *362*(1), 1-7.

Pearson, J. R., & Regad, T. (2017). Targeting cellular pathways in glioblastoma multiforme. *Signal transduction and targeted therapy*, *2*, 17040.

Phillips, M. M., Sheaff, M. T., & Szlosarek, P. W. (2013). Targeting arginine-dependent cancers with arginine-degrading enzymes: opportunities and challenges. *Cancer research and treatment: official journal of Korean Cancer Association*, *45*(4), 251-262.

Polivka, J., Rohan, V., Topolcan, O., & Ferda, J. (2012). New molecularly targeted therapies for glioblastoma multiforme. *Anticancer research*, *32*(7), 2935-2946.

Prasad, G., & Haas-Kogan, D. A. (2009). Radiation-induced gliomas. *Expert review of neurotherapeutics*, *9*(10), 1511-1517.

Ramirez, Y. P., Weatherbee, J. L., Wheelhouse, R. T., & Ross, A. H. (2013). Glioblastoma multiforme therapy and mechanisms of resistance. *Pharmaceuticals*, *6*(12), 1475-1506.

Ravikanth, R. (2017). Advanced magnetic resonance imaging of glioblastoma multiforme. *Journal of neurosciences in rural practice*, *8*(3), 439-440.

Roy, S., Lahiri, D., Maji, T., & Biswas, J. (2015). Recurrent glioblastoma: where we stand. *South Asian journal of cancer*, *4*(4), 163-173.

Ryan, K. M., Phillips, A. C., & Vousden, K. H. (2001). Regulation and function of the p53 tumor suppressor protein. *Current opinion in cell biology*, *13*(3), 332-337.

Salvati, M., Frati, A., Russo, N., Caroli, E., Polli, F. M., Minniti, G., & Delfini, R. (2003). Radiation-induced gliomas: report of 10 cases and review of the literature. *Surgical neurology*, *60*(1), 60-67.

Santana-Codina, N., Mancias, J. D., & Kimmelman, A. C. (2017). The Role of Autophagy in Cancer. *Annual Review of Cancer Biology*, *1*(1), 19–39.

Seymour, T., Nowak, A., & Kakulas, F. (2015). Targeting aggressive cancer stem cells in glioblastoma. *Frontiers in oncology*, *5*, 159.

Stone, E. M., Glazer, E. S., Chantranupong, L., Cherukuri, P., Breece, R. M., Tierney, D. L., ... & Georgiou, G. (2010). Replacing Mn2+ with Co2+ in human arginase I enhances cytotoxicity toward L-arginine auxotrophic cancer cell lines. *ACS chemical biology*, *5*(3), 333-342.

Szlosarek, P. W. (2014). Arginine deprivation and autophagic cell death in cancer. *Proceedings of the National Academy of Sciences*, *111*(39), 14015-14016.

Tamimi, A. F., & Juweid, M. (2017). Epidemiology and Outcome of Glioblastoma. In S. De Vleeschouwer (Ed.), *Glioblastoma*. Brisbane (AU): Codon Publications. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK470003/

Tanowitz, H. B., Machado, F. S., & Albanese, C. (2013). An expanded role for Caveolin-1 in brain tumors. *Cell Cycle*, *12(*10), 1485-1486.

Urbańska, K., Sokołowska, J., Szmidt, M., & Sysa, P. (2014). Glioblastoma multiforme–an overview. *Contemporary oncology*, *18*(5), 307-312.

Valdés-Rives, S. A., Casique-Aguirre, D., Germán-Castelán, L., Velasco-Velázquez, M. A., & González-Arenas, A. (2017). Apoptotic Signaling Pathways in Glioblastoma and Therapeutic Implications. *BioMed research international*, 2017.

Watanabe, R., Wei, L., & Huang, J. (2011). mTOR signaling, function, novel inhibitors, and therapeutic targets. *Journal of nuclear medicine*, *52*(4), 497-500.

Weathers, S. P., & Gilbert, M. R. (2014). Advances in treating glioblastoma. *F1000prime reports*, *6*.

Yan, Y., Xu, Z., Dai, S., Qian, L., Sun, L., & Gong, Z. (2016). Targeting autophagy to sensitive glioma to temozolomide treatment. *Journal of Experimental & Clinical Cancer Research : CR*, *35*.

Yang, Y., Shao, N., Luo, G., Li, L., Zheng, L., Nilsson-Ehle, P., & Xu, N. (2010). Mutations of PTEN gene in gliomas correlate to tumor differentiation and short-term survival rate. *Anticancer research*, *30*(3), 981-985.

Yang, Z. J., Chee, C. E., Huang, S., & Sinicrope, F. A. (2011). The role of autophagy in cancer: therapeutic implications. *Molecular cancer therapeutics*, *10*(9), 1533-1541.

Yoon, J. K., Frankel, A. E., Feun, L. G., Ekmekcioglu, S., & Kim, K. B. (2013). Arginine deprivation therapy for malignant melanoma. *Clinical pharmacology: advances and applications*, *5*, 11-19.

Zakalskiy, A. E., Zakalska, O. M., Rzhepetskyy, Y. A., Potocka, N., Stasyk, V., Horak, D., & Gonchar, M. V. (2012). Overexpression of (His) 6-tagged human arginase I in Saccharomyces cerevisiae and enzyme purification using metal affinity chromatography. *Protein expression and purification*, *81*(1), 63-68.

Zhuang, W., Long, L., Zheng, B., Ji, W., Yang, N., Zhang, Q., & Liang, Z. (2012). Curcumin promotes differentiation of glioma-initiating cells by inducing autophagy. *Cancer science*, *103*(4), 684-690.