Lebanese American University

ROS-Mediated Autophagic Death in Glioblastoma Cells Upon Treatment by Human Recombinant Arginase I (Co)- PEG5000 [HuArgI (Co)-PEG5000]

By

Maya Abdelkhalek

A thesis
submitted in partial fulfillment of the requirements for the
degree of Master of Science in Biological Sciences

School of Arts and Sciences

August 2021

© 2021

Maya Abdelkhalek

All rights reserved

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS APPROVAL FORM

Student Name: Maya Abdelkhalek I.D. #: 201905666

Thesis Title: ROS-Mediated Autophagic Death in Glioblastoma Cells Upon Treatment by Human Rec

Program: Master of Science in Biological Sciences

Department: Department of Natural Sciences

School: School of Arts and Sciences

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

Master of Science in the major of Biological Sciences

Thesis Advisor's Name: Dr. Ralph Abi-Habib

Signature: [redacted] Date: 09 / 08 / 2021
Day Month Year

Committee Member's Name: Dr. Dan Georgess

Signature: [redacted] Date: 09 / 08 / 2021
Day Month Year

Committee Member's Name: Dr. Michella Ghassibe Sabbagh

Signature: [redacted] Date: 09 / 08 / 2021
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) the non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwide in print and electronic formats and in any medium, including but not limited to audio or video. You agree that LAU may, without changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree that LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the submission contains material for which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to grant LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within the text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED BY 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: Maya Abdelkhalek

Signature: [redacted] Date: 26 / 07 / 2021
(Day / Month / Year)

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: Maya Abdelkhalek

Signature: [redacted] Date: 26 / 07 / 2021
(Day / Month / Year)

Acknowledgements

What I have achieved would not have been here without the support and motivation of many people.

I would greatly give my appreciation to the most inspiring advisor Dr. Ralph Abi Habib for him being supportive, motivative. Despite all what happened in the past two years, he was always there to help in anything, and this made the work with him a pleasant as well as productive. Adding to that, he was always guiding my research process and this is greatly valued.

I would like to thank my committee members Dr. Dan Georgess and Dr. Michella Ghassibe Sabbagh for their involvement in my thesis.

A big thanks goes to Dr. Oula El Atat for the appreciated time she offered to us in sharing her knowledge with us, helping us, being always there for us, and finally in making the lab environment too friendly.

To my lab mates, I really need to thank you because I would not have make it without your presence beside me in happy and sad times; starting with the cutest and loveliest lab partner out there one could have Rania Soukarieh, and to Fatemah Haidar, Chantal Fayyad, and George El Hajj.

To my family, my loving dad Makram, and mom Ghada thank you for your endless support and for always pushing me towards success and by that I will always make you proud. To my brother Shadi, thank you that you are my source of joy and support that will never change.

To my friends Jana, Ghida, Sarah, Rawan, Sara Ob., Mirna, and Nour thank you for always encouraging me, and believing in me since day one. There are no words that can express how much you mean to me and how much I love you.

Finally, thank you, GOD, for all your blessings.

ROS-Mediated Autophagic Death in Glioblastoma Cells Upon Treatment by Human Recombinant Arginase I (Co)- PEG5000 [HuArgI (Co)-PEG5000]

Maya Abdelkhalek

ABSTRACT

Targeting cancers; that exhibit amino acid auxotrophy, such as glioblastoma multiforme, through starvation of amino acids is becoming a promising therapy. The efficiency of this targeted therapy is based on the idea of killing cancer cells without damaging normal cells that are capable of synthesizing specific amino acids being targeted. In this study, we examine the mechanism of HuArgI (Co)-PEG5000-induced cell death in both partially and completely auxotrophic GBM cell lines. Our results first showed a significant increase in HuArgI (Co)-PEG5000 treated cells for both U251 and A172 in autophagosome formation; suggesting activation of autophagy in response to arginine deprivation. Secondly, the results also showed in both U251 and A172 cells, an increase in the percentage of the survival of cells co-treated with HuArgI (Co)-PEG5000 and chloroquine; indicating that autophagy is contributing to cell death at late time points in GBM cells.

Moreover, only U251 cells showed an overexpression in Argininosuccinate Synthetase-1 (ASS-1); the main key player in L-arginine biosynthesis, in case of arginine deprivation and the rescue was detected upon addition of exogenous L-citrulline.

Addition of N-acetyl-cysteine (NAC); being a reactive oxygen species (ROS) scavenger, led to a significant decrease in the cytotoxicity of arginine deprivation to

U251 and A172 cells, indicating that ROS generation reversed the cytotoxicity of arginine deprivation to GBM cells. Finally, we have also shown that in U251 cells the autophagosome formation; thus, death by autophagy is mediated by ROS generation. Taken together, ROS plays an essential role in the mechanism of autophagy activation in some arginine deprived GBM cells, where we have ROS-induced autophagy.

Keywords: Glioblastoma Multiforme, HuArgI (Co)-PEG5000, Arginine Deprivation, Cytotoxicity, Autophagy, ROS-induced Autophagy, Death by Autophagy.

TABLE OF CONTENTS

Chapter **Page**

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-FU: 5-fluorouracil

ADCD: autophagy-dependent cell death

ADI: arginine deiminase

AKT: protein kinase B

ALL: acute lymphoblastic leukemia

AML: Acute myeloid leukemia

ARG1: arginase I

AS: argininosuccinate

ASL: argininosuccinate lyase

ASS-1: argininosuccinate synthetase-1

ATCC: American Type Culture Collection

ATM: Ataxia-telangiectasia-mutated

ATR: Ataxia telangiectasia and Rad3-related

AVi: immature autophagic vacuole

BBB: blood brain barrier

BV: Bevacizumab

CDK4: Cyclin-dependent kinase 4

CHK1: Checkpoint kinase 1

CHK2: Checkpoint kinase 2

CNS: central nervous system

COX-2: cyclooxygenase-2

CQ: chloroquine

CRC: colorectal cancer

DMEM: Dulbecco's modified Eagle's medium

DNA: deoxyribonucleic acid

EFS: event-free survival

EGFR: epidermal growth factor receptor

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

GBM: Glioblastoma Multiforme

GBM-SCs: glioblastoma stem cells

GSCs: glioma stem cells.

hArg: human arginase

HCC: hepatocellular carcinoma

HuArg I: human l-arginase I

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

IC50: inhibitory concentration 50

IDH: isocytrate dehydrogenase

LC3: Microtubule-associated protein light chain 3

m-Abs: monoclonal antibodies

MAPK: mitogen activated-protein kinase

MDM2: Mouse double minute 2 homolog

MG: malignant glioma

MGMT: Methylguanine methyltransferase

NAC: N-acetylcysteine

OS: overall survival

OTC: ornithine transcarbamoylase

PC: pancreatic carcinoma

PDGFR: platelet derived growth factor receptor

PEG: polyethylene glycol

PI: propidium iodide

PI3K: phosphatidylinositol-3-OH kinase

PTEN: phosphatase and tensin homolog

RB1: Retinoblastoma

RB1-p16INK4a: Cyclin-dependent kinase inhibitor 2A pathway

RFI: ratio of fluorescence intensity

ROS: reactive oxygen species

RTK: receptor tyrosine kinase

Sc-RNAseq: single cell RNA sequencing

T-ALL: lymphoblastic T cell leukemia

TCGA: The Cancer Genome Atlas

TMZ: Temozolomide

TP53: tumor protein p53

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptors

WHO: world health organization

Chapter One

Introduction

1.1 Gliomas

Gliomas, a very broad term that defines different tumors of a neuroepithelial origin and initiating from the glial cells or can also initiate from the supporting cells of the central nervous system (Davis, 2018). Briefly, glial cells are considered to be the most abundant type of cells when studying the central nervous system, and these cells play a role in surrounding, insulating, and supplying nutrients and oxygen to different neurons (Davis, 2018). As for the classification of gliomas, they can be categorized into different groups, including astrocytomas (Glioblastoma Multiforme), oligodendrogliomas, ependymomas, mixed gliomas, and others like brain stem and optic nerve gliomas (Ostrom et al., 2017). Gliomas account for approximately 24% of all primary brain tumors and central nervous system tumors; when it comes to histology these tumors diverge from benign ependymoma tumors reaching grade IV GBM being the most aggressive and deadly form (Ostrom et al., 2015).

1.2 GBM

1.2.1 Classification and Signaling pathways

As determined by the WHO and according to the genetic and histological features, the grading of tumors ranges from I to IV (Louis et al., 2007). The focus will be on Glioblastoma Multiforme which is a grade IV, and is one of the most aggressive malignant brain tumors. They can be classified into two groups: the glio-

sarcoma and the giant cell glioblastoma (Reifenberger & Collins, 2004). This tumor can be developed as a primary GBM via *de novo* or from a secondary glioblastoma that is less malignant (Kleihues & Ohgaki, 1999). Before showing the difference between primary and secondary types, here are the other grades and their characteristics. Starting with grade I including pilocytic astrocytoma, they are known to be slow non-malignant tumors where surgical resection is used as a cure (Ostrom et al., 2017; *Prognosis | CERN Foundation*, n.d.; *Quick Brain Tumor Facts*, n.d.; Vigneswaran et al., 2015). Grade II tumors such as diffuse astrocytomas are known to have high level of differentiation, increased levels in cellularity and thus high ability to turn into a malignant form reaching the glioblastomal form (Sarkar et al., 2009). As for Grade III, and for instance anaplastic astrocytomas are characterized with abnormal mitosis with a substantial increase in proliferation and cellularity, as well as the presence of nuclear atypia that are multinucleated (Sarkar et al., 2009). On the genetic level, grade II and III tumors demonstrate high levels of mutation in the TP53 gene and/ or overexpression in the levels of p53 gene (94%), unlike in grade I tumors where these mutations are rare (Park et al., 2017). Note that, TP53 is located in 17p13 and is a tumor suppressor gene that encodes the nuclear protein p53; that contributes in the regulation of the cell cycle (Davis, 2018). However, Grade III tumors may also have deletions in chromosomes 6, 9p, 11p, 19q, and 22q, as well as mutations in the RB1 (retinoblastoma) gene (K et al., 1996). Surgical reduction as a cure is not possible in this case due to the deep infiltration in the brain (Furnari et al., 2007; Galli et al., 2004).

With respect to the genetic level, GBM can be formed by two different molecular types. The first type, which is the primary *de novo*, is known to be triggered by the amplification of EGFR, 7p12 (epidermal growth factor receptor) vs.

the secondary glioblastoma type that mainly initiates from the mutations that are caused in the TP53 gene as previously mentioned (Deimling et al., 1995; Lang et al., 1994). The majority of the GBMs are of the primary type, thus originating from new cells that lack any of the previous diseases that are considered to be as low-grade diseases (Furnari et al., 2007). Unlike, the secondary form that originates mainly from grades II or III gliomas (Galli et al., 2004). Approximately in 25-30% of the primary GBM several p53 mutations were observed and in 60-70% of secondary GBM cases, rendering this mutation to be the most molecular detected anomaly in GBMS (England et al., 2013). After DNA damage takes place, we will have activation of Ataxia-telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), that in turn trigger checkpoint kinase 1 and checkpoint kinase 2 (CHK1 and CHK2) respectively (Lukashchuk & Vousden, 2007; Vousden & Prives, 2009). This results in p53 phosphorylation, henceforth leading to TP53 related genes transactivation, with the increase in the regulation of Mouse double minute 2 homolog (MDM2) upregulation (Lukashchuk & Vousden, 2007; Vousden & Prives, 2009). This represents a negative feedback loop where we have downregulation of p53. Pathways that contribute to the proliferation of cells, their senescence, migration, apoptosis, and cell-cell interaction have to do with the signaling pathways that are also involved in GBM (Preusser et al., 2011). And these include molecular amplification and/or mutational activation of different genes, such as phosphatidylinositol-3-OH kinase (PI3K) pathway activation, receptor tyrosine kinase (RTK) genes activation, p53 inactivation, and retinoblastoma suppressor gene pathways inactivation (Furnari et al., 2007). All of these have been shown to be involved in approximately more than 80 percent of glioblastoma multiforme (Preusser et al., 2011). In particular, primary GBMs show overexpression of the

EGFR along with PDGFR (RTKs) oncogenes, and mutation in TP53 and PTEN tumor suppressor genes that in turn activate the Ras/MAPK and P13K/Akt pathways (Mischel & Cloughesy, 2003).

All of what was mentioned previously about the classification, refers to the WHO 2007 Classification. Another sorting of glioblastoma was done based on the information about IDH mutation (P & D, 2018). Glioblastomas are accordingly separated (according to the WHO 2016 Classification) into 3 different glioblastoma forms: IDH-wildtype, glioblastoma: IDH-mutant, and GBM: NOS (P & D, 2018). GBM, IDH-wild type; which accounts for approximately 90 percent of the cases, correspond principally to the clinically defined primary or *de novo* GBM; this form is majorly present in the elderly patients with an age being over 55 years (Kleihues & Ohgaki, 1999; Ohgaki & Kleihues, 2013). As for the GBM, IDH-mutant type (accounts for about 10percent of the cases) resembles greatly to the so-called secondary GBM, having a history of prior lower grade glioma, and occurring specially in the GBM patients that are younger in age (Kleihues & Ohgaki, 1999; Ohgaki & Kleihues, 2013). Finally, GBM, NOS type, which is a diagnosis that is held in reserve for those tumors where the complete evaluation of IDH cannot be accomplished (Tamimi & Juweid, 2017).

1.2.2 Characteristics, Diagnosis, Symptoms, and Risk Factors

GBM being one type of malignant gliomas, accounts for almost 80% of all primary brain gliomas, and around 60% of all the adult brain tumors (Agnihotri et al., 2013; Denysenko et al., 2010; Messali et al., 2014; Schwartzbaum et al., 2006). Saying that, out of 100,000 people approximately 5-6 cases are diagnosed with primary malignant gliomas (MGs), where GBM accounts for most of these MG cases (Alifieris & Trafalis, 2015). Grade IV tumors including GBM show the most

innovative characteristics of malignancy from vascular proliferation, to necrosis, as well as resistance to chemotherapeutics along with radio-therapy (Furnari et al., 2007; Galli et al., 2004). Patients having a secondary/IDH-mutant glioblastoma are commonly younger in age and have a slightly better prognosis than those with IDH-wildtype glioblastoma (Ohgaki & Kleihues, 2013).

Reaching the clinical displays of GBM, these mainly depend on different things. They depend on the location of the tumor, size, resectability, multifocality, advanced age, comorbidities, and the general conditions of the patient (Nieder et al., 2005). Thus, different symptoms of GBM may be encountered, and the most common ones include: nausea, vomiting, aphasia, seizures, hemiparesis, headaches, personality disturbances, parethesia, visual and mood disturbances which in turn all signify an increase in the pressure of the intracranial area (Yuile et al., 2006). It was also shown that the genotypes that increase the risk of having asthma are linked with having a decrease in the risk of glioblastoma; thus, a minor risk of having gliomas has been shown to be related to allergies or other atopic diseases, such as: eczema, asthma, and psoriasis (Brenner et al., 2007; Scheurer et al., 2011; Schwartzbaum et al., 2006).

From the prognosis side, remarkably GBM has a poor prognosis, and this may be due to the early invasive GBM properties that target the CNS; thus, preventing the surgical cure in this case (Khasraw & Lassman, 2010; Schwartzbaum et al., 2006). GBM patients are expected to have 14-16 months as median survival, along with a percentage being lower than 5 as having a 2 and a half year survival expectancy, and less than 5% for a 5-year expected survival period (Khasraw & Lassman, 2010; Schwartzbaum et al., 2006; Stupp et al., 2005; Tamimi & Juweid, 2017). As for the recurrency of GBM it is also associated with a weaker prognosis,

having a 3-6 months as an expected median survival (along with the use of the traditional chemotherapy) (Gruber & Buster, 2004). Something to note here is that these rates of survival were not improved during the past three decades by more than few weeks even when dealing with the different therapeutic approaches used today as will be talked about later (Stupp et al., 2005; Tamimi & Juweid, 2017).

1.2.3 GBM Progression

Glioblastoma is characterized by intra-tumoral phenotypic as well as genetic heterogeneity as mentioned before; thus, different studies devised an integrated genomic and functional strategy to attain single cell-derived tumor clones directly from GBM patient to identify the mechanisms of drug resistance and aggressive clone behavior (Meyer et al., 2015). The analysis of the genomics of the single clones helped identify the genes that are associated with clonal phenotypes, and the integration of this analysis at a clonal level will be crucial for understanding therapeutic resistance of this tumor and evolution, leading to the discovery of novel driver mechanisms as well as cancer treatments that are clone-specific (Meyer et al., 2015). Another study, explored a path where the GSCs progressively renovated to form the invasive type of cells, and this path is termed the “stem-to-invasion path”, where cells revealed gradual expression of the invasion signatures associated with GBM and a decline in the expression of the stem cell markers of GBM (Pang et al., 2019). In the same study they also analyzed the molecular cascades underlying that path and thus identified key factors that have a major role in controlling the fulfilment of the tumor cells’ invasive potential, including long noncoding RNAs and transcription factors; providing new understandings into the progression of GBM, and supporting the model of cancer stem cells along with efficient implications for therapeutically targeting GBM (Pang et al., 2019). In other words, the heterogeneity

of GBM cells is one of the major reasons in the tumor progression and failure of the treatment, so here comes the advantage of using single-cell RNA sequencing (scRNA-seq) to study the transcriptomes (Stegle et al., 2015). This has been applied in different tumors, including head, neck, brain, breast tumors, and blood diseases (Bajikar et al., 2017; Puram et al., 2017; Zhao et al., 2017). Tumor heterogeneity plays a great challenge in the treatment of GBM, which has to do with the complex microenvironment of the tumor and the heterogeneity of the GBM cells, it is vital for researchers to know how different GBM cell types interact with neoplasm cells and identify their lineage and phenotypes (Darmanis et al., 2017). So, in order to have better treatment options for the future, researchers have been focusing on targeting different GBM genes (Z. Li et al., 2018; J. Liu et al., 2013; Xiao et al., 2014).

Different studies about GBM, indicated the existence of a cell population (GBM stem cells, GBM-SCs) that decreases the capability of adult stem cells to self-renew themselves and have the capability to survive chemotherapeutics and the radiation-therapy treatments (Maugeri-Saccà et al., 2013). These stem cells are characterized with their tumor accessibility, rapid rates of proliferation, and they are associated with resistance to treatment of GBM and poor prognosis (Maugeri-Saccà et al., 2013).

For all of these reasons, what available therapies can do; is to prolong the lives of GBM patients more to reach one year and infrequently past the two years (Hui-Wen, 2009).

1.2.4 Treatments for GBM

The options for the treatments of GBM are somehow limited. Operating a resection of the tumor through surgery, followed by radio-therapy and temozolomide is the typical treatment used currently for GBM (Karachi et al., 2018). Before

continuing, temozolomide which crosses the Blood brain barrier (BBB) is a prodrug (alkylating agent) and known as a chemotherapy that is taken by mouth (Karachi et al., 2018). To make it clear how does Temozolomide works; it adds a CH_3 group to both pyrimidine and purine in the DNA; thus, leading to cell damage reaching apoptotic stage (Jihong et al., 2011). Not only that, the methyl guanine methyl transferase (MGMT) DNA repair system that is found in GBM patients lead to about 55 percent of these patients are thought to have resistance to temozolomide (Hegi et al., 2005). What happens next is the transferring of the methyl group from guanine by MGMT leading to the repair of the DNA damage in the tumor cells (Karachi et al., 2018). To solve this problem of resistance, combinatorial therapies were tested on GBM patients. For instance, some studies administered the combination of TMZ with capecitabine, an oral fluoropyrimidine prodrug that is eventually transformed to 5-fluorouracil (5- FU) in tumor cells (Dolan et al., 1990; Fine et al., 2005). This will solve the problem by depleting MGMT mRNa and the protein, causing the disruption of the repair activity of the MGMT discussed previously (Dolan et al., 1990; Fine et al., 2005). Another example, the combination of TMZ with vaccine immunotherapy, promotes an increase in the levels of interleukin 2 that are found in the serum; in turn, causing the naïve T cells to be changed to fully matured memory T cells as well as the expansion of CD8 T cells in rodent studies, resulting in the slight improvement of the survival of tumor bearing mice (Sanchez-Perez et al., 2013). This combination caused the improvement in a T cell dependent manner. Now, the grouping of TMZ with dendritic cells that are pulsed with tumor-antigen, triggered anti-tumor immune responses and slight improvement in the survival in GL-261 rodent model studied, and was due to an increase in the antigen cross-priming (Kim et al., 2010). Briefly, the immunotherapy allows the immune system to first distinguish, mark, and finally

put the tumor cells to death, but this had some breakthroughs in GBM due to its heterogeneity, causing it to be an intrinsic immunological quiet disease (E. K. Liu et al., 2020). To solve this problem with heterogeneity of the epigenetic and genetic alterations found in GBM, three trademark paths are commonly poorly regulated, representing possible therapeutical targets; including: receptor tyrosine kinase (RTK)/Ras/ phosphoinositide 3-kinase (PI3K), retinoblastoma (Rb), and p53 that was discussed before (McLendon et al., 2008). What can be done with these alterations is that they can be targeted with the main objective is to inhibit these driver pathways by the use of different monoclonal antibodies, or small molecule inhibitors (E. K. Liu et al., 2020). Focusing on the RTK/PI3K/AKT signaling pathway, since it is key pathway in the contribution in the level of progression and the development of different tumors, including GBM; thus, as a potentially targetable pathway and a regulatory molecule, Akt oncogenic role is essential to be studied (Xia et al., 2019). The study identified three isoforms of AKT (Akt 1, 2, and 3), and it was shown that Circ-Akt3 is slightly expressed in glioblastoma tissues when distinguished from the expression in normal tissues of the brain, it encodes a 174 amino acids (aa) innovative protein, noting that the overexpressing AKT3-174aa triggered a significant decrease in the proliferation of the cells, resistance to radiation, and tumorigenicity of the glioblastoma tumor cells *in vivo* (Xia et al., 2019). It was shown that the decrease in the expression of the circ-RNA encoding for the AKT3 gene leads to tumorigenesis of GBM, thus; reestablishing AKT3-174aa whereas hindering the stimulated AKT may carry more benefits for certain GBM patients (Xia et al., 2019).

As mentioned previously, dealing with monoclonal antibodies (mAbs) can be followed in order to prevent tumor driver pathways; this class included bevacizumab

(BV), that plays a role in targeting vascular endothelial growth factor (VEGF) and by that causes the blocking of angiogenesis; with an occupied FDA approval for the treatment of GBM patients (Gilbert et al., 2014). One limiting factor that should be kept in mind when dealing with the efficacy of the therapeutics of mAbs may be incomplete penetration of the BBB, due to their large size (E. K. Liu et al., 2020). In fact, VEGF pathway hindering permits for the rebuilding of the irregular vasculature present in the tumor cells to a more like regular condition, just by diminishing the volume of the blood cerebral found in the surrounding area of the tumor and thus decreasing the vascular permeability, in addition to the decrease in the peritumoral edema (Takano et al., 2013). Moreover, the use for example of cyclooxygenase-2 (COX-2) inhibitors remains debatable where a positive outcome was seen in reducing the gliomagenesis both *in vivo* and *in vitro* (Fujita et al., 2011). In clinical settings, the use of COX-2 inhibitor was shown to be unrelated to risk of glioma (Seliger et al., 2016).

1.2.5 The Need for Innovative Targeted Treatment

As mentioned previously, GBM survival is poor; as only few patients survive for two and a half years after diagnosis on one hand, and less than five percent of the patients survive for five years post diagnosis (Tamimi & Juweid, 2017). Moreover, the survival rates for GBM patients have not shown any statistical significant improvement in the last three decades (Tamimi & Juweid, 2017).

Although, until today more therapies have been established including: genetic and epigenetic therapies as well as immune therapies; until these days, GBM remains in a way or another not fully curable even when applying neuro-surgical resection, followed by chemotherapy and radiotherapy combined with TMZ as a standard way to try to cure GBM patients (Agnihotri et al., 2013; Denysenko et al., 2010; Messali

et al., 2014). In conclusion, GBM has been known to be biologically heterogeneous along with different risk groups as well as different profiles of prognosis, that's why the standard of the 100% curable treatment is yet not individualized nor targeted. Moreover, the current used treatment displays relapse high rates and is at the same time is no longer showing any improvement in the overall survival (OS) rates. From here, we can notice the importance for a better selective/targetable treatment for glioblastoma multiforme (GBM) with better efficiency in curing GBM patients.

1.3 The Metabolism of Cancer and the Deprivation of Amino Acids

As a hallmark of malignancy of cancer cells, the metabolic reprogramming had been extensively recognized; where the metabolism and uptake of different amino acids are abnormally upregulated in different cancers which exhibit dependance on specific amino acids (Wei et al., 2021). Knowing that amino acids help in the proliferation and survival of cancer cells under different conditions, including: oxidative, genotoxic, and nutritional stress; targeting the metabolism of amino acids is becoming a potential therapeutical method for cancer patients (Wei et al., 2021). Enzymatic mutations involved in the metabolism of tumor cells trigger the different observed modifications (Tsun & Possemato, 2015). One significant set of these mutations are the ones that finally cause auxotrophy of amino acids making the cancer cells incapable of synthesizing specific semi-essential or non-essential amino acids that are fully synthesized in cells functioning normally; thus, the cancer cells are now more depending on the extracellular environment to supply their need for amino acid as for the survival of the cells (Long et al., 2017; Pavlova & Thompson, 2016).

Talking briefly about amino acids, they are divided into essential amino acids within them are the semi-essential and non-essential ones; where the essential ones cannot be synthesized *de novo* and the semi-essential are these which can be

synthesized *de novo* but not in sufficient amounts; thus, require supplementary diet under specific conditions such as in early development, increased cell proliferation, and infections; while the non-essential ones can be synthesized *de novo* (Tabe et al., 2019). Thus, targeting cancerous cells that display amino acid auxotrophy through deprivation of amino acids using certain enzymes is becoming interesting that it would potentially target tumor cells with causing no effect on the normal cells that are able to produce the targetable amino-acids (Fung & Chan, 2017; Tabe et al., 2019). This therapy of starvation of a specific amino acid is being a promising approach to selectively treat patients with different types of cancer, showing low toxicity and acceptable therapeutical window (Geck & Toker, 2016). Several studies studied the non-essential amino acid metabolism and were assessed for being a targeted treatment in the therapy of starvation (Panosyan et al., 2017). For instance, the pathways for the synthesis of asparagine (treatment approved by FDA), asparaginase, is offered as a treatment for acute lymphoblastic leukemia (ALL) (Fernandes et al., 2017). In addition to that, other studies showed that methionine, alanine, asparagine- glutamine, tryptophan deprivation are being promising in the targeting of GBM patients (Panosyan et al., 2017), as well as cystine (Chung et al., 2005), and serine/glycine (Engel et al., 2020).

From here, one can notice the importance of developing treatments depending on the depletion of specific amino acids in order to treat different cancer types. Here the focus will mainly be on the depletion of arginine, and the efficiency of this targeted starvation on the treatment of GBM patients. So, the upcoming part will be about arginine metabolism, how to deprive this amino acid, and what will it lead to.

1.4 Metabolism of Arginine and its Deprivation

Briefly, arginine is considered to be a versatile amino acid which can be produced *de novo* by normal cells through the reactions of the urea cycle by ornithine; and also, being obtained through certain diets making arginine a semi-essential amino acid, or from intestinal renal biosynthesis (Fung & Chan, 2017; Geck & Toker, 2016; Husson et al., 2003). In addition to that, arginine has different essential biological functions that one should know about, including: cell survival and progression, immune regulation, production of metabolites as urea, nitric oxide, and polyamines, and protein synthesis along with their modification (Albaugh et al., 2017; Jahani et al., 2018; Szefel et al., 2019). As for the urea cycle and the synthesis of arginine, this synthesis mainly depends on two major enzymes; argininosuccinate synthetase 1 (ASS-1) and argininosuccinate lyase (ASL), and these catalyze the citrulline conversion and produce arginine (Manca et al., 2011). Additionally, the condensation of L-citrulline and aspartate to form argininosuccinate is catalyzed by ASS-1, and then argininosuccinate splits to release both L-arginine and fumarate via a catalysis reaction through ASL that acts downstream of AS (Albaugh et al., 2017). After, there is the enzyme arginase that is only present in liver cells, will convert L-arginine into L-ornithine and urea, and then this is returned back to L-citrulline with the help of an enzyme called ornithine trans-carbamoylase (OTC); to form arginine again and the cycle will be repeated all over from the beginning **(Figure 1)** (Geck & Toker, 2016; Jahani et al., 2018; Morris, 2016; Zou et al., 2019).

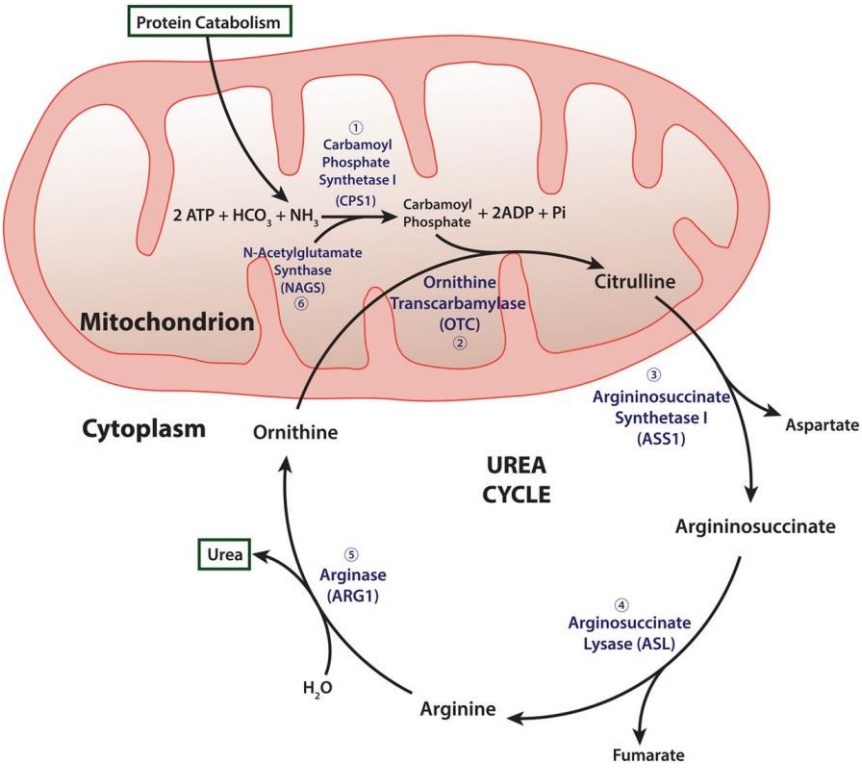


Figure 1 Arginine metabolism and the urea cycle

By a two-step process and with the help of ASS-1 and ASL arginine is produced starting from citrulline. Then the transformation of arginine occurs by ARG1 into urea and ornithine that will be in turn converted back to form citrulline by OTC. (Modified from: Blair et al., 2015)

When talking about the process of synthesis of arginine, one should highly know that ASS-1 is the rate limiting enzyme on one hand, and the activity as well as the amount of ASS-1 on the other hand have a major role in the L-arginine biosynthesis (Long et al., 2017). The expression of ASS-1 fluctuates between different tumor cells, and the deficit in ASS-1 expression is the basis of arginine auxotrophy in cancer cells representing the source of arginine starvation targeted approach. As was done in our lab, the study categorized the GBM cell lines into either partially auxotrophic cells or completely auxotrophic cell lines relying on the expression of ASS-1, where the complete auxotrophic cell lines are classified together where they totally lack the expression of ASS-1, vs the partial auxotrophic cell lines that are grouped together and are characterized to have low expression of ASS-1 along with a high rate in the proliferation of cells; thus, these cells are now considered to be reliant

on sources of arginine that are extracellular. In addition to that, the studies done in our lab also showed that the exogenous L-citrulline is commonly used as well to distinguish between complete and partial arginine auxotrophy after the addition of exogenous L-citrulline and its ability to inverse the reliance of the cell on arginine (Mauldin et al., 2012). The depletion of arginine induced by arginase showed substantial enhancement in treating different tumor types including, lymphoblastic T cell leukemia (T-ALL), hepatocellular carcinoma (HCC), pancreatic carcinoma, and glioblastoma multiforme (GBM) (Cp et al., 2010; Glazer et al., 2011; Khoury et al., 2015). This shows that the deprivation of arginine signifies that this therapy can be a targetable approach to treat different tumors. In the therapy of arginine starvation, the degradation of the extracellular arginine could be done by two different enzymes; starting with the bacterial arginine deaminase (ADI) and the human arginase (hArg) (Tabe et al., 2019). Different studies have shown that the degradation of arginine to citrulline by ADI was efficient in preventing the proliferation of different tumor cells including lung cancer, melanoma, hepatocellular carcinoma, and renal cell carcinoma (Feun et al., 2008; J. Liu et al., 2014), and it was shown that these tumors share a deficiency in the OTC or ASS-1 that are completely auxotrophic (*The Human Protein Atlas,* n.d.). Nonetheless, there is a limitation when treating the tumor with ADI, since it provokes in human cells an immune response as it is derived from prokaryotes in specific *Mycoplasma arginini* and this is known by antigenicity (Ensor et al., 2002; Zhang et al., 2015), with ADI having a short half-life (4 hours) and this limits the efficacy in treating the tumor.

1.5 Recombinant Human Arginase

The pegylated form (ADI-PEG20) was developed to decrease the immunogenicity of the drug as well as to elongate the enzyme's half-life (6 days)

(Ensor et al., 2002). Studies dealing with ADI-PEG20 monotherapy, showed significant reduction in the progress of the intracranial area when in ASS1 negative GBM cell lines and extension in the endurance of the mice having ASS-1 negative GBM type without any detectable toxicity effect (Przystal et al., 2018). Although, this therapy was efficient in some tumors, but resistance was developed to ADI-PEG20 monotherapy (Tabe et al., 2019).

On the other hand, there is another arginine depleting strategy with the ARGase which plays a role in converting arginine to orthinine and urea (Cheng et al., 2007). In fact, human arginase has two subtypes, ARGase I and ARGase II; with a similarity of 60% between their amino acid sequence (Tabe et al., 2019). Starting with arginase I, it is found in liver cells of humans and is a cytosolic enzyme; while arginase II is found in other tissues, a mitochondrial enzyme that has no contribution in the urea cycle (Krebs et al., 1973; Morris et al., 1997). Just like ADI, the recombinant ARGase was also pegylated to elongate its half-life from few hours to about 4 days (Tabe et al., 2019). And since arginase I (ARGI) isoform has to do with the urea cycle, then it was the only one to be tested for the arginine starvation therapy. ARGI differs from ADI, that it is of human origin and it doesn't provoke the immune response or antigenicity (Mussai et al., 2015). The focus will be on Arginase I. It consists of 332 amino acids and a 34.7 kDa as a molecular weight (Stasyk et al., 2015). This human L-arginase I (hArgI) relies on two manganese ion cofactors with an ideal activity at pH 9.5, but with a low activity at the physiological pH and this is due to the fact that these two manganese ions have a tendency to be quickly replaced from the enzyme when present in serum and thus inactivating it (Glazer et al., 2011; Stone et al., 2010). Several modifications were done to the native human arginase I that it has low efficiency at the neutral pH in the human serum with a short-life being about 4.5 hours and this is

because of the rapid loss of the two manganese ions. Some of these modifications, include the substitution of the two manganese ions in the enzyme's active site with two Cobalt ions (Co2+); thus, improving the catalytic activity of the enzyme and this showed a robust electrostatic correlation with amino acids that are cationic, increasing the amino acid binding affinity to the enzyme (enhanced the serum stability) (D'Antonio & Christianson, 2011). A second modification was done to prolong the persistence of the enzyme, so the conjugation of HuArgI (Co) to polyethylene glycol was performed, generating the pegylated, Cobalt-substitute recombinant human arginase [HuArgI (Co)-PEG5000] (Glazer et al., 2011). In addition to that, adding these hydrophilic PEG (polyethylene glycol) chains (was FDA approved) that are non-toxic, also increase the serum stability by protecting from degradation using the metabolic enzymes (Harris & Chess, 2003; Veronese & Pasut, 2005). Consequently, [HuArgI(Co)-PEG5000] was developed **(Figure 2)** with a catalytic activity being higher than before by 10 folds, stability of the serum higher by 5 folds, decrease in immunogenicity, and at neutral pH there is a decrease in the dissociation constant (Stasyk et al., 2015; Stone et al., 2010). Promising effects were shown when using [HuArgI (Co)-PEG5000] to target the auxotrophy of arginine in different tumor types. Some of which include, hepatocellular carcinoma (HCC) (Glazer et al., 2011), pancreatic carcinoma (Glazer et al., 2011; Khalil & Abi-Habib, 2020), lymphoblastic T cell leukemia (T-ALL) (Cp et al., 2010), acute lymphoblastic leukemia (AML) (Tanios et al., 2013), glioblastoma multiforme (GBM) (Khoury et al., 2015; Pavlyk et al., 2015), renal cell carcinoma (Yoon et al., 2007), prostate cancer (Hsueh et al., 2012), colorectal cancer (CRC) (Al-Koussa et al., 2019), ovarian carcinoma

(Nasreddine et al., 2020), breast cancer (Wang et al., 2014).

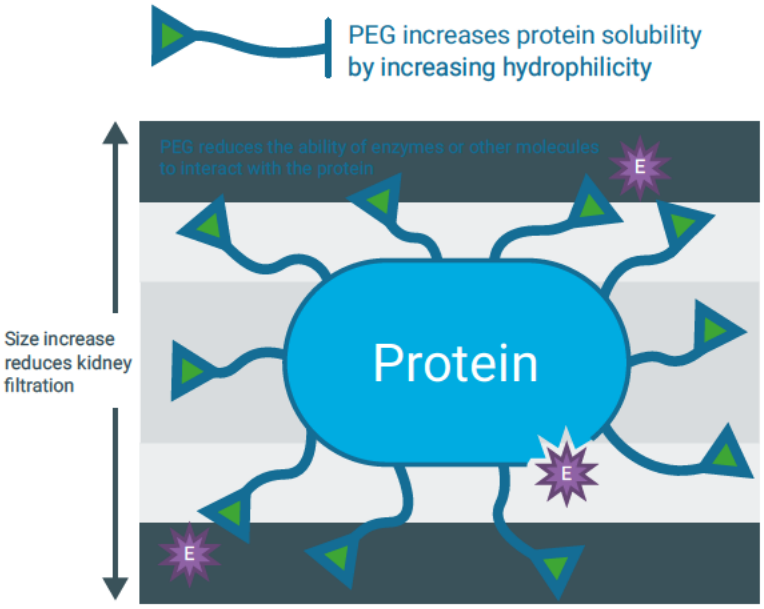


Figure 2 Why are some proteins PEGylated?

Modified from: BioPharmaSpec, n.d. PEGylation of Proteins.

A study done in our lab has showed the cell cycle analysis studies that revealed the deprivation of arginine in GBM cells did not lead to any arrest in the cell cycles, confirming what was mentioned before about the cytotoxicity of arginase to the GBM cells (Khoury et al., 2015).

1.6 Autophagy

1.6.1 Definition, and mechanism

The ancient Greek term from which autophagy was derived first is "self-eating", or in other words it is a cellular autodigestion process that takes place intracellularly (Mizushima & Komatsu, 2011). Autophagy is considered to be the major system of the degradation of the intracellular cytoplasmic materials mostly organelles and macromolecules such as proteins, glycogen, mitochondria, along with

lipid droplets, Golgi apparatus, and the endoplasmic reticulum (Bialik et al., 2018; Galluzzi et al., 2017). To note something, the main role of autophagy is not only to eliminate the cytoplasmic constituents but also to recycle them to deliver new building blocks including fatty acids, sugars, nucleosides, and amino acids as well as energy (Levy et al., 2017). Apart from being triggered at a constant rate in cells with a normal cellular renewal percentage and under homeostatic settings, autophagy might be induced by a variety and physiological circumstances, as well as stressful settings like nutrition deprivation (Bento et al., 2016; D'Arcy, 2019). In other words, whether autophagy is being protective or not, it is mainly triggered by different stressful settings including hypoxia, nutrient deprivation, metabolic stress, and reactive oxygen species (ROS) (Onorati et al., 2018). To add on that, starvation induced autophagy is a kind of autophagy that is initiated in response to nutrition deprivation and can take place in tumor cells experiencing exponential development or when the microenvironment of the tumor is deprived of certain supplements and nutrients (Mizushima & Klionsky, 2007; Ravanan et al., 2017). Autophagy involves the delivery of cytoplasmic material to lysosomes for destruction. Autophagy can be categorized into three types according to the delivery mechanism, including the chaperone mediated autophagy, micro-autophagy, and the macro-autophagy being the common type referred to as autophagy (Bhat et al., 2018a). Focusing on macro-autophagy, which is a multistep method characterized by the establishment of a phagophore or an isolation membrane; a structure with a double membrane that elongates and thus encloses the cytoplasmic components. After that, this phagophore will form a vesicle called the autophagosome or known by the immature vacuole (AVi), that then combines or fuses with the lysosome leading to the formation of autolysosome. In the autolysosome, the hydrolytic enzymes will degrade the isolated cytoplasmic

components that will be finally recycled to be used by the cell again **(Figure 3)** (Berg et al., 1998; Kabeya et al., 2000; Levine & Kroemer, 2008; Nakamura & Yoshimori, 2017).

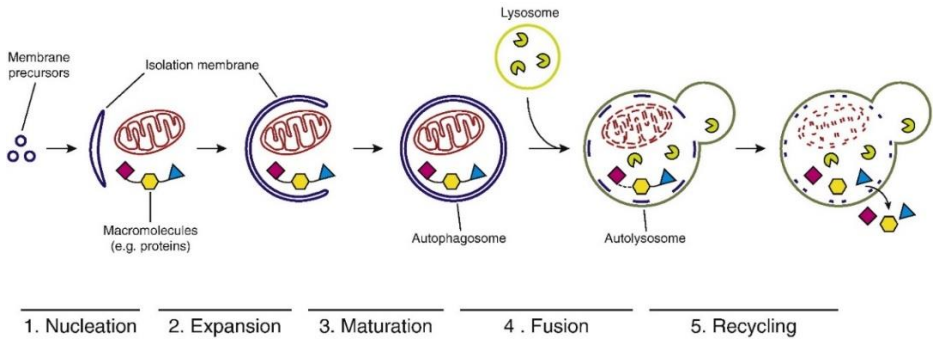


Figure 3 Mechanism of Autophagy

1.6.2 Activation and pathway

Modified from: Zaffagnini & Martens, 2016.

Autophagy can also be categorized as selective for the cargo that is targeted for degradation or non-selective (Gatica et al., 2018). Briefly, many of the cytoplasmic components will be sequestered to the autophagosomes during the non-selective autophagy; however, when it comes to the selective autophagy, specific cargos are being targeted such as protein aggregates, pathogens, damaged mitochondria to the autophagosomes (Shaid et al., 2013). Now, to selectively degrade the mitochondria, and this is known by mitophagy which is important for the maintenance of homeostasis and the quality control of the mitochondria, there must happen an interaction between the mitochondria and the autophagosomal membrane with the help of the autophagic receptors (Anding & Baehrecke, 2017; Lemasters, 2005; Um & Yun, 2017). These autophagic receptors bind to the LC3 on the autophagosomal membrane on one side and to the mitochondria on the other side, in turn this recruits the damaged mitochondria to the autophagosomes (Yoo & Jung, 2018; Zaffagnini & Martens, 2016). This targeted mitochondrial recognition by the autophagosome through the LC3

adapters can occur via direct interaction between the LC3 and their receptors found on the mitochondria or by ubiquitin-independent or dependent pathways (Chu, 2019; Palikaras et al., 2018). So, the autophagy activation (considered as selective or not), can be triggered by the reactive oxygen species (ROS), which are created in greater quantities in the mitochondria under a variety of stressors (Essick & Sam, 2010). ROS can regulate autophagy by working on different transcriptional and post-transcriptional pathways, as well as mitophagy when at high concentrations (Frank et al., 2012; Shefa et al., 2019; Xu et al., 2017). When it comes to mitophagy activation, this is done by high ROS concentrations that will act on many proteins that in turn will be involved in the selective targeting of the mitochondria to reach a new destination, which is the autophagosomes (Frank et al., 2012; Shefa et al., 2019). However, when it comes to the activation of autophagy by ROS regulation at the transcriptional level and due to ROS accumulation this leads to the activation of specific transcription factors that will in turn lead to the increase in transcription levels of proteins that are related to autophagy (L. Li et al., 2015). Moving to the regulation by ROS at the post-transcription level, it can act upstream on different pathways that have to do with the regulation of autophagy such as, AMPK-mTOR, MAPK/ERK, and the PI3K-Akt pathways (Gersey et al., 2019; Marin et al., 2016; Seyed et al., 2020; Zhang et al., 2014).

LC3, is known to be a microtubule-associated protein light chain 3, and is a 17 kDa protein. So, what happens during autophagy, the pre-autophagosome starts to engulf the cytoplasmic organelles that will later become an enclosed double membrane autophagosome. After that, LC3-I (cytosolic form) becomes lapidated and is transformed to LC3-II that is being recruited on both faces of the autophagosome (Tanida et al., 2008). Next, there will happen a combination between the

autophagosome and the lysosome to form an autolysosome, and finally the LC3-II being oriented on the cytosolic side will be de-lapidated; thus, released into the cytosol on one hand, and the intra-autophagosomal components will be hydrolyzed and degraded after (including the intra-autophagosomal LC3-II) (Tanida et al., 2005).

1.6.3 Autophagy; the double-edged sword, and cancer

Autophagy was first discovered to be a protective mechanism that permits cells to withstand stressful situations (He et al., 2018). Yet, as more evidence accumulates, autophagy appears to play specific roles in cell death modulation, depending on the context of the cell and the level of autophagy activation taking place (Galluzzi et al., 2017; Khalil & Abi-Habib, 2020). So, when it comes to cancer autophagy plays a somehow complex role. In other words, during the development phase of cancer, autophagy can act as a tumor suppressor, whereas during the cancer progression phase, autophagy plays an ensuring survival role (Kondo et al., 2005). To add on that, autophagy plays a tumor suppressor role because there is expression of beclin 1 (BECN1) which induces autophagy by inhibiting the tumorigenic potential (Kondo et al., 2005). On other hand, in case of abnormal glycolytic metabolism and high proliferation rate, rendering the cancer cells to be under a greater stress when being compared to normal untreated cells, leading to the rely of the cancer cells on autophagy to survive the stressful conditions (Amaravadi et al., 2011). As for the continuous activation of autophagy, this leads to a turnover of the cellular proteins and organelles that finally crosses the threshold at one point and results in the death of these cancer cells (Yang et al., 2011). Adding on the cell death role that autophagy plays, this role can be divided into three groups: autophagy-dependent cell death (ADCD) and this type is independent of necrosis and apoptosis, autophagy-associated cell death and here it is recommended that cell death and autophagy are coinciding, and finally the

third type which is the autophagy-mediated cell death proposing that the cell death taking place in this case is triggered by autophagy (Bhat et al., 2018b; Galluzzi et al., 2018b; Yan et al., 2019). Cell death that is dependent on the activation of autophagy was defined by the Nomenclature Committee of Cell Death as "a form of regulated cell death that mechanistically depends on the autophagic machinery (or components thereof)" (Galluzzi et al., 2018a). Key components of the macro-autophagy process are used by the cell and this in turn helps in ell survival even though there might be some differences found when it comes to the duration and the rate of the autophagic flux, as well as for the fate of the components being engulfed that will be then degraded or recycled (Bialik et al., 2018; Denton & Kumar, 2019). A large number of research nowadays, are presenting evidence for autophagy dependent cell death as a consequence of a variety of factors in different cancer cells. For example it was shown that depriving cancer cells from arginine or from different amino acids leads to death by autophagy in different cell lines including prostate cancer cells as was shown by Changou et al. (2014), pancreatic cancer cells (Khalil & Abi-Habib, 2020), ovarian cancer cells studied by Nasreddine et al. (2020), HeLa cell line (J. Liu et al., 2013) and other types as well. In addition to that, when exposing different cancer cell lines to 2-merceptoethanol or to hydrogen peroxide this will lead to the death of these cells by autophagy as was evident and shown in human embryonic kidney cell lines, certain cervical cancer cell lines, and glioblastoma cell lines (Chen et al., 2008). Moreover, it was also shown that when treating cancer cells by different natural molecules such as resveratrol, this will lead to ADCD in prostate cancerous cells that are apoptosis-deficient along with glioma cells being cancerous and malignant (Lian et al., 2011; Voss et al., 2010).

1.7 Previous work done on GBM

Studies on GBM done in our lab by khoury et al. showed that different cell lines of glioblastoma are auxotrophic (complete or partial) for arginine and there is sensitivity to arginine deprivation through the use of HuArgI (Co)-PEG5000 (2015). This was indicated by the inability of L-citrulline to save the cells from the cytotoxicity caused by arginine depletion (resembling the complete arginine auxotrophic cells). They also needed to examine the effect of arginine depletion by HuArgI (Co)-PEG5000 on the cell cycle of different types of GBM cell lines. No effect on the cell cycle arrest was detected after depriving the cells from arginine, confirming the cytotoxicity of HuArgI(Co)-PEG5000-induced arginine starvation with anti-proliferative effects that were shown in these treated GBM cells after detecting an increase in the pre-G0 to G1 ratio that represents the dead cells population when at the maximum tested concentrations of HuArgI (Co)-PEG5000 (Khoury et al., 2015). Furthermore, they also analyzed the type of cell death in treated GBM cells, that they tested for caspase activation, annexin V, and staining for PI at two time points only (24 and 48 hours). They found out that cell death is non-apoptotic and that there was no activations of caspases in all nine tested GBM cell lines (Khoury et al., 2015). After, autophagy inhibition using chloroquine (CQ) resulted in an increase in the sensitivity of treated GBM cells to the induced arginine starvation; thus, there is an activation in the mechanism of autophagy (Khoury et al., 2015). Finally, the expression of ASS-1 by the single cell intracellular staining using the flow cytometer was studied, and 7 out of 9 cell lines were found to be positive for the expression of ASS-1 (with a ratio of fluorescence intensity (RFI) greater than 2, including U251) and these are considered to be partially auxotrophic, and 2 out of 9 tested GBM cells were considered to be negative for the expression of ASS-1 with an RFI value being less than or equal to 2

(including A172) and have complete arginine auxotrophy (these cells will not be saved by the addition of the exogenous L-citrulline) (Khoury et al., 2015).

1.8 Objectives

Adding to what was previously done in our lab; in this study, our aim is to examine the mechanism of cell death that is caused by HuArgI (Co)-PEG5000 in auxotrophic GBM cell lines (partial vs. complete) by studying the activation of autophagy in addition to the impact of this activation. This was done by investigating the outcome of the inhibition of autophagy on the sensitivity of different GBM cell lines to HuArgI (Co)-PEG5000. Additionally, we aspire to study the progression of the levels of expression of ASS-1 in arginine depleted GBM cells over time. Finally, we aim to examine the role played by the reactive oxygen species in the activation of autophagy in arginine depleted GBM cell lines.

Chapter Two

Materials and Methods

2.1 HuArgI (Co)-PEG5000 Purification and expression

Pegylated human recombinant Arginase I cobalt [HuArgI (Co)-PEG5000] (Pegzilarginase) was a gift from Aeaglea BioTherapeutics (Austin, TX, USA).

2.2 Cell Culture

The effectiveness of [HuArgI (Co)-PEG5000] was tested on two human GBM cell lines: U251, and A172. These cell lines were obtained from ATCC (American Type Culture Collection) and cultured in culture media called DMEM; supplemented with 10% Fetal Bovine Serum (heat inactivated, Sigma), 1% L-Glutamine (Biowest, Sigma), and 1% penicillin/streptomycin (Biowest). The cultured cell lines were incubated at 37°C and 5% CO_2.

2.3 Cytotoxicity Assay (Proliferation inhibition Assay)

To test the sensitivity of GBM cell lines to HuArgI (Co)-PEG5000, with or without chloroquine (CQ 25 μM) used as an exogenous inhibitor for autophagy, and N-acetylcysteine (NAC) being the scavenger of reactive oxygen species (ROS), proliferation inhibition assays were performed. In brief, aliquots of 1.2×10^5 cells/ml were plated in a flat-bottom 96-well plate (Corning Inc. Corning, NY) with 100 μL cell culture medium/well. NAC was added at a concentration of 10 μM. After that, 24 μL HuArgI (Co)-PEG5000 was added in media to each well from a round-bottom 96-well plate (Corning Inc. Corning, NY), and after the serial dilutions this led to the concentrations ranging from 10^{-7} reaching 10^{-13} M.

This is followed by incubation for 24, 48, 72, 96, and 120 hours at 37ºC and 5% CO_2. After the end of each time point, 40 μl of XTT cell proliferation reagent (Roche, Basel, Switzerland) were added to each of the 96 wells and the plate was incubated for 4 hours. Then, the absorbance was read at 450 nm using a Varioskan Flash plate reader (Thermo Fisher Scientific, Waltham, MA). The percent maximal absorbance was plotted against the non-linear regression and the log of concentration along with a variable of slope sigmoidal dose-response curve that was made with the IC50 (inhibitory concentration 50) of HuArgI (Co)-PEG5000 with or without NAC.

2.4 Autophagy Assay

$2x10^5$ cells/well were plated in a flat-bottom 6-well plate (Corning Inc. Corning, NY) with 2 ml of cell culture medium/well to detect autophagosome formation. Some wells contained cells only, others with HuArgI (Co)-PEG5000 with a concentration of 10^{-8} M, in the presence or not of chloroquine of 25 μM as the used concentration, in addition to NAC that was added at a concentration of 10 μM in some wells. After that, plates were incubated for five different time points: 24, 48, 72, 96 and 120 hours at 37ºC and 5% CO_2. The protocol followed was according to the manual of the company (ENZO product manual - CYTO-ID® Autophagy Detection Kit, 2016 - Catalog No. ENZ-51031 - Part D. Live Cell Analysis by Flow Cytometry). In brief, cells were washed with PBS, trypsinized and each condition was collected separately, to add after the cyto-id to the cells/condition and this was followed by an incubation period for 30 mins in the incubator. Finally, the cells were washed twice with PBS and the results were read using the C6 flow cytometer.

2.5 Expression of ASS-1

To assess the expression levels of Argininosuccinate Synthetase 1 (ASS-1) intracellular staining on flow cytometry was performed as described previously (Kassab et al, 2013). Aliquots of $2x10^5$ cells/well were plated in flat-bottom 6-well plate (Corning Inc. Corning, NY) in 2 mL of cell culture medium. Cells were either treated or not with HuArgI (Co)-PEG5000 with a concentration of 10^{-8} M, in the presence or not of L-citrulline which was added at a concentration of 11.4 mM. Afterward, plates were incubated for five different time points: 24, 48, 72, 96 and 120 hours at 37ºC and 5% CO_2. In short-term, cells were collected first and then incubated in antibody binding buffer consisting of PBS, 1% FBS, and 0.05% Triton-X 100 in addition to a 1/100 dilution of anti-ASS-1 mouse monoclonal antibody (Abcam, UK) for 1 hour at 37 °C and in dark. Isotypic control contained cells that were incubated with a mouse IgG (Abcam, UK). Once done, this was followed by an incubation for 30 minutes along with a 1/100 dilution of a FITC-conjugated rabbit anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Finally, the cells were washed using phosphate buffered saline (PBS) and were ready for reading using the C6 flow cytometer (BD Accuri, Ann Arbor, MI). Cells were gated and ASS-1 staining was detected on FL1-H. To determine the positivity for ASS-1 presence, the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the stained cells and the MFI of the isotypic control cells was used. An RFI ≤ 2 was considered negative for the expression of ASS-1 while an RFI > 2 was considered positive. In addition to that, cells were analyzed on forward scatter versus FL1-H in order to determine the ASS-1 positive cells percentage.

2.6 Protein Analysis

2.6.1 Protein Quantification

Aliquots of 1x106 cells/well were plated in flat-bottom 6-well plate (Corning Inc. Corning, NY) in 2 mL of cell culture medium. Cells were either treated or not with HuArgI (Co)-PEG5000 with a concentration of 10^{-8} M, in the presence or not of CQ with a concentration of 25 μM. Following that, the samples were washed with PBS, and after supplemented with a

protease inhibitor cocktail which is mixed with a lysis buffer (Millipore, RIPA buffer). Then, the collection of the supernatants as whole-cell lysates was done, and protein concentrations was determined using the Lowry assay (Biorad).

2.6.2 Western Blot

After that, the separation of whole-cell lysates using 12 % SDS-PAGE gel, then transferred to PVDF membrane (Biorad), followed by the incubation of the membranes with a primary antibody being specific for LC3 (1:3000 dilution) expression (Abcam). This was followed by serial washes of the membranes and incubation with the appropriate secondary antibody: Anti-Rabit IgG (Promega). The visualization of the bands of the immunoreactive proteins was done with the help of enhanced chemiluminescence western-blotting detection reagents (GE Healthcare UK) and finally the quantification is done using ChemiDoc XRS+ imaging system (Biorad).

Chapter Three

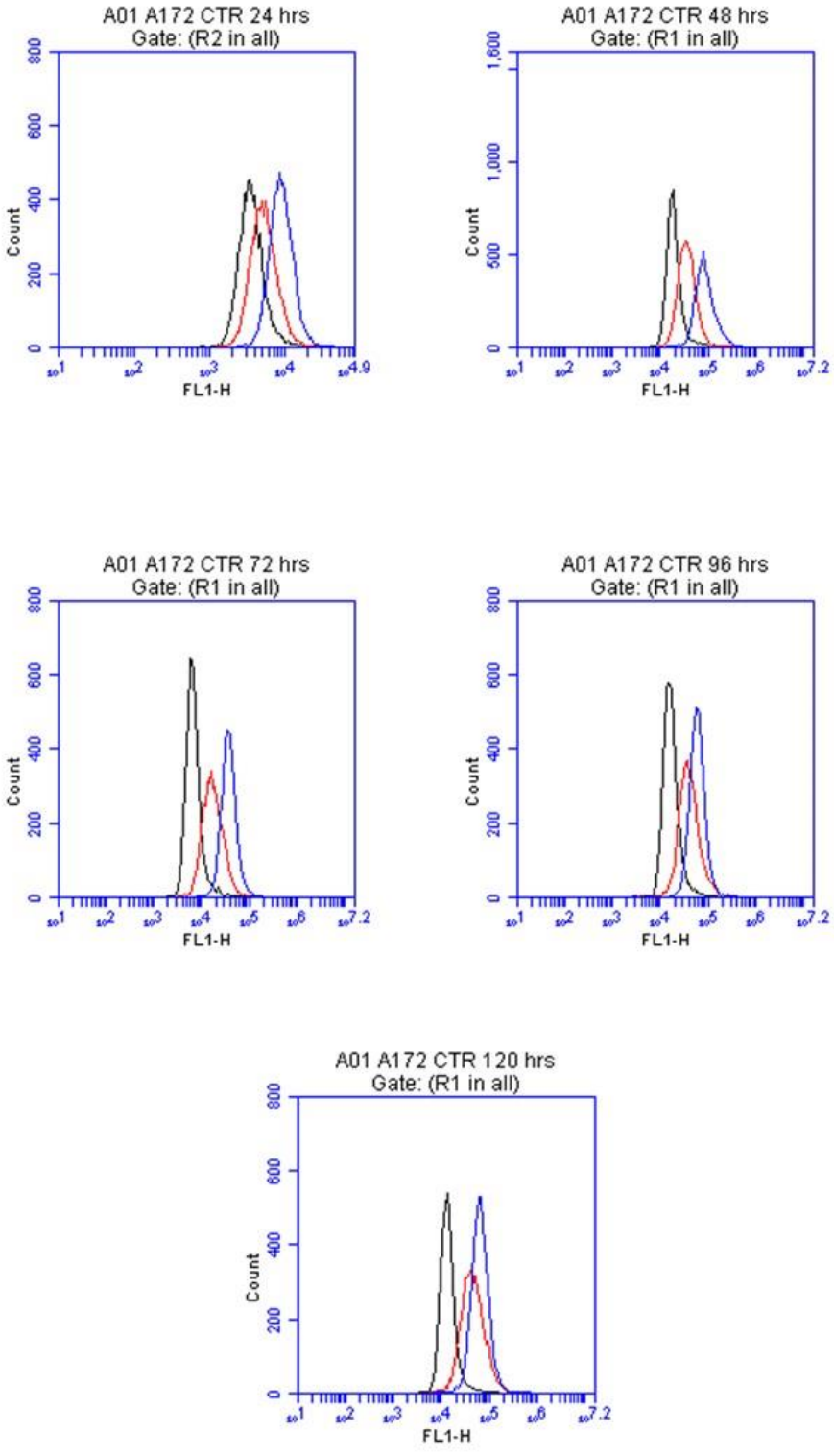
Results

3.1 Autophagy Activation

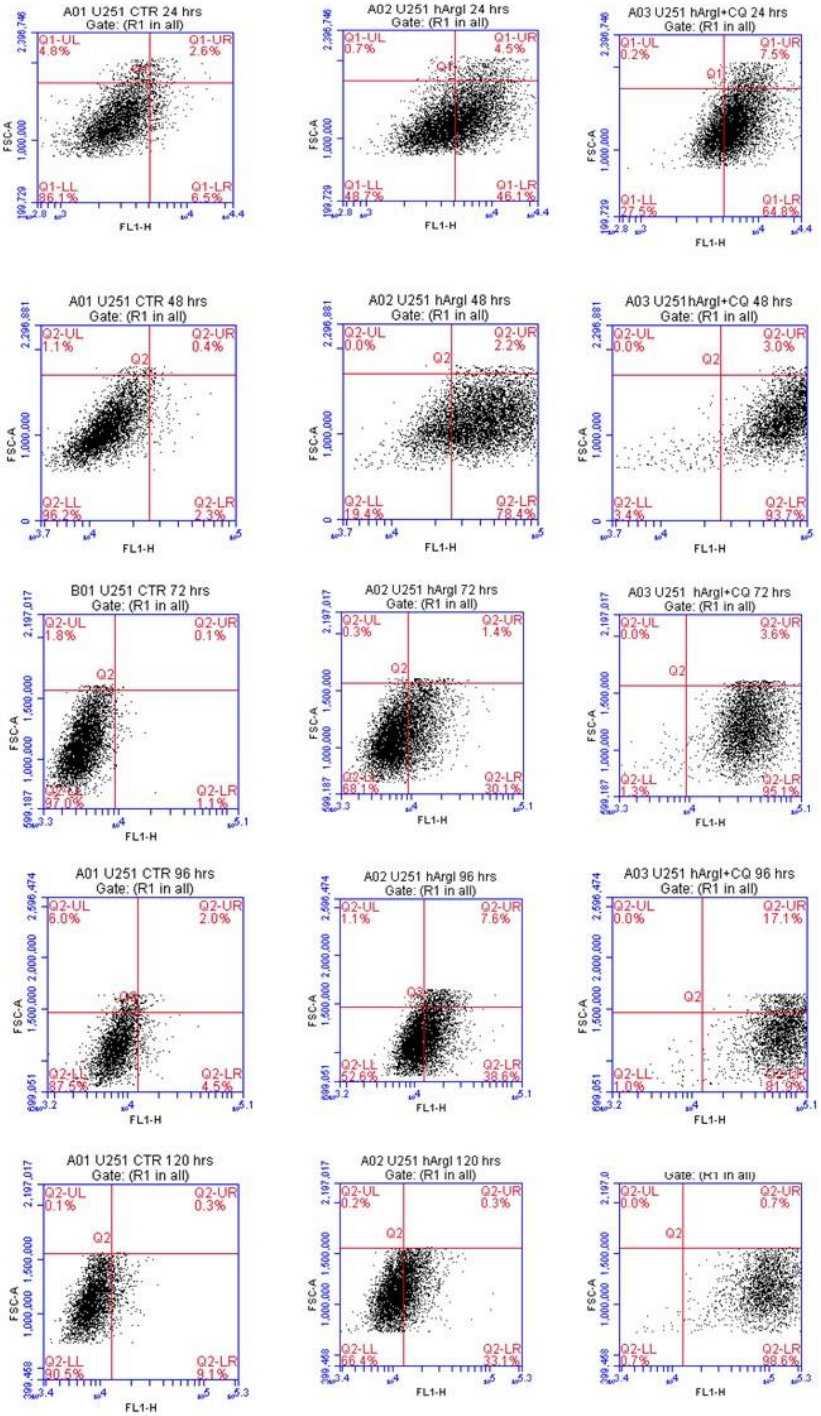
We detected the formation of autophagosomes in untreated cells and compared them to that found in cells treated with HuArgI (Co)-PEG5000 having a 10^{-7} M concentration, in order to study the effect of the depletion of arginine on the activation of autophagy in GBM. We observed the activation of autophagy in one partially auxotrophic cell line (U251), along with one completely auxotrophic cell line (A172) at early and late time points (24, 48, 72, 96, and 120 hours) after treatment.

First autophagosome formation was detected in both A172 and U251 at 24 hours after depriving the cells from arginine by HuArgI (Co)-PEG5000 when being distinguished from control untreated cells. As for the upcoming time points and starting from 48 hours reaching 120 hours post treatment with the PEGylated arginase isoform used in both A172 and U251 cells, a significant increase was determined in the percentage of the positively stained cells for autophagosomes as compared to untreated cells. Note that, this percentage slightly decreased in U251 treated cells after 96- and 120-hours post-treatment. In addition, after adding chloroquine (CQ) which is known as a downstream inhibitor for autophagy at a 25 μM concentration, it was clearly shown that in most of the cases there was autophagosome accumulation where we have a significant rise in the percentage of cells that are positive for the staining of autophagosomes when being compared to incubated cells with HuArgI(Co)-PEG5000 only.

A


A01 A172 CTR 24 hrs
Gate: (R2 in all)
Count
FL1-H
A01 A172 CTR 48 hrs
Gate: (R1 in all)
Count
FL1-H
A01 A172 CTR 72 hrs
Gate: (R1 in all)
Count
FL1-H
A01 A172 CTR 96 hrs
Gate: (R1 in all)
Count
FL1-H
A01 A172 CTR 120 hrs
Gate: (R1 in all)
Count
FL1-H

B


A01 U251 CTR 24 hrs
Gate: (R1 in all)
Q1-UL 4.8%
Q1-UR 2.6%
Q1-LL 86.1%
Q1-LR 6.5%
A02 U251 hArgI 24 hrs
Gate: (R1 in all)
Q1-UL 0.7%
Q1-UR 4.5%
Q1-LL 48.7%
Q1-LR 46.1%
A03 U251 hArgI+CQ 24 hrs
Gate: (R1 in all)
Q1-UL 0.2%
Q1-UR 7.5%
Q1-LL 27.5%
Q1-LR 64.8%
A01 U251 CTR 48 hrs
Gate: (R1 in all)
Q2-UL 1.1%
Q2-UR 0.4%
Q2-LL 96.2%
Q2-LR 2.3%
A02 U251 hArgI 48 hrs
Gate: (R1 in all)
Q2-UL 0.0%
Q2-UR 2.2%
Q2-LL 19.4%
Q2-LR 78.4%
A03 U251hArgI+CQ 48 hrs
Gate: (R1 in all)
Q2-UL 0.0%
Q2-UR 3.0%
Q2-LL 3.4%
Q2-LR 93.7%
B01 U251 CTR 72 hrs
Gate: (R1 in all)
Q2-UL 1.8%
Q2-UR 0.1%
Q2-LR 1.1%
A02 U251 hArgI 72 hrs
Gate: (R1 in all)
Q2-UL 0.3%
Q2-UR 1.4%
Q2-LR 30.1%
A03 U251 hArgI+CQ 72 hrs
Gate: (R1 in all)
Q2-UL 0.0%
Q2-UR 3.6%
Q2-LL 1.3%
Q2-LR 95.1%
A01 U251 CTR 96 hrs
Gate: (R1 in all)
Q2-UL 6.0%
Q2-UR 2.0%
Q2-LR 4.5%
A02 U251 hArgI 96 hrs
Gate: (R1 in all)
Q2-UL 1.1%
Q2-UR 7.6%
Q2-LR 39.6%
A03 U251 hArgI+CQ 96 hrs
Gate: (R1 in all)
Q2-UL 0.0%
Q2-UR 17.1%
Q2-LL 1.0%
A01 U251 CTR 120 hrs
Gate: (R1 in all)
Q2-UL 0.1%
Q2-UR 0.3%
Q2-LL 90.5%
Q2-LR 9.1%
A02 U251 hArgI 120 hrs
Gate: (R1 in all)
Q2-UL 0.2%
Q2-UR 0.3%
Q2-LL 66.4%
Q2-LR 33.1%
Q2-UL 0.0%
Q2-UR 0.7%
Q2-LL 0.7%
Q2-LR 98.6%
FSC-A
FL1-H

B′

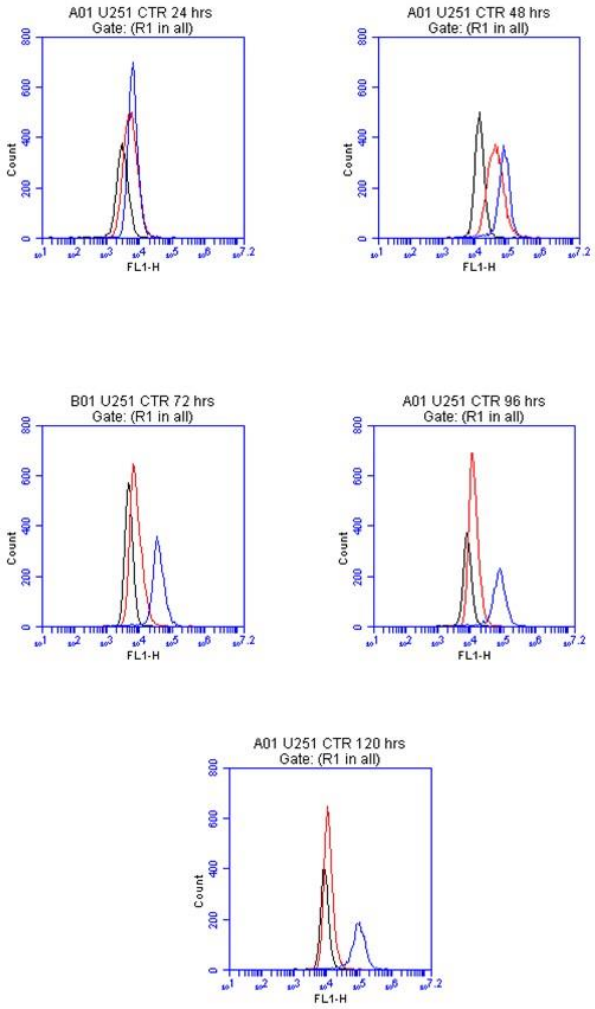
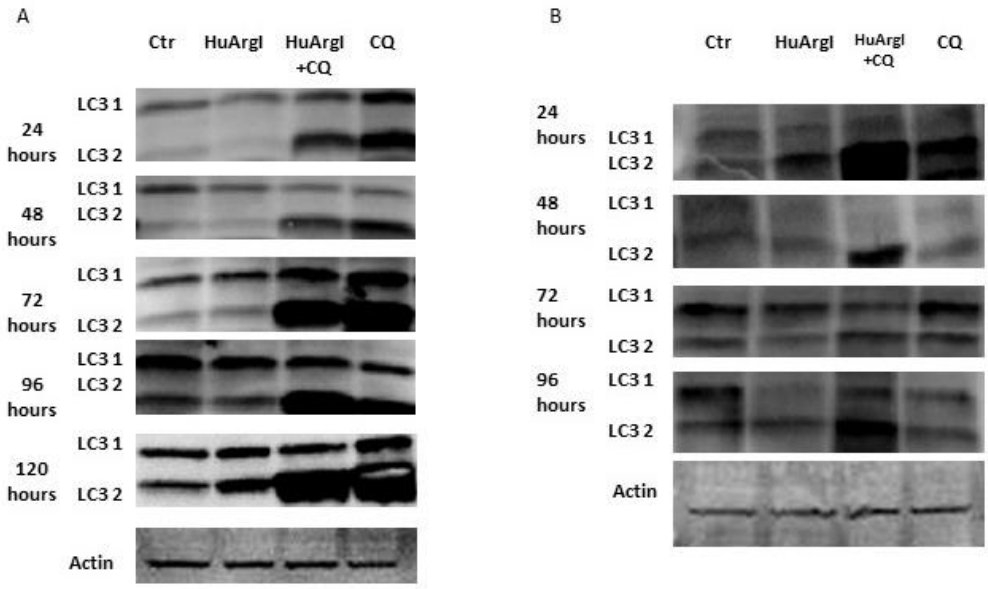


Figure 4 Formation of Autophagosomes in A172 (A, A'), U251 cell line (B, B') at 24, 48, 72, 96 and 120 hours after treatment.

Control untreated samples (black curve) are to the left, treated cells with HuArgI (Co)-PEG5000 (10^{-7} M) (red curve) in the middle and co-treated cells with HuArgI (Co)-PEG5000 (10^{-7} M) and CQ (25μM) (blue curve) to the right.

And in order to confirm that, we then did some western blots to check for LC3 I and LC3 II protein levels in both U251 and A172 cell lines at 24, 48, 72, 96, and 120 hours in untreated cells as our control, cells treated with HuArgI (Co)-PEG5000, cells co-incubated with both HuArgI (Co)-PEG5000 and CQ of 25 uM, or

cell treated with CQ only. When comparing the ratios of LC3 II to LC3 I that shows the formation of autophagosome formation in arginase treated cells and the accumulation of autophagosomes in cells co-treated with arginase and CQ we can notice the slight increase in the ratio in U251 arginase treated cells and a significant increase in this ratio as well in U251 cells co-treated with HuArgI (Co)-PEG5000 and CQ starting from early time points to reach 120 hours and this increase was highly significant at 96 hours. However, when it comes to A172 cells the increase in the ratio was slightly detected at late time points and this might be due to the fact that this is a semi-quantitative method and the detected increase in the ratio in co-treated cells with chloroquine and HuArgI (Co)-PEG5000 was seen in late time points showing the accumulation of autophagosomes in these cells when compared to untreated cells.



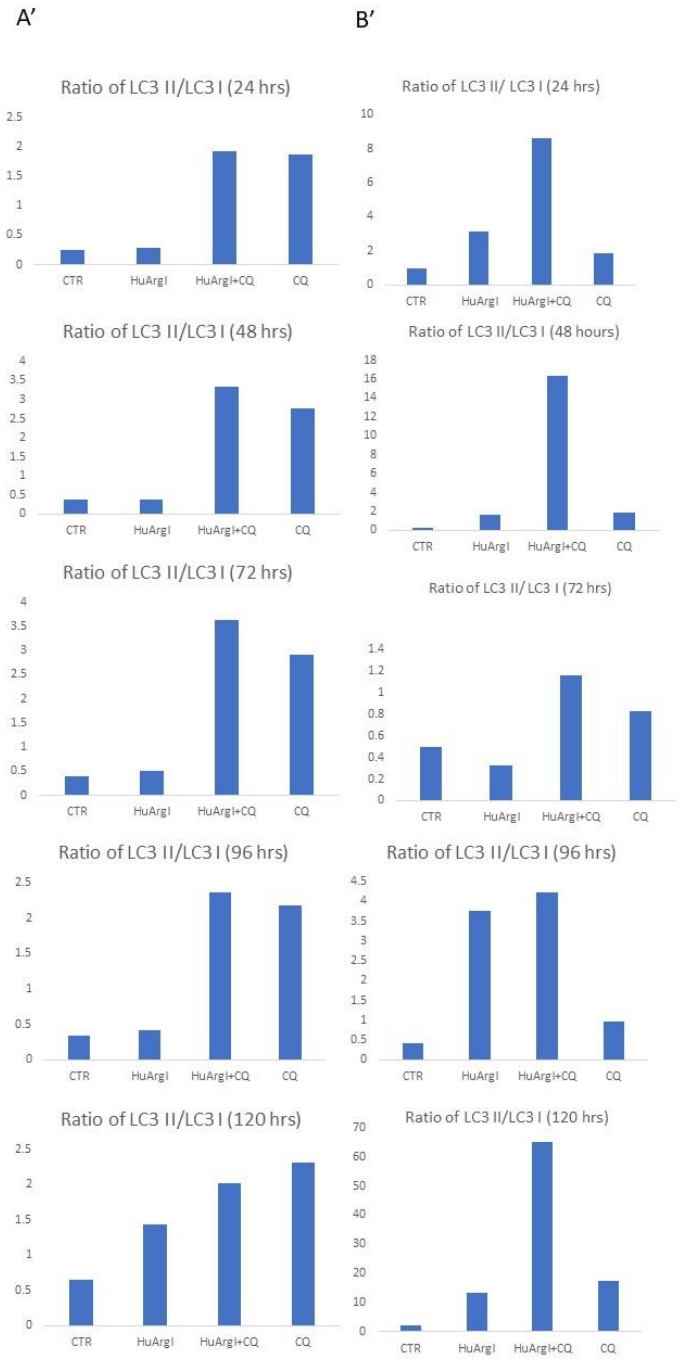


Figure 5 Western Blot showing the conversion of LC3 I to LC3II in A172 cell (a), and U251 cell line (b) thus the autophagosome formation at different time points post-treatment; with representative bar graphs of the LC3 data.

Control samples are shown to the left, followed by samples treated with HuArgI (Co)-PEG5000 (10^{-7} M), then samples co-treated with HuArgI (Co)-PEG5000 (10^{-7} M) and CQ (25μM), and samples treated with CQ alone to the right.

3.2 Inhibiting Autophagy and its Effect on GBM Cell's Sensitivity to the Deprivation of Arginine

To examine the effect of inhibiting autophagy on the sensitivity of both U251 and A172 cells after being treated with HuArgI (Co)-PEG5000, we added the inhibitor of autophagy, chloroquine of 25 μM, and see if it is able to rescue the arginine depleted cells.

Starting with A172 cells, when comparing cells treated with HuArgI (Co)-PEG5000 only to cells co-incubated with both chloroquine and HuArgI (Co)-PEG5000, we detected an increase in the percentage of surviving A172 cells, respectively from 19 to 31 percent at 96 hours, and from 16 to 68 percent at 120 hours. This reveals a reduction in the sensitivity of A172 treated cells with HuArgI (Co)-PEG5000. Moving to U251 cells, we performed the same steps, where the co-incubation with chloroquine also showed a decrease in the sensitivity of the cells to HuArgI (Co)-PEG5000. This was obvious after we have detected the increase from 10 to 45 percent at 72 hours, 10 to 50 percent at 96 hours, and from 11 to 38 at 120 hours in the percentage of surviving cells when comparing cells incubated with HuArgI (Co)-PEG5000 only to cells treated with both HuArgI (Co)-PEG5000 along with chloroquine. (Note here that U251 cell line misses one time point (24 hours) and the A172 cell line misses also one time point (72 hours); so, these are preliminary data that will be completed and repeated soon).

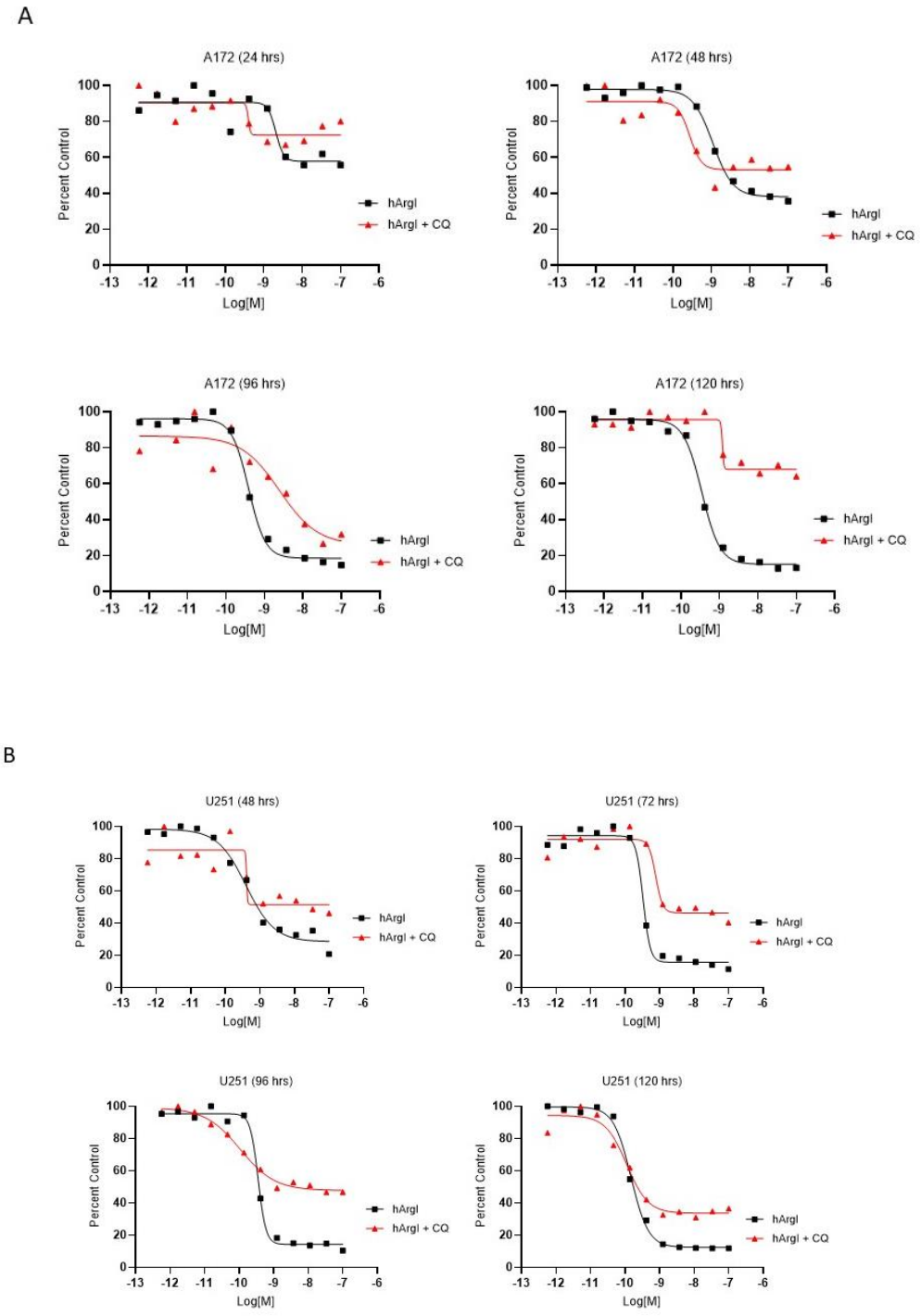


Figure 6 The sensitivity of GBM cells to HuArgI (Co)-PEG5000 along with the inhibition of autophagy using CQ after 24, 48, 72, 96, and 120 hours of treatment.

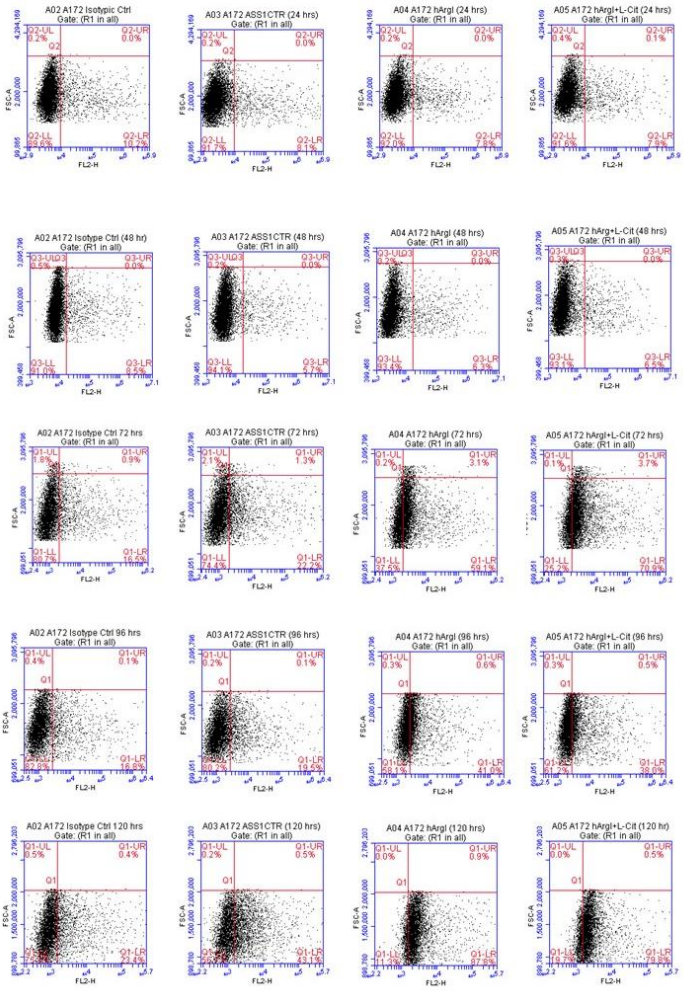
Curves for the non-linear regression of A172 cells (A), and U251 cells (B) where incubated cells with HuArgI (Co)-PEG5000 only (black) vs. co-incubation with CQ (red).

These results show that, at late time points, in both U251 and A172 cells, chloroquine was able to rescue the cells from death (observed in cells treated with HuArgI (Co)-PEG5000 only). We indicated that arginine starvation by HuArgI (Co)-PEG5000 leads to the activation of autophagy as mentioned in the previous result, that in turn causes the death of the glioblastoma multiforme cells being tested (death by autophagy).

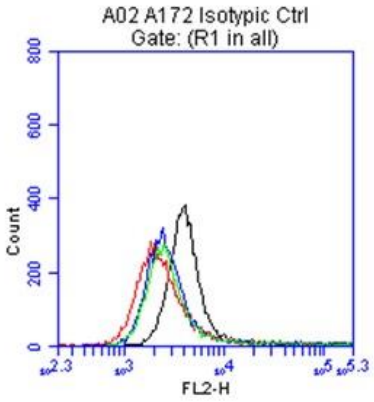
3.3 ASS-1 Levels after Prolonged Arginine Deprivation

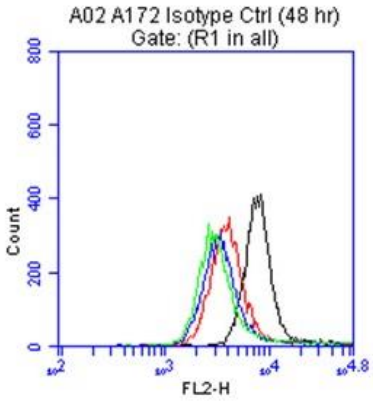
In our lab, we had already studied the level of ASS-1 in untreated cells as mentioned previously and found out that some of the studied GBM cell lines were partially auxotrophic for arginine and others were completely auxotrophic (positive for ASS-1 expression with a RFI value greater than 2 and negative for the expression of ASS-1 with a RFI value less than or equal to 2 respectively). To study the effect of arginine starvation on the progression of the expression of Argininosuccinate synthase-1 (ASS-1) over time, we studied the levels of ASS-1 in control cells that are untreated, cells that are treated with HuArgI (Co)-PEG5000 alone with a 10^{-7} M concentration, and in combination with L-citrulline with an 11.4 mM concentration at 24, 48, 72, 96, and 120 hours after the treatment.

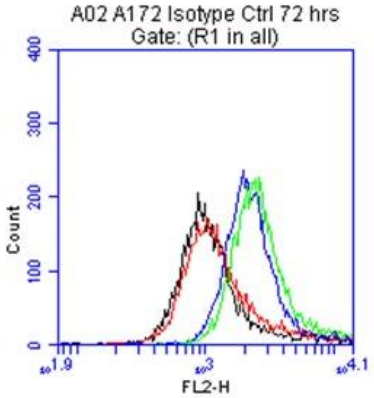
A

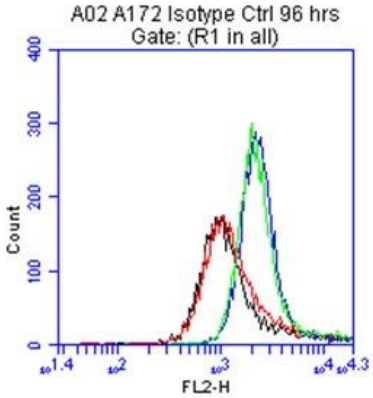

A02 A172 Isotypic Ctrl
Gate: (R1 in all)
A03 A172 ASS1CTR (24 hrs)
Gate: (R1 in all)
A04 A172 hArgI (24 hrs)
Gate: (R1 in all)
A05 A172 hArgI+L-Cit (24 hrs)
Gate: (R1 in all)
A02 A172 Isotype Ctrl (48 hr)
Gate: (R1 in all)
A03 A172 ASS1CTR (48 hrs)
Gate: (R1 in all)
A04 A172 hArgI (48 hrs)
Gate: (R1 in all)
A05 A172 hArg+L-Cit (48 hrs)
Gate: (R1 in all)
A02 A172 Isotype Ctrl 72 hrs
Gate: (R1 in all)
A03 A172 ASS1CTR (72 hrs)
Gate: (R1 in all)
A04 A172 hArgI (72 hrs)
Gate: (R1 in all)
A05 A172 hArgI+L-Cit (72 hrs)
Gate: (R1 in all)
A02 A172 Isotype Ctrl 96 hrs
Gate: (R1 in all)
A03 A172 ASS1CTR (96 hrs)
Gate: (R1 in all)
A04 A172 hArgI (96 hrs)
Gate: (R1 in all)
A05 A172 hArgI+L-Cit (96 hrs)
Gate: (R1 in all)
A02 A172 Isotype Ctrl 120 hrs
Gate: (R1 in all)
A03 A172 ASS1CTR (120 hrs)
Gate: (R1 in all)
A04 A172 hArgI (120 hrs)
Gate: (R1 in all)
A05 A172 hArgI+L-Cit (120 hr)
Gate: (R1 in all)
FSC-A
FL2-H

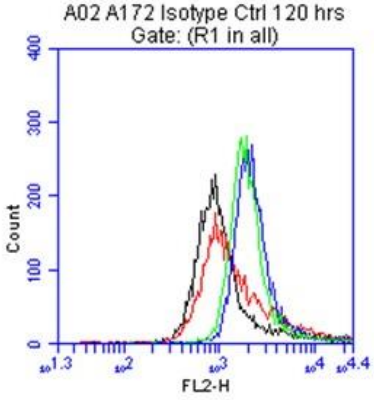
A’


A02 A172 Isotypic Ctrl
Gate: (R1 in all)
Count
FL2-H

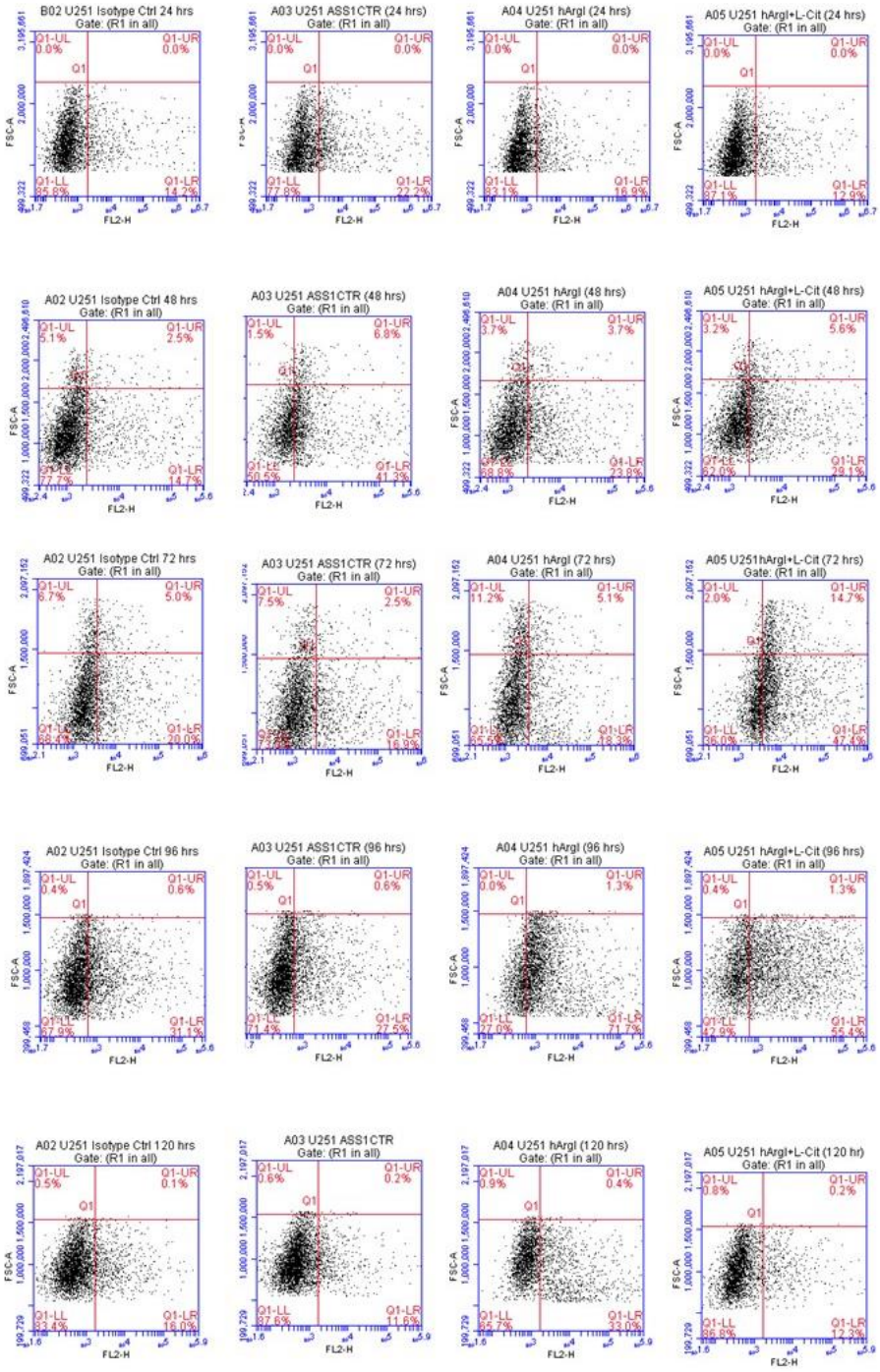

A02 A172 Isotype Ctrl (48 hr)
Gate: (R1 in all)
Count
FL2-H


A02 A172 Isotype Ctrl 72 hrs
Gate: (R1 in all)
Count
FL2-H

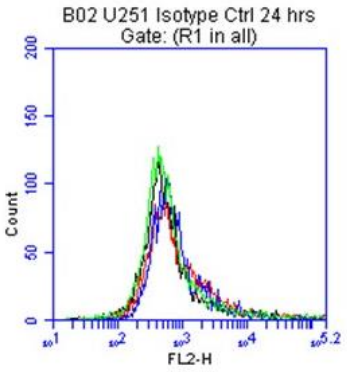

A02 A172 Isotype Ctrl 96 hrs
Gate: (R1 in all)
Count
FL2-H

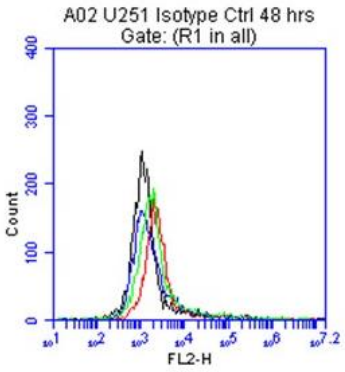

A02 A172 Isotype Ctrl 120 hrs
Gate: (R1 in all)
Count
FL2-H

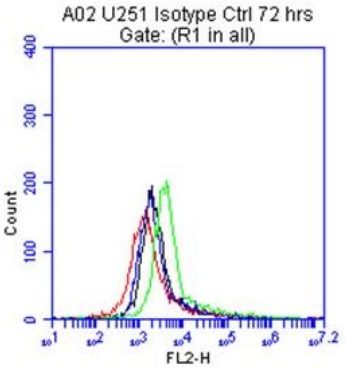
B

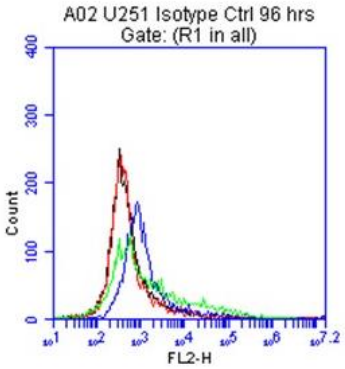

B02 U251 Isotype Ctrl 24 hrs
Gate: (R1 in all)
A03 U251 ASS1CTR (24 hrs)
Gate: (R1 in all)
A04 U251 hArgI (24 hrs)
Gate: (R1 in all)
A05 U251 hArgI+L-Cit (24 hrs)
Gate: (R1 in all)
A02 U251 Isotype Ctrl 48 hrs
Gate: (R1 in all)
A03 U251 ASS1CTR (48 hrs)
Gate: (R1 in all)
A04 U251 hArgI (48 hrs)
Gate: (R1 in all)
A05 U251 hArgI+L-Cit (48 hrs)
Gate: (R1 in all)
A02 U251 Isotype Ctrl 72 hrs
Gate: (R1 in all)
A03 U251 ASS1CTR (72 hrs)
Gate: (R1 in all)
A04 U251 hArgI (72 hrs)
Gate: (R1 in all)
A05 U251hArgI+L-Cit (72 hrs)
Gate: (R1 in all)
A02 U251 Isotype Ctrl 96 hrs
Gate: (R1 in all)
A03 U251 ASS1CTR (96 hrs)
Gate: (R1 in all)
A04 U251 hArgI (96 hrs)
Gate: (R1 in all)
A05 U251 hArgI+L-Cit (96 hrs)
Gate: (R1 in all)
A02 U251 Isotype Ctrl 120 hrs
Gate: (R1 in all)
A03 U251 ASS1CTR
Gate: (R1 in all)
A04 U251 hArgI (120 hrs)
Gate: (R1 in all)
A05 U251 hArgI+L-Cit (120 hr)
Gate: (R1 in all)
FSC-A
FL2-H

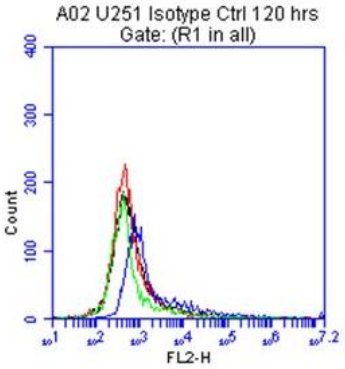
B’


B02 U251 Isotype Ctrl 24 hrs
Gate: (R1 in all)
Count
FL2-H


A02 U251 Isotype Ctrl 48 hrs
Gate: (R1 in all)
Count
FL2-H


A02 U251 Isotype Ctrl 72 hrs
Gate: (R1 in all)
Count
FL2-H


A02 U251 Isotype Ctrl 96 hrs
Gate: (R1 in all)
Count
FL2-H


A02 U251 Isotype Ctrl 120 hrs
Gate: (R1 in all)
Count
FL2-H

C

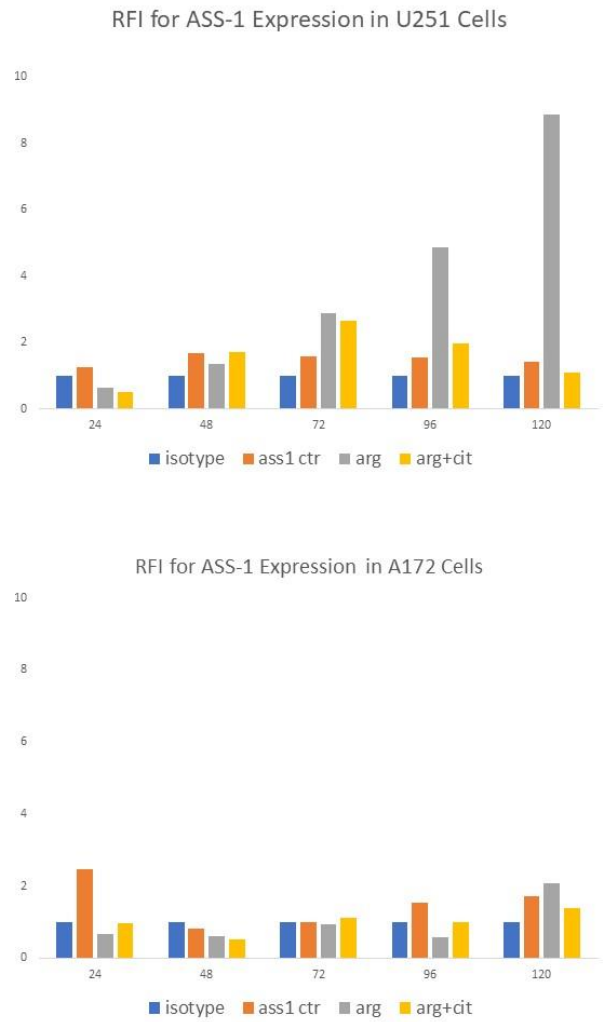


Figure 7 ASS-1 expression in A172 and U251 cells

In A172 (A, A'), U251 cell line (B, B'): from left to right represented as isotype control (Black curve), ASS-1 control (Red curve), treated with HuArgI(Co)-PEG5000 (10^{-7} M) (Blue curve), or treated with HuArgI(Co)-PEG5000 and L-citrulline (11.4 mM) (green curve) at 24, 48, 72, and 96 hours after treatment; and (C) representative bar graphs.

In U521 cells, untreated control cells (red curve) show no expression of ASS-1 enzymes when being compared to the isotypic control (black curve). Arginine

starvation using HuArgI (Co)-PEG5000 (blue curve) leads to ASS-1 enzyme overexpression at late time points starting from 72, 96, and 120 hours up to 2.9, 4.9, and 8.9 folds respectively, when compared to control cells. Upon adding L-citrulline it was clearly shown the reverse in the expression of ASS-1 to reach back the levels as before treatment (green curve). Note to mention here, the overexpression of ASS-1 did not affect cytotoxicity because the cells are still dying at 120 hours after treatment.

However, in A172 cells, control untreated cells (red curve) display a greater level of the enzyme ASS-1 when being compared to the isotopic control (black curve) at 24 hours. For the treated cells with HuArgI (Co)-PEG5000 (blue curve) no change was detected in the ASS-1 enzyme levels until the late time point 120 hours where a slight increase in the level of ASS-1 enzyme was shown (by 2.1 folds), when compared to untreated control cells. Moreover, no reduction in the ASS-1 upon the addition of L-citrulline (green curve) was detected.

This shows that in A172 cells we have ASS-1 expression as a consequence of HuArgI (Co)-PEG5000-mediated arginine starvation at late time points while in U251 cells there is overexpression of ASS-1 as a consequence of arginine starvation through HuArgI (Co)-PEG5000.

3.4 Impact of ROS Scavenging on the Activation of Autophagy in GBM

To check if the activation of autophagy is dependent on ROS, and to see if we have formation of autophagosomes, we started with a 5mM concentration of N-acetyl cysteine (NAC) and then increased this concentration to reach 10 mM.

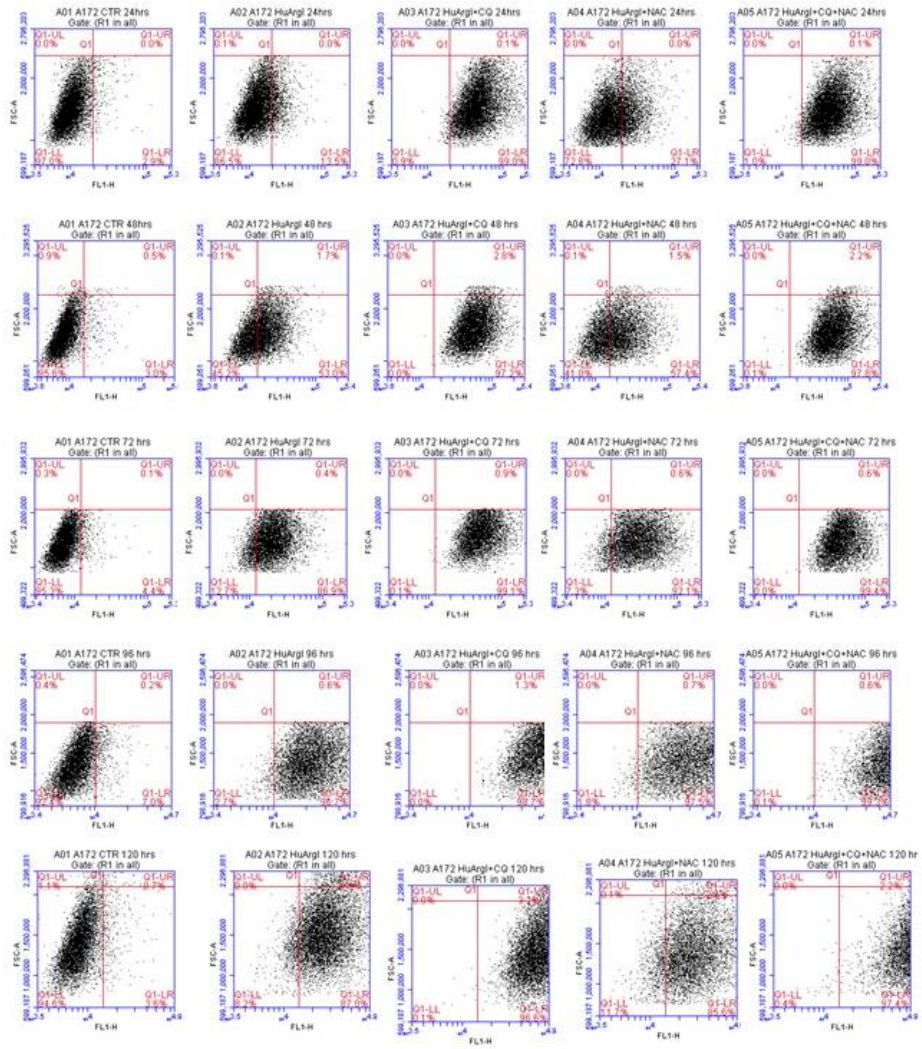
We observed the formation of autophagosomes in untreated control cells, cells incubated with HuArgI (Co)-PEG5000, and finally cells co-incubated with both

HuArgI (Co)-PEG5000 and NAC for five time points (24, 48, 72, 96, and 120 hours) in U251 cells that are partially auxotrophic, and A172 cells that are completely auxotrophic. Starting with the concentration of 5 mM of NAC; in A172 cells treated with NAC, no change in the extent of autophagy activation was detected, in comparison to that observed in HuArgI (Co)-PEG5000 treated cells at all time points. And because of the autophagic flux, sometimes it is not able for us to see the difference between arginase and arginase with NAC, so we compared the cells co-treated with arginase and CQ to the cells treated with arginase, CQ, and NAC to check for the decrease in the flux; and still no change was detected in the accumulation of autophagosomes.

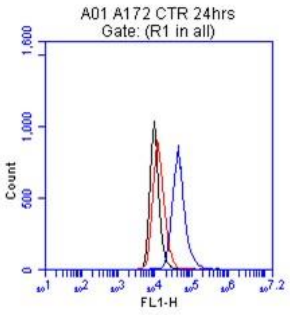
However, in U251 cells incubated with 5 mM of NAC, a slight decrease in the extent of autophagosome formation was detected, when compared to that obtained in cells incubated with HuArgI (Co)-PEG5000 only at different time points starting 24, 48, and 96 hours.

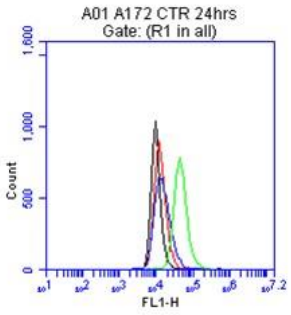
To continue, we increased the concentration of NAC to 10 mM and followed the same conditions as mentioned before. No change was detected in the autophagosome formation when comparing cells that are treated with HuArgI (Co)-PEG5000 to that treated with both HuArgI (Co)-PEG 5000 and NAC; as well as in A172 cells treated with arginase and CQ to A172 cells treated with arginase, CQ, and NAC. On the other hand, a significant decrease in the extent of the formation of autophagosomes was detected, when being compared to the formation of autophagosomes detected in treated cells with HuArgI (Co)-PEG5000 only, in addition to the comparison between U251 cells treated with arginase and CQ and U251 cells treated with arginase, CQ, and NAC this decrease was also observed.

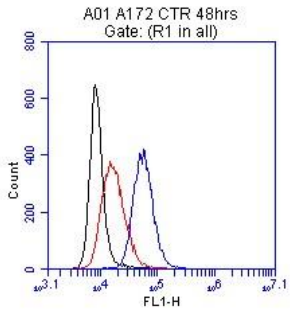
A

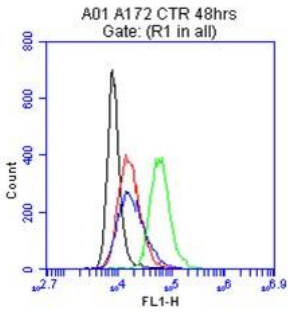

A01 A172 CTR 24hrs
A02 A172 HuArgI 24hrs
A03 A172 HuArgI+CQ 24hrs
A04 A172 HuArgI+NAC 24hrs
A05 A172 HuArgI+CQ+NAC 24hrs
A01 A172 CTR 48hrs
A02 A172 HuArgI 48 hrs
A03 A172 HuArgI+CQ 48 hrs
A04 A172 HuArgI+NAC 48 hrs
A05 A172 HuArgI+CQ+NAC 48 hrs
A01 A172 CTR 72 hrs
A02 A172 HuArgI 72 hrs
A03 A172 HuArgI+CQ 72 hrs
A04 A172 HuArgI+NAC 72 hrs
A05 A172 HuArgI+CQ+NAC 72 hrs
A01 A172 CTR 96 hrs
A02 A172 HuArgI 96 hrs
A03 A172 HuArgI+CQ 96 hrs
A04 A172 HuArgI+NAC 96 hrs
A05 A172 HuArgI+CQ+NAC 96 hrs
A01 A172 CTR 120 hrs
A02 A172 HuArgI 120 hrs
A03 A172 HuArgI+CQ 120 hrs
A04 A172 HuArgI+NAC 120 hrs
A05 A172 HuArgI+CQ+NAC 120 hr

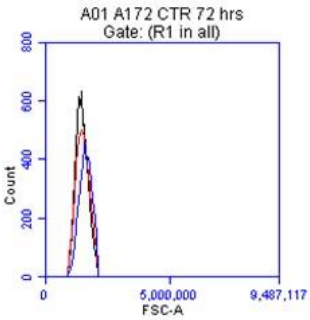
A’

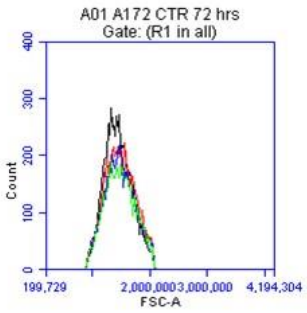

A01 A172 CTR 24hrs
Gate: (R1 in all)
Count
FL1-H

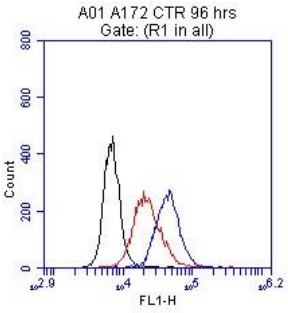

A01 A172 CTR 24hrs
Gate: (R1 in all)
Count
FL1-H

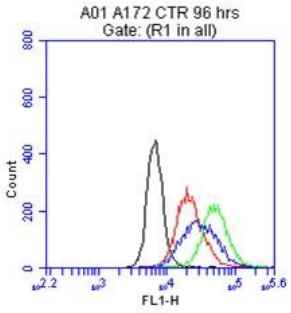

A01 A172 CTR 48hrs
Gate: (R1 in all)
Count
FL1-H

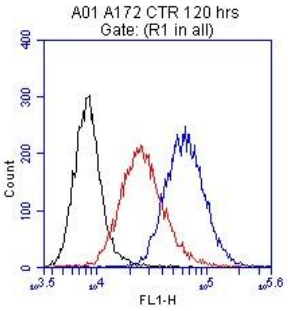

A01 A172 CTR 48hrs
Gate: (R1 in all)
Count
FL1-H

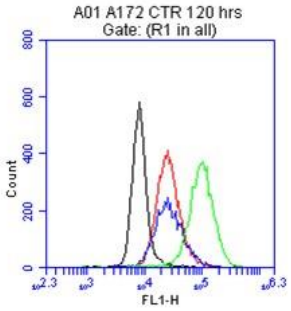

A01 A172 CTR 72 hrs
Gate: (R1 in all)
Count
0
5,000,000
9,487,117
FSC-A


A01 A172 CTR 72 hrs
Gate: (R1 in all)
Count
199,729
2,000,000
3,000,000
4,194,304
FSC-A

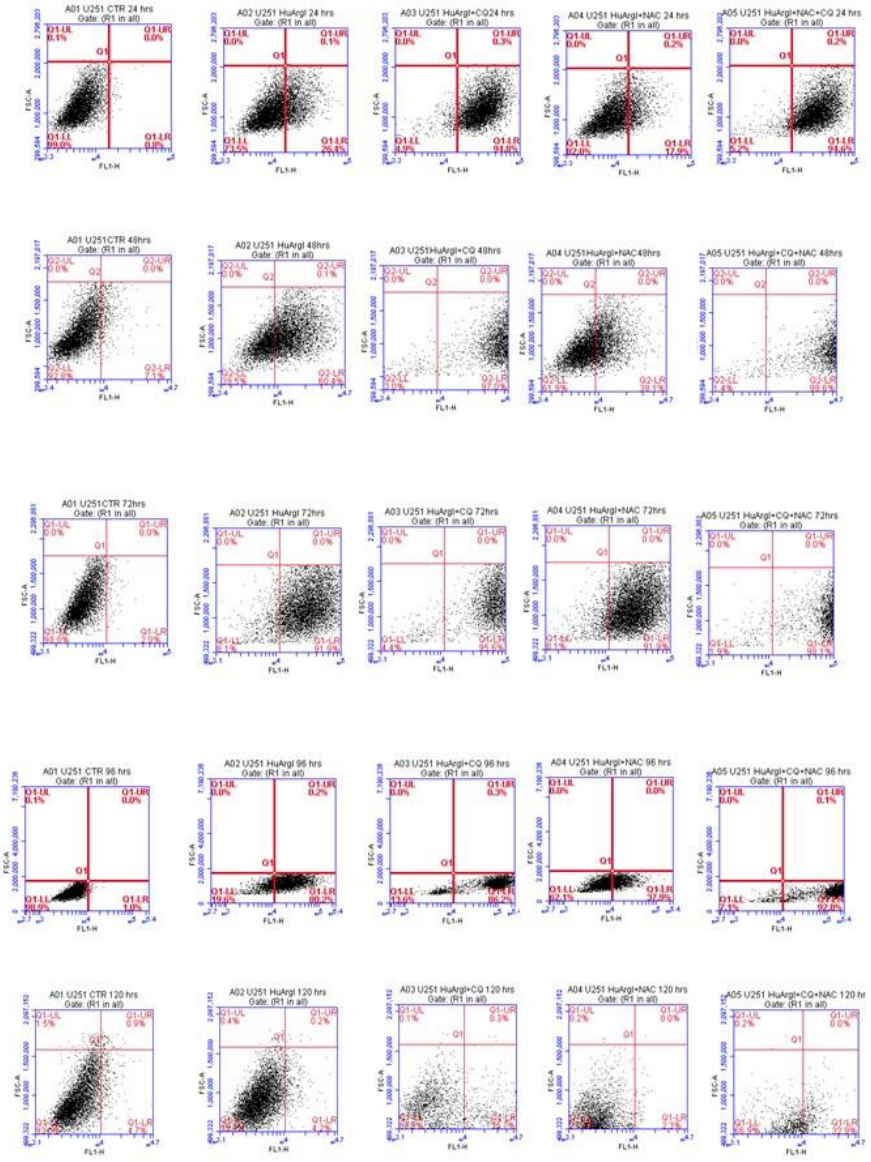

A01 A172 CTR 96 hrs
Gate: (R1 in all)
Count
FL1-H


A01 A172 CTR 96 hrs
Gate: (R1 in all)
Count
FL1-H

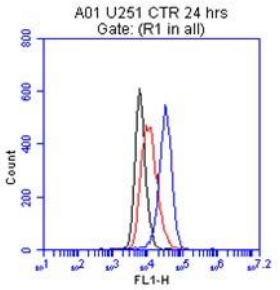

A01 A172 CTR 120 hrs
Gate: (R1 in all)
Count
FL1-H

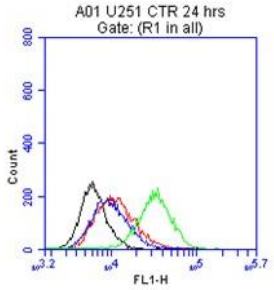

A01 A172 CTR 120 hrs
Gate: (R1 in all)
Count
FL1-H

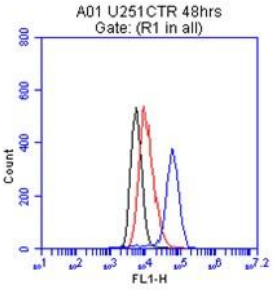
B

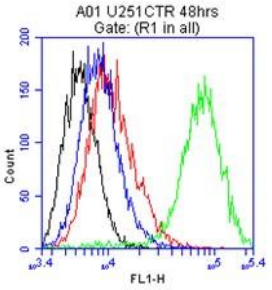

A01 U251 CTR 24 hrs
Gate: (R1 in all)
A02 U251 HuArgI 24 hrs
Gate: (R1 in all)
A03 U251 HuArgI+CQ24 hrs
Gate: (R1 in all)
A04 U251 HuArgI+NAC 24 hrs
Gate: (R1 in all)
A05 U251 HuArgI+NAC+CQ 24 hrs
Gate: (R1 in all)
A01 U251CTR 48hrs
Gate: (R1 in all)
A02 U251 HuArgI 48hrs
Gate: (R1 in all)
A03 U251HuArgI+CQ 48hrs
Gate: (R1 in all)
A04 U251HuArgI+NAC48hrs
Gate: (R1 in all)
A05 U251 HuArgI+CQ+NAC 48hrs
Gate: (R1 in all)
A01 U251CTR 72hrs
Gate: (R1 in all)
A02 U251 HuArgI 72hrs
Gate: (R1 in all)
A03 U251 HuArgI+CQ 72hrs
Gate: (R1 in all)
A04 U251 HuArgI+NAC 72hrs
Gate: (R1 in all)
A05 U251 HuArgI+CQ+NAC 72hrs
Gate: (R1 in all)
A01 U251 CTR 96 hrs
Gate: (R1 in all)
A02 U251 HuArgI 96 hrs
Gate: (R1 in all)
A03 U251 HuArgI+CQ 96 hrs
Gate: (R1 in all)
A04 U251 HuArgI+NAC 96 hrs
Gate: (R1 in all)
A05 U251 HuArgI+CQ+NAC 96 hrs
Gate: (R1 in all)
A01 U251 CTR 120 hrs
Gate: (R1 in all)
A02 U251 HuArgI 120 hrs
Gate: (R1 in all)
A03 U251 HuArgI+CQ 120 hrs
Gate: (R1 in all)
A04 U251 HuArgI+NAC 120 hrs
Gate: (R1 in all)
A05 U251 HuArgI+CQ+NAC 120 hr
Gate: (R1 in all)

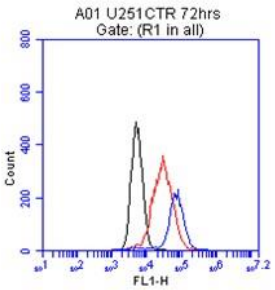
B’

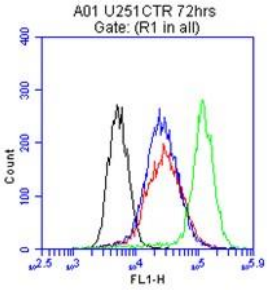

A01 U251 CTR 24 hrs
Gate: (R1 in all)
Count
FL1-H

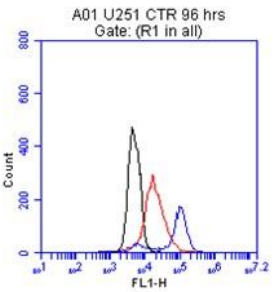

A01 U251 CTR 24 hrs
Gate: (R1 in all)
Count
FL1-H

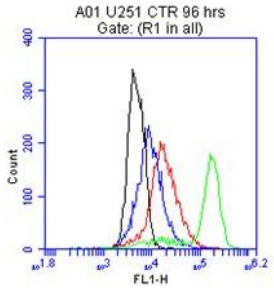

A01 U251CTR 48hrs
Gate: (R1 in all)
Count
FL1-H

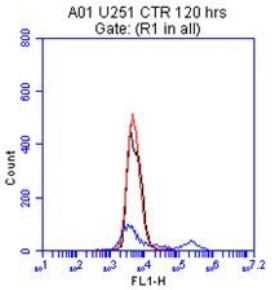

A01 U251CTR 48hrs
Gate: (R1 in all)
Count
FL1-H

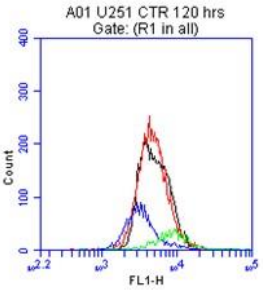

A01 U251CTR 72hrs
Gate: (R1 in all)
Count
FL1-H


A01 U251CTR 72hrs
Gate: (R1 in all)
Count
FL1-H

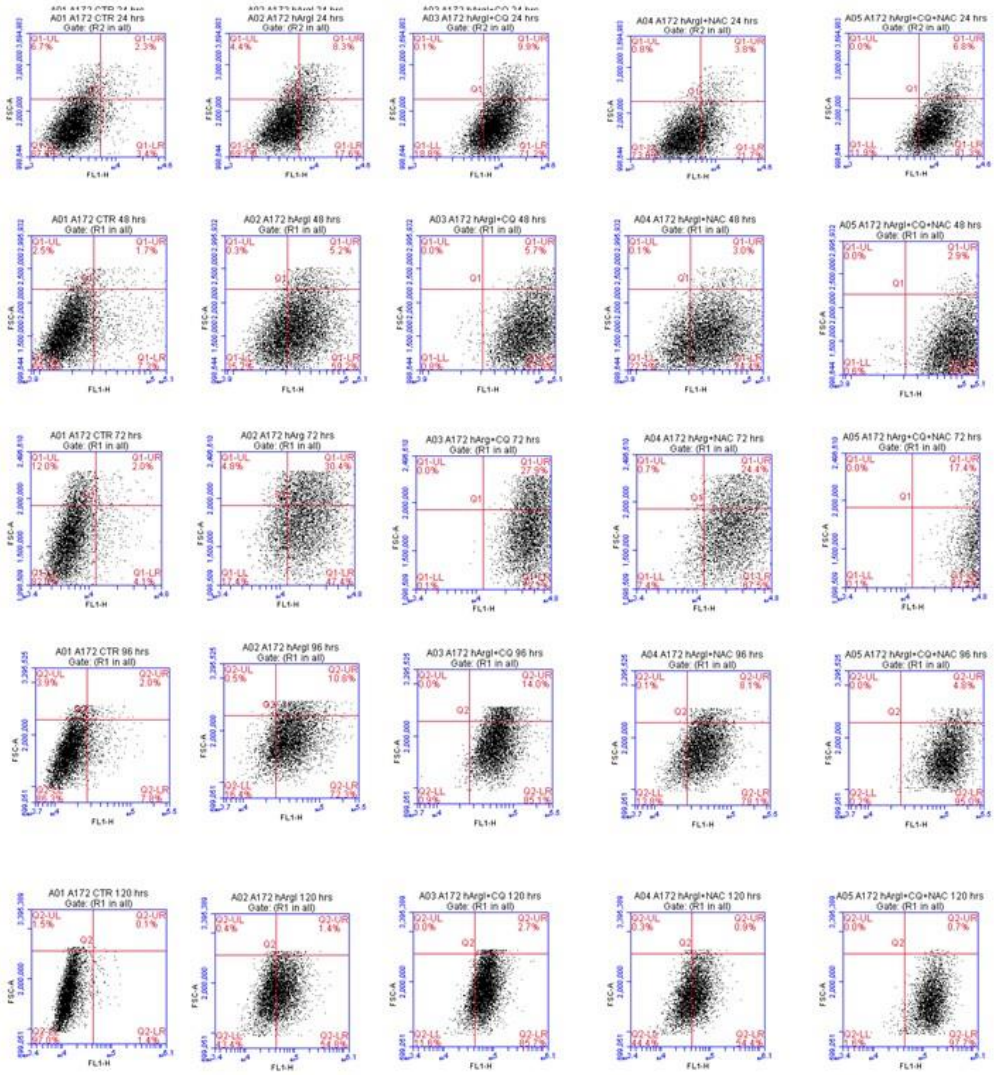

A01 U251 CTR 96 hrs
Gate: (R1 in all)
Count
FL1-H


A01 U251 CTR 96 hrs
Gate: (R1 in all)
Count
FL1-H

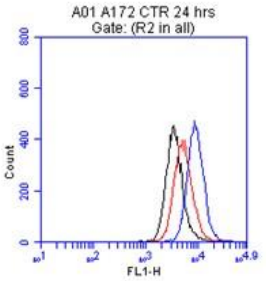

A01 U251 CTR 120 hrs
Gate: (R1 in all)
Count
FL1-H

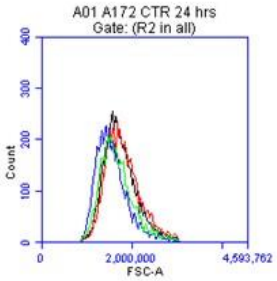

A01 U251 CTR 120 hrs
Gate: (R1 in all)
Count
FL1-H

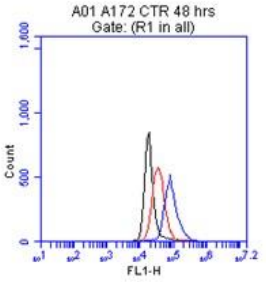
C

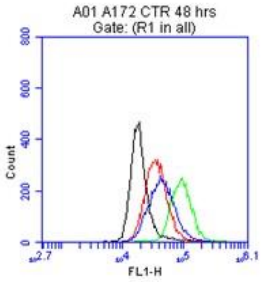

A01 A172 CTR 24 hrs
A02 A172 hArgI 24 hrs
A03 A172 hArgI+CQ 24 hrs
A04 A172 hArgI+NAC 24 hrs
A05 A172 hArgI+CQ+NAC 24 hrs
A01 A172 CTR 48 hrs
A02 A172 hArgI 48 hrs
A03 A172 hArgI+CQ 48 hrs
A04 A172 hArgI+NAC 48 hrs
A05 A172 hArgI+CQ+NAC 48 hrs
A01 A172 CTR 72 hrs
A02 A172 hArg 72 hrs
A03 A172 hArg+CQ 72 hrs
A04 A172 hArg+NAC 72 hrs
A05 A172 hArg+CQ+NAC 72 hrs
A01 A172 CTR 96 hrs
A02 A172 hArgI 96 hrs
A03 A172 hArgI+CQ 96 hrs
A04 A172 hArgI+NAC 96 hrs
A05 A172 hArgI+CQ+NAC 96 hrs
A01 A172 CTR 120 hrs
A02 A172 hArgI 120 hrs
A03 A172 hArgI+CQ 120 hrs
A04 A172 hArgI+NAC 120 hrs
A05 A172 hArgI+CQ+NAC 120 hrs

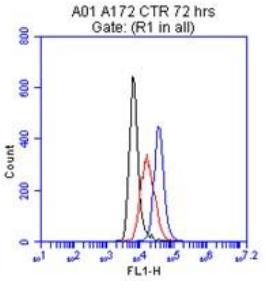
C’

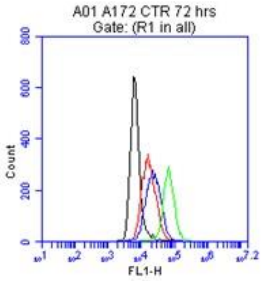

A01 A172 CTR 24 hrs
Gate: (R2 in all)
Count
FL1-H

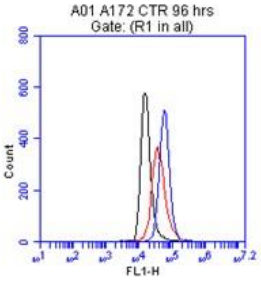

A01 A172 CTR 24 hrs
Gate: (R2 in all)
Count
0
2,000,000
4,593,762
FSC-A

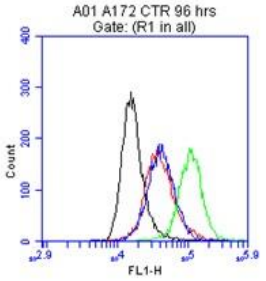

A01 A172 CTR 48 hrs
Gate: (R1 in all)
Count
FL1-H

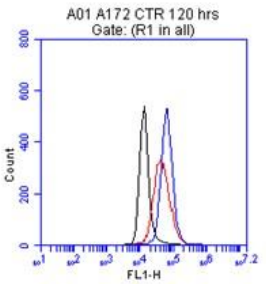

A01 A172 CTR 48 hrs
Gate: (R1 in all)
Count
FL1-H

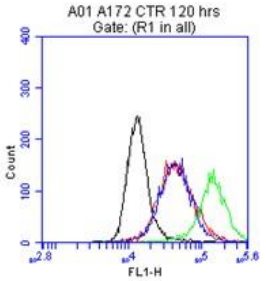

A01 A172 CTR 72 hrs
Gate: (R1 in all)
Count
FL1-H


A01 A172 CTR 72 hrs
Gate: (R1 in all)
Count
FL1-H

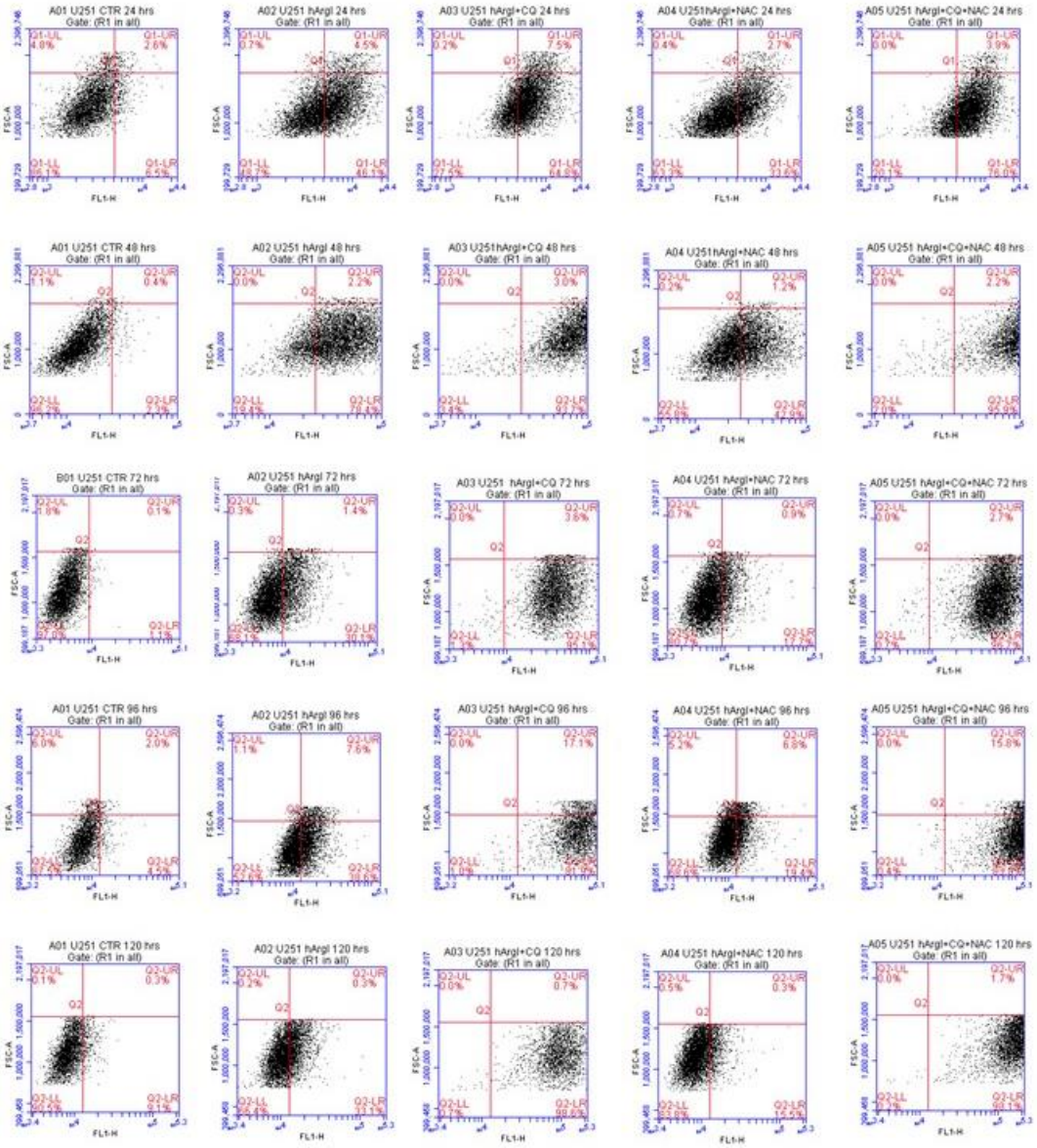

A01 A172 CTR 96 hrs
Gate: (R1 in all)
Count
FL1-H


A01 A172 CTR 96 hrs
Gate: (R1 in all)
Count
FL1-H


A01 A172 CTR 120 hrs
Gate: (R1 in all)
Count
FL1-H


A01 A172 CTR 120 hrs
Gate: (R1 in all)
Count
FL1-H

D


A01 U251 CTR 24 hrs
Gate: (R1 in all)
A02 U251 hArgI 24 hrs
Gate: (R1 in all)
A03 U251 hArgI+CQ 24 hrs
Gate: (R1 in all)
A04 U251hArgI+NAC 24 hrs
Gate: (R1 in all)
A05 U251 hArgI+CQ+NAC 24 hrs
Gate: (R1 in all)
A01 U251 CTR 48 hrs
Gate: (R1 in all)
A02 U251 hArgI 48 hrs
Gate: (R1 in all)
A03 U251hArgI+CQ 48 hrs
Gate: (R1 in all)
A04 U251hArgI+NAC 48 hrs
Gate: (R1 in all)
A05 U251 hArgI+CQ+NAC 48 hrs
Gate: (R1 in all)
B01 U251 CTR 72 hrs
Gate: (R1 in all)
A02 U251 hArgI 72 hrs
Gate: (R1 in all)
A03 U251 hArgI+CQ 72 hrs
Gate: (R1 in all)
A04 U251 hArgI+NAC 72 hrs
Gate: (R1 in all)
A05 U251 hArgI+CQ+NAC 72 hrs
Gate: (R1 in all)
A01 U251 CTR 96 hrs
Gate: (R1 in all)
A02 U251 hArgI 96 hrs
Gate: (R1 in all)
A03 U251 hArgI+CQ 96 hrs
Gate: (R1 in all)
A04 U251 hArgI+NAC 96 hrs
Gate: (R1 in all)
A05 U251 hArgI+CQ+NAC 96 hrs
Gate: (R1 in all)
A01 U251 CTR 120 hrs
Gate: (R1 in all)
A02 U251 hArgI 120 hrs
Gate: (R1 in all)
A03 U251 hArgI+CQ 120 hrs
Gate: (R1 in all)
A04 U251 hArgI+NAC 120 hrs
Gate: (R1 in all)
A05 U251 hArgI+CQ+NAC 120 hrs
Gate: (R1 in all)
FSC-A
FL1-H

D’

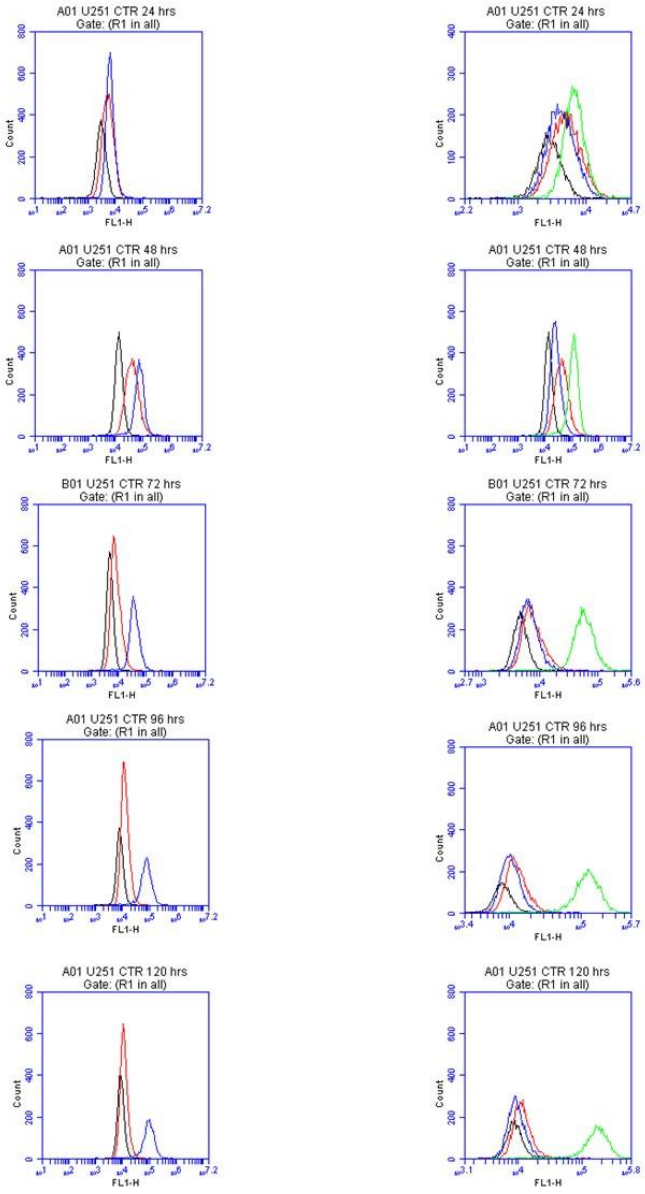


Figure 8 ROS scavenging and autophagosome formation in A172, and U251 cell lines

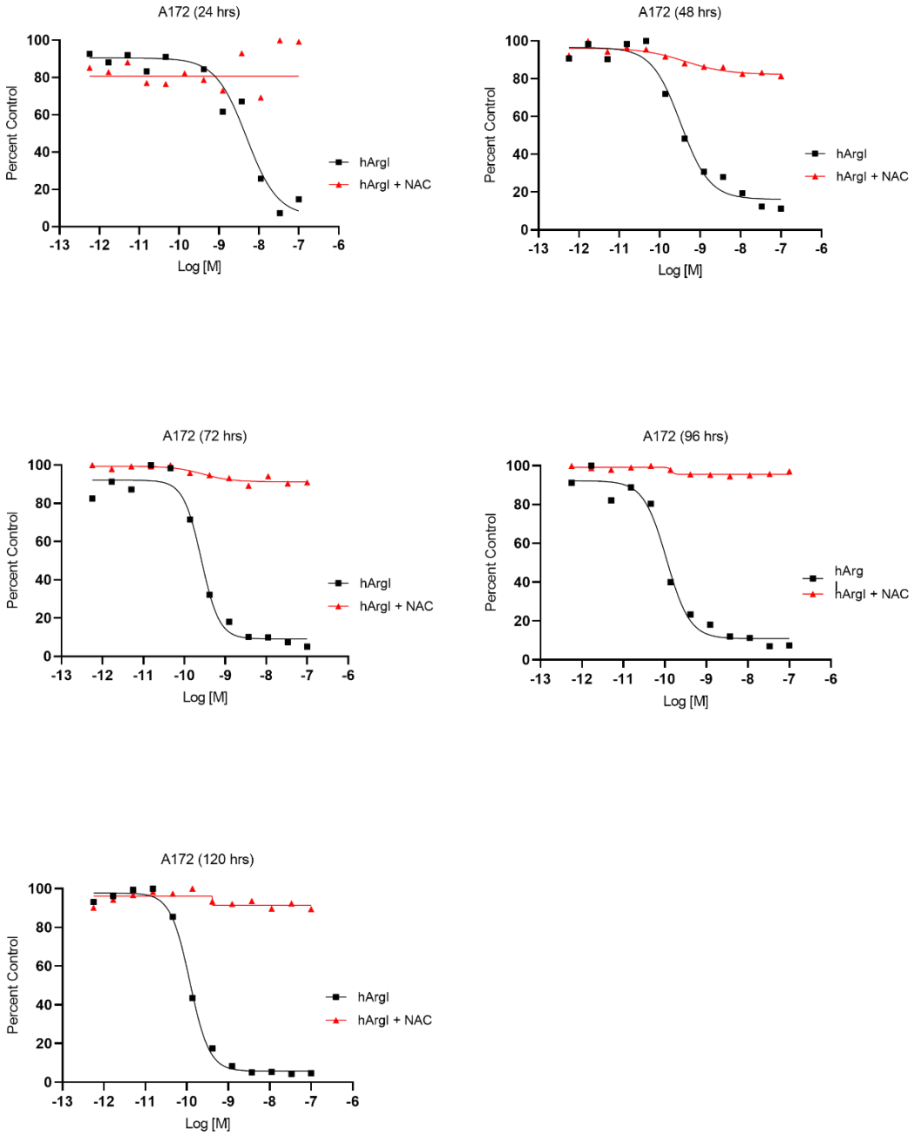
ROS scavenging and autophagosome formation in A172(A, A’), U251(B, B’) cell line with 5mM of NAC vs. A172(C, C’), and U251(D, D’) with NAC concentration being 10 mM: from left to right: control untreated (Black curve), treated with HuArgI (Co)-PEG5000 (10^{-7} M) (Red curve), co-treated with HuArgI (Co)-PEG5000 and NAC or with HuArgI (Co)-PEG5000 and CQ (25 uM) (Blue curve), and cells treated with arginase, NAC, and CQ (Green curve) at 24, 48, 72, 96 and 120 hours after treatment.

This indicates that in U251 cell line, the autophagosome formation is ROS dependent but not in A172 cells.

3.5 Effect of ROS scavenging on the cytotoxicity mediated by HuArgI (Co)-PEG5000 in GBM cells

Adding to that, we then tested the effect of NAC on the autophagic death that is mediated by HuArgI (Co)-PEG5000. We examined the effect of the ROS scavenger; NAC, on the cytotoxicity in both partially (U251) and completely (A172) auxotrophic cell lines at five diverse time points starting from 24 hours and reaching 120 hours post-treatment. When dealing with cells depleted of arginine, NAC is known to be a ROS scavenger, with a concentration of 10^{-7} M. In both U251 and A172 tested cells, upon the co-incubation with N-acetylcysteine (NAC) and HuArgI (Co)-PEG5000, we detected a significant decrease in the two cell lines' sensitivity to the cytotoxic effect of HuArgI (Co)-PEG5000 at early time points as well as late time points when being compared to treated cells with HuArgI (Co)-PEG5000 enzyme only.

A


A172 (24 hrs)
Percent Control
Log [M]
hArgI
hArgI + NAC
A172 (48 hrs)
Percent Control
Log [M]
hArgI
hArgI + NAC
A172 (72 hrs)
Percent Control
Log [M]
hArgI
hArgI + NAC
A172 (96 hrs)
Percent Control
Log [M]
hArg
hArgI + NAC
A172 (120 hrs)
Percent Control
Log [M]
hArgI
hArgI + NAC

B

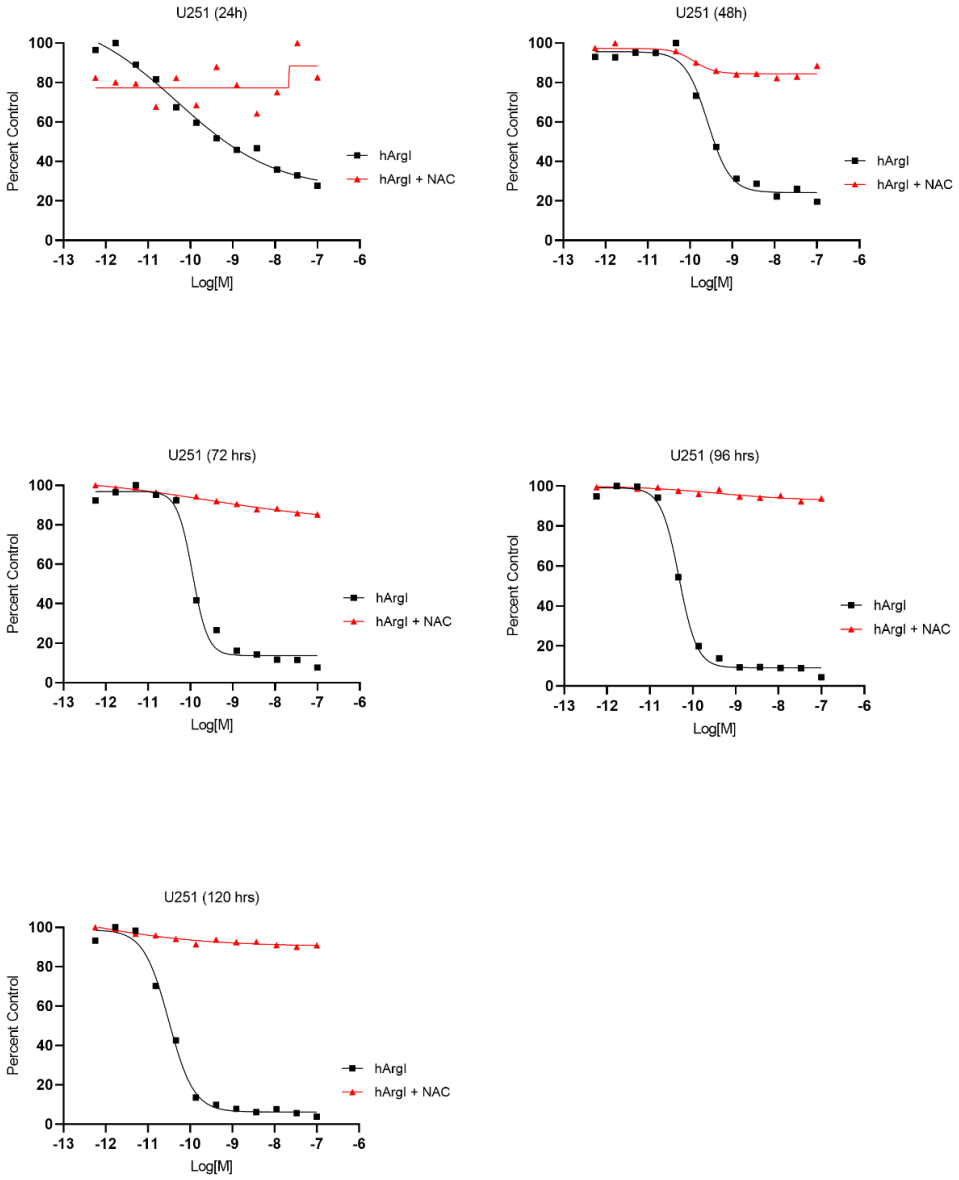


Figure 9 Cytotoxicity of HuArgI (Co)-PEG5000 alone and with NAC to A172, and U251 at 24, 48, 72, 96, and 120 hours after treatment

This dramatic decrease in the sensitivity of the cells to the cytotoxic effect caused by HuArgI (Co)-PEG5000 upon the addition of NAC being a ROS scavenger, shows that ROS is involved in death by autophagy but not necessary in autophagosome formation.

Chapter Four

Discussion

Since GBM is considered to have very low survival rate as mentioned previously, studying new targeted therapies for glioblastoma multiforme is increasing. One of these new therapies is dealing with the depletion of arginine using specific enzymes, and as shown by different studies this therapy is promising when treating GBM (*in vivo*, as well as *in vitro* studies) (Fiedler et al., 2015).

Glioblastoma cell lines' sensitivity to induced arginine starvation by HuArgI(Co)-PEG5000 was confirmed in our lab previously, with some of the cell lines of GBM are considered to be partially auxotrophic for arginine and others are completely auxotrophic depending on ASS-1 expression (Khoury et al., 2015). It has also been confirmed in our lab that different cell lines of GBM have a significant sensitivity to HuArgI (Co)-PEG5000-induced arginine depletion as a long-term effect (not published), showing the vital significance of arginine starvation, caused by HuArgI (Co)-PEG5000, as a novel therapeutical treatment to be targeted in case of GBM.

Saying that, since the activation of autophagy in response to the depletion of arginine is highly examined; in this study, we have shown that the depletion of arginine, mediated by HuArgI (Co)-PEG5000, leads in both GBM cell lines (U251 and A172) to autophagy activation that lasted up to 120 hours following the treatment. After the addition of chloroquine to cells depleted from arginine, an increase in the autophagosome's level was detected in most of the cases. What chloroquine does is that it leads to the interruption of the fusion of autophagosomes with the lysosomes;

thus, the accumulation of autophagosomes due to the blocking of the downstream processing which describes the results that we have obtained. All of this allows for a better detection of the autophagic flux seen in both U251 and A172 cell lines (Mauthe et al., 2018).

Secondly, to see if the cell death taking place is mediated by autophagy, we added chloroquine (inhibits autophagy) and observed its effect on the sensitivity of the GBM cells that are deprived of arginine. In both U251 and A172 cells, we detected an increase in the percentage of the surviving cells. This indicates a decrease in the sensitivity to the deprivation of the amino acid, arginine. Moreover, this shows that the death caused by the HuArgI (Co)-PEG5000 enzyme is due to autophagy (death by autophagy at late time points in GBM cells).

We then showed that upon the addition of L-citrulline, we have a reverse in the activation of autophagy in U251 cells (partially auxotrophic) but not in A172 treated cells (completely auxotrophic), further verifying that autophagy activation is certainly caused by the depletion of arginine. In fact, what happens is that adding the exogenous L-citrulline, via the urea cycle, rescues the cells from arginine depletion and replenishes the levels of arginine in these tumor cells (Plunkett, 2015; Wheatley et al., 2005). This shows that the addition of exogenous L-citrulline can lead to the reverse in the effect caused by HUArgI(Co)-PEG5000 in the partially auxotrophic cells (U251) and not in the completely auxotrophic cell line (A172), and because this was seen then autophagy activation is definitely induced by HuArgI(Co)-PEG5000 enzyme. The same findings were detected by Tanios et al., (2013) upon the same study performed but on AML cells. In the partially auxotrophic cell lines, a reverse in the effect of HuArgI (Co)-PEG5000 upon adding the exogenous source of L-citrulline but not in complete auxotrophic AML cell lines (Tanios et al., 2013).

The role played by autophagy is paradoxical, it can play a protective role or a deleterious one, and this is still not verified with different publications showing one of the two roles played by autophagy in different tumor types following diverse treatments. To explain that, there are some differences detected at the cellular level (heterogeneity) in tumors and the extent of the activation of autophagy (Fulda & Kögel, 2015; Galluzzi et al., 2017). Many studies some of which are from our lab are showing the two opposing roles that autophagy can play and its effect on cell death on different types of tumors (Fulda & Kögel, 2015; Galluzzi et al., 2017; Khalil & Abi-Habib, 2019; Nasreddine et al., 2019).

Adding to that, many studies have shown the ability of different tumor cells being auxotrophic for arginine to have an overexpression in the level of ASS-1 in case of arginine depletion (Delage et al., 2010; Miraki-Moud et al., 2015; Szlosarek et al., 2014). Similar to that, after doing this study we have detected that GBM cells that are partially auxotrophic (U251 cells) show an overexpression in the levels of ASS-1 after 72 hours from being treated by HuArgI(Co)-PEG5000 and reaching 120 hours, and a reversal in the ASS-1 expression was detected (levels lowered to before HuArgI(Co)-PEG5000 treatment) upon the addition of exogenous L-citrulline in partially auxotrophic cells; as a consequence, ASS-1 overexpression was shown to be due to the depletion of arginine caused by HuArgI(Co)-PEG5000 addition.

To further continue, the activation of autophagy, generated by the depletion of arginine might be because of the regulation of ROS (that can interfere in autophagy mechanism) (Xu et al., 2017), we investigated the role of ROS when it comes to activating autophagy in GBM cells incubated with HuArgI(Co)-PEG5000. So, after trying two different concentrations of NAC (5mM vs. 10 mM), ROS scavenger, which leads to the neutralization of ROS, we showed that NAC was able to reverse autophagy

activation in partially auxotrophic GBM cells (U251) depleted from arginine (the effect was clearer when dealing with 10 mM of NAC- dose dependent to fully reverse autophagy in these treated cells), while in A172 cells even the highest concentration of NAC did not show any effect. This indicates that autophagy cell death is dependent on ROS in some arginine deprived cells (partially auxotrophic but not completely auxotrophic cells). Moreover, after the addition of NAC, we detected a significant decrease in the cytotoxicity of both U251 and A172 cells to the cytotoxic effect of HuArgI (Co)-PEG5000 starting from 24 hours and reaching 120 hours after treatment. This indicates, that NAC was able to inhibit autophagy and then it inhibits cell death, verifying that link between autophagy and cell death.

To strengthen our findings, in future studies we have to perform the same experiments on more completely and partially auxotrophic GBM cell lines, as well as using different pegylated arginase enzymes and the mitochondrial isoform of NAC and see if we have the same effects. Furthermore, we need to study the signaling pathway of the activation of autophagy induced by the depletion of arginine. Where we have to detect the possible markers being downstream or upstream of the major autophagy regulator mTOR.

Altogether, we have detected that the death of the GBM cells that is induced by the deprivation of arginine in the presence of HuArgI (Co)-PEG5000 is due to the activation of autophagy, and this might be dependent on the reactive oxygen species. As a consequence, depriving tumor cells of certain amino acids is becoming a promising selective therapy when treating cancer patients, where it was shown in this study that it has a low toxicity and a therapeutical window that is really acceptable.

References

Agnihotri, S., Burrell, K. E., Wolf, A., Jalali, S., Hawkins, C., Rutka, J. T., & Zadeh, G. (2013). Glioblastoma, a Brief Review of History, Molecular Genetics, Animal Models and Novel Therapeutic Strategies. *Archivum Immunologiae et Therapiae Experimentalis*, *61*(1), 25–41. https://doi.org/10.1007/s00005-012-0203-0

Albaugh, V. L., Pinzon-Guzman, C., & Barbul, A. (2017). Arginine—Dual roles as an onconutrient and immunonutrient. *Journal of Surgical Oncology*, *115*(3), 273–280. https://doi.org/10.1002/jso.24490

Alifieris, C., & Trafalis, D. T. (2015). Glioblastoma multiforme: Pathogenesis and treatment. *Pharmacology & Therapeutics*, *152*, 63–82. https://doi.org/10.1016/j.pharmthera.2015.05.005

Al-Koussa, H., Al-Haddad, M., Abi-Habib, R., & El-Sibai, M. (2019). Human Recombinant Arginase I [HuArgI (Co)-PEG5000]-Induced Arginine Depletion Inhibits Colorectal Cancer Cell Migration and Invasion. *International Journal of Molecular Sciences*, *20*(23). https://doi.org/10.3390/ijms20236018

Amaravadi, R. K., Lippincott-Schwartz, J., Yin, X.-M., Weiss, W. A., Takebe, N., Timmer, W., DiPaola, R. S., Lotze, M. T., & White, E. (2011). Principles and Current Strategies for Targeting Autophagy for Cancer Treatment. *Clinical Cancer Research*, *17*(4), 654–666. https://doi.org/10.1158/1078-0432.CCR-10-2634

Anding, A. L., & Baehrecke, E. H. (2017). Cleaning House: Selective Autophagy of Organelles. *Developmental Cell*, *41*(1), 10–22. https://doi.org/10.1016/j.devcel.2017.02.016

Bajikar, S. S., Wang, C.-C., Borten, M. A., Pereira, E. J., Atkins, K. A., & Janes, K. A. (2017). Tumor-Suppressor Inactivation of GDF11 Occurs by Precursor Sequestration in Triple-Negative Breast Cancer. *Developmental Cell*, *43*(4), 418-435.e13. https://doi.org/10.1016/j.devcel.2017.10.027

Bento, C. F., Renna, M., Ghislat, G., Puri, C., Ashkenazi, A., Vicinanza, M., Menzies, F. M., & Rubinsztein, D. C. (2016). Mammalian Autophagy: How Does It Work? *Annual Review of Biochemistry*, *85*(1), 685–713. https://doi.org/10.1146/annurev-biochem-060815-014556

Berg, T. O., Fengsrud, M., Strømhaug, P. E., Berg, T., & Seglen, P. O. (1998). Isolation and Characterization of Rat Liver Amphisomes: EVIDENCE FOR FUSION OF AUTOPHAGOSOMES WITH BOTH EARLY AND LATE ENDOSOMES *. *Journal of Biological Chemistry*, *273*(34), 21883–21892. https://doi.org/10.1074/jbc.273.34.21883

Bhat, P., Kriel, J., Shubha Priya, B., Basappa, Shivananju, N. S., & Loos, B. (2018). Modulating autophagy in cancer therapy: Advancements and challenges for cancer cell death sensitization. *Biochemical Pharmacology*, *147*, 170–182. https://doi.org/10.1016/j.bcp.2017.11.021

Bialik, S., Dasari, S. K., & Kimchi, A. (2018). Autophagy-dependent cell death – where, how and why a cell eats itself to death. *Journal of Cell Science*, *131*(jcs215152). https://doi.org/10.1242/jcs.215152

BioPharmaSpec. (n.d.). PEGylation of Proteins | Protein Characterization Services. *Https://Biopharmaspec.Com/*. Retrieved 2 July 2021, from https://biopharmaspec.com/biopharmaceuticals/pegylated-proteins/

Blair, N. F., Cremer, P. D., & Tchan, M. C. (2015). Urea cycle disorders: A life-threatening yet treatable cause of metabolic encephalopathy in adults. Practical Neurology, 15(1), 45–48. https://doi.org/10.1136/practneurol-2014-000916

Brenner, A. V., Butler, M. A., Wang, S. S., Ruder, A. M., Rothman, N., Schulte, P. A., Chanock, S. J., Fine, H. A., Linet, M. S., & Inskip, P. D. (2007). Single-nucleotide polymorphisms in selected cytokine genes and risk of adult glioma. *Carcinogenesis*, *28*(12), 2543–2547. https://doi.org/10.1093/carcin/bgm210

Changou, C. A., Chen, Y.-R., Xing, L., Yen, Y., Chuang, F. Y. S., Cheng, R. H., Bold, R. J., Ann, D. K., & Kung, H.-J. (2014). Arginine starvation-associated atypical cellular death involves mitochondrial dysfunction, nuclear DNA leakage, and chromatin autophagy. *Proceedings of the National Academy of Sciences*, *111*(39), 14147–14152. https://doi.org/10.1073/pnas.1404171111

Chen, Q., Xie, W., Kuhn, D. J., Voorhees, P. M., Lopez-Girona, A., Mendy, D., Corral, L. G., Krenitsky, V. P., Xu, W., Moutouh-de Parseval, L., Webb, D. R., Mercurio, F., Nakayama, K. I., Nakayama, K., & Orlowski, R. Z. (2008). Targeting the p27 E3 ligase SCFSkp2 results in p27- and Skp2-mediated cell-cycle arrest and activation of autophagy. *Blood*, *111*(9), 4690–4699. https://doi.org/10.1182/blood-2007-09-112904

Cheng, P. N.-M., Lam, T.-L., Lam, W.-M., Tsui, S.-M., Cheng, A. W.-M., Lo, W.-H., & Leung, Y.-C. (2007). Pegylated Recombinant Human Arginase (rhArg-peg5,000mw) Inhibits the In vitro and In vivo Proliferation of Human Hepatocellular Carcinoma through Arginine Depletion. *Cancer Research*, *67*(1), 309–317. https://doi.org/10.1158/0008-5472.CAN-06-1945

Chu, C. T. (2019). Mechanisms of selective autophagy and mitophagy: Implications for neurodegenerative diseases. *Neurobiology of Disease*, *122*, 23–34. https://doi.org/10.1016/j.nbd.2018.07.015

Chung, W. J., Lyons, S. A., Nelson, G. M., Hamza, H., Gladson, C. L., Gillespie, G. Y., & Sontheimer, H. (2005). Inhibition of Cystine Uptake Disrupts the Growth of Primary Brain Tumors. *Journal of Neuroscience*, *25*(31), 7101–7110. https://doi.org/10.1523/JNEUROSCI.5258-04.2005

Cp, H., K, M., La, L.-B., J, Z., R, S., C, V., J, C., & Pc, R. (2010). Pegylated arginase I: A potential therapeutic approach in T-ALL. *Blood*, *115*(25), 5214–5221. https://doi.org/10.1182/blood-2009-12-258822

D'Antonio, E. L., & Christianson, D. W. (2011). Crystal Structures of Complexes with Cobalt-Reconstituted Human Arginase I,. *Biochemistry*, *50*(37), 8018–8027. https://doi.org/10.1021/bi201101t

D'Arcy, M. S. (2019). Cell death: A review of the major forms of apoptosis, necrosis and autophagy. *Cell Biology International*, *43*(6), 582–592. https://doi.org/10.1002/cbin.11137

Darmanis, S., Sloan, S. A., Croote, D., Mignardi, M., Chernikova, S., Samghababi, P., Zhang, Y., Neff, N., Kowarsky, M., Caneda, C., Li, G., Chang, S. D., Connolly, I. D., Li, Y., Barres, B. A., Gephart, M. H., & Quake, S. R. (2017). Single-Cell RNA-Seq Analysis of Infiltrating Neoplastic Cells at the Migrating Front of Human Glioblastoma. *Cell Reports*, *21*(5), 1399–1410. https://doi.org/10.1016/j.celrep.2017.10.030

Davis, M. E. (2018). Epidemiology and Overview of Gliomas. *Seminars in Oncology Nursing*, *34*(5), 420–429. https://doi.org/10.1016/j.soncn.2018.10.001

Deimling, A. von, Louis, D. N., & Wiestler, O. D. (1995). Molecular pathways in the formation of gliomas. *Glia*, *15*(3), 328–338. https://doi.org/10.1002/glia.440150312

Delage, B., Fennell, D. A., Nicholson, L., McNeish, I., Lemoine, N. R., Crook, T., & Szlosarek, P. W. (2010). Arginine deprivation and argininosuccinate synthetase expression in the treatment of cancer. *International Journal of Cancer*, *126*(12), 2762–2772. https://doi.org/10.1002/ijc.25202

Denton, D., & Kumar, S. (2019). Autophagy-dependent cell death. *Cell Death & Differentiation*, *26*(4), 605–616. https://doi.org/10.1038/s41418-018-0252-y

Denysenko, T., Gennero, L., Roos, M. A., Melcarne, A., Juenemann, C., Faccani, G., Morra, I., Cavallo, G., Reguzzi, S., Pescarmona, G., & Ponzetto, A. (2010). Glioblastoma cancer stem cells: Heterogeneity, microenvironment and related therapeutic strategies. *Cell Biochemistry and Function*, *28*(5), 343–351. https://doi.org/10.1002/cbf.1666

Dolan, M. E., Moschel, R. C., & Pegg, A. E. (1990). Depletion of mammalian O6-alkylguanine-DNA alkyltransferase activity by O6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proceedings of the National Academy of Sciences*, *87*(14), 5368–5372. https://doi.org/10.1073/pnas.87.14.5368

Engel, A. L., Lorenz, N. I., Klann, K., Münch, C., Depner, C., Steinbach, J. P., Ronellenfitsch, M. W., & Luger, A.-L. (2020). Serine-dependent redox homeostasis regulates glioblastoma cell survival. *British Journal of Cancer*, *122*(9), 1391–1398. https://doi.org/10.1038/s41416-020-0794-x

England, B., Huang, T., & Karsy, M. (2013). Current understanding of the role and targeting of tumor suppressor p53 in glioblastoma multiforme. *Tumor Biology*, *34*(4), 2063–2074. https://doi.org/10.1007/s13277-013-0871-3

Ensor, C. M., Holtsberg, F. W., Bomalaski, J. S., & Clark, M. A. (2002). Pegylated Arginine Deiminase (ADI-SS PEG20,000 mw) Inhibits Human Melanomas and Hepatocellular Carcinomas in Vitro and in Vivo. *Cancer Research*, *62*(19), 5443–5450.

Essick, E. E., & Sam, F. (2010). Oxidative Stress and Autophagy in Cardiac Disease, Neurological Disorders, Aging and Cancer. *Oxidative Medicine and Cellular Longevity*, *3*(3), 168–177. https://doi.org/10.4161/oxim.3.3.12106

Fiedler, T., Strauss, M., Hering, S., Redanz, U., William, D., Rosche, Y., Classen, C. F., Kreikemeyer, B., Linnebacher, M., & 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. https://doi.org/10.1080/15384047.2015.1026478

Fernandes, H. S., Teixeira, C. S. S., Fernandes, P. A., Ramos, M. J., & Cerqueira, N. M. F. S. A. (2017). Amino acid deprivation using enzymes as a targeted therapy for cancer and viral infections. *Expert Opinion on Therapeutic Patents*, *27*(3), 283–297. https://doi.org/10.1080/13543776.2017.1254194

Feun, L., You, M., Wu, C. J., Kuo, M. T., Wangpaichitr, M., Spector, S., & Savaraj, N. (2008). Arginine Deprivation as a Targeted Therapy for Cancer. *Current Pharmaceutical Design*, *14*(11), 1049–1057.

Fine, R. L., Fogelman, D. R., & Schreibman, S. M. (2005). Effective treatment of neuroendocrine tumors With temozolomide and capecitabine. *Journal of Clinical Oncology*, *23*(16_suppl), 4216–4216. https://doi.org/10.1200/jco.2005.23.16_suppl.4216

Frank, M., Duvezin-Caubet, S., Koob, S., Occhipinti, A., Jagasia, R., Petcherski, A., Ruonala, M. O., Priault, M., Salin, B., & Reichert, A. S. (2012). Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1823(12), 2297–2310. https://doi.org/10.1016/j.bbamcr.2012.08.007

Fujita, M., Kohanbash, G., Fellows-Mayle, W., Hamilton, R. L., Komohara, Y., Decker, S. A., Ohlfest, J. R., & Okada, H. (2011). COX-2 Blockade Suppresses Gliomagenesis by Inhibiting Myeloid-Derived Suppressor Cells. *Cancer Research*, *71*(7), 2664–2674. https://doi.org/10.1158/0008-5472.CAN-10-3055

Fulda, S., & Kögel, D. (2015). Cell death by autophagy: Emerging molecular mechanisms and implications for cancer therapy. *Oncogene*, *34*(40), 5105–5113. https://doi.org/10.1038/onc.2014.458

Fung, M. K. L., & Chan, G. C.-F. (2017). Drug-induced amino acid deprivation as strategy for cancer therapy. *Journal of Hematology & Oncology*, *10*(1), 144. https://doi.org/10.1186/s13045-017-0509-9

Furnari, F. B., Fenton, T., Bachoo, R. M., Mukasa, A., Stommel, J. M., Stegh, A., Hahn, W. C., Ligon, K. L., Louis, D. N., Brennan, C., Chin, L., DePinho, R. A., & Cavenee, W. K. (2007). Malignant astrocytic glioma: Genetics, biology, and paths to treatment. *Genes & Development*, *21*(21), 2683–2710. https://doi.org/10.1101/gad.1596707

Galli, R., Binda, E., Orfanelli, U., Cipelletti, B., Gritti, A., Vitis, S. D., Fiocco, R., Foroni, C., Dimeco, F., & Vescovi, A. (2004). Isolation and Characterization of Tumorigenic, Stem-like Neural Precursors from Human Glioblastoma. *Cancer Research*, *64*(19), 7011–7021. https://doi.org/10.1158/0008-5472.CAN-04-1364

Galluzzi, L., Baehrecke, E. H., Ballabio, A., Boya, P., Bravo-San Pedro, J. M., Cecconi, F., Choi, A. M., Chu, C. T., Codogno, P., Colombo, M. I., Cuervo, A. M., Debnath, J., Deretic, V., Dikic, I., Eskelinen, E.-L., Fimia, G. M., Fulda, S., Gewirtz, D. A., Green, D. R., … Kroemer, G. (2017). Molecular definitions of autophagy and related processes. *The EMBO Journal*, *36*(13), 1811–1836. https://doi.org/10.15252/embj.201796697

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., Andrews, D. W., Annicchiarico-Petruzzelli, M., Antonov, A. V., Arama, E., Baehrecke, E. H., Barlev, N. A., Bazan, N. G., Bernassola, F., Bertrand, M. J. M., Bianchi, K., … Kroemer, G. (2018). Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death & Differentiation*, *25*(3), 486–541. https://doi.org/10.1038/s41418-017-0012-4

Gatica, D., Lahiri, V., & Klionsky, D. J. (2018). Cargo recognition and degradation by selective autophagy. *Nature Cell Biology*, *20*(3), 233–242. https://doi.org/10.1038/s41556-018-0037-z

Geck, R. C., & Toker, A. (2016). Nonessential amino acid metabolism in breast cancer. *Advances in Biological Regulation*, *62*, 11–17. https://doi.org/10.1016/j.jbior.2016.01.001

Gersey, Z., Osiason, A. D., Bloom, L., Shah, S., Thompson, J. W., Bregy, A., Agarwal, N., & Komotar, R. J. (2019). Therapeutic Targeting of the Notch Pathway in Glioblastoma Multiforme. *World Neurosurgery*, *131*, 252-263.e2. https://doi.org/10.1016/j.wneu.2019.07.180

Gilbert, M. R., Dignam, J. J., Armstrong, T. S., Wefel, J. S., Blumenthal, D. T., Vogelbaum, M. A., Colman, H., Chakravarti, A., Pugh, S., Won, M., Jeraj, R., Brown, P. D., Jaeckle, K. A., Schiff, D., Stieber, V. W., Brachman, D. G., Werner-Wasik, M., Tremont-Lukats, I. W., Sulman, E. P., … Mehta, M. P. (2014). A Randomized Trial of Bevacizumab for Newly Diagnosed Glioblastoma. *New England Journal of Medicine*, *370*(8), 699–708. https://doi.org/10.1056/NEJMoa1308573

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.

Gruber, M. L., & Buster, W. P. (2004). Temozolomide in Combination With Irinotecan for Treatment of Recurrent Malignant Glioma. *American Journal of Clinical Oncology*, *27*(1), 33–38. https://doi.org/10.1097/01.coc.0000045852.88461.80

Harris, J. M., & Chess, R. B. (2003). Effect of pegylation on pharmaceuticals. *Nature Reviews Drug Discovery*, *2*(3), 214–221. https://doi.org/10.1038/nrd1033

He, L., Zhang, J., Zhao, J., Ma, N., Kim, S. W., Qiao, S., & Ma, X. (2018). Autophagy: The Last Defense against Cellular Nutritional Stress. *Advances in Nutrition*, *9*(4), 493–504. https://doi.org/10.1093/advances/nmy011

Hegi, M. E., Diserens, A. C., Gorlia, T., Hamou, M. F., Tribolet, N. D., Weller, M., Kros, J. M., Hainfellner, J. A., Mason, W., Mariani, L., Bromberg, J. E. C., Hau, P., Mirimanoff, R. O., Cairncross, J. G., Janzer, R. C., & Stupp, R. (2005). MGMT gene silencing and benefit from temozolomide in glioblastoma. *New England Journal of Medicine*, *352*(10), 997–1003. https://doi.org/10.1056/NEJMoa043331

Hsueh, E. C., Knebel, S. M., Lo, W.-H., Leung, Y.-C., Cheng, P. N.-M., & Hsueh, C.-T. (2012). Deprivation of arginine by recombinant human arginase in prostate cancer cells. *Journal of Hematology & Oncology*, *5*(1), 17. https://doi.org/10.1186/1756-8722-5-17

Hui-Wen, L. (2009). EGFR-Targeted Therapy in Malignant Glioma: Novel Aspects and Mechanisms of Drug Resistance. *Current Molecular Pharmacology*, *3*(1), 37–52.

Husson, A., Brasse-Lagnel, C., Fairand, A., Renouf, S., & Lavoinne, A. (2003). Argininosuccinate synthetase from the urea cycle to the citrulline–NO cycle. *European Journal of Biochemistry*, *270*(9), 1887–1899. https://doi.org/10.1046/j.1432-1033.2003.03559.x

Jahani, M., Noroznezhad, F., & Mansouri, K. (2018). Arginine: Challenges and opportunities of this two-faced molecule in cancer therapy. *Biomedicine & Pharmacotherapy*, *102*, 594–601. https://doi.org/10.1016/j.biopha.2018.02.109

Jihong, Z., Malcolm, F. G. S., & Tracey, D. B. (2011). Temozolomide: Mechanisms of Action, Repair and Resistance. *Current Molecular Pharmacology*, *5*(1), 102–114.

K, I., Ee, S., Hm, G., & Vp, C. (1996). Human glioblastomas with no alterations of the CDKN2A (p16INK4A, MTS1) and CDK4 genes have frequent mutations of the retinoblastoma gene. *Oncogene*, *13*(5), 1065–1072.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., & Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO Journal*, *19*(21), 5720–5728. https://doi.org/10.1093/emboj/19.21.5720

Karachi, A., Dastmalchi, F., Mitchell, D. A., & Rahman, M. (2018). Temozolomide for immunomodulation in the treatment of glioblastoma. *Neuro-Oncology*, *20*(12), 1566–1572. https://doi.org/10.1093/neuonc/noy072

Khalil, N., & Abi-Habib, R. J. (2020). [HuArgI (co)-PEG5000]-induced arginine deprivation leads to autophagy dependent cell death in pancreatic cancer cells. *Investigational New Drugs*, *38*(5), 1236–1246. https://doi.org/10.1007/s10637-019-00883-4

Khasraw, M., & Lassman, A. B. (2010). Advances in the Treatment of Malignant Gliomas. *Current Oncology Reports*, *12*(1), 26–33. https://doi.org/10.1007/s11912-009-0077-4

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. https://doi.org/10.1007/s11060-014-1698-5

Kim, T.-G., Kim, C.-H., Park, J.-S., Park, S.-D., Kim, C. K., Chung, D.-S., & Hong, Y.-K. (2010). Immunological Factors Relating to the Antitumor Effect of Temozolomide Chemoimmunotherapy in a Murine Glioma Model. *Clinical and Vaccine Immunology*, *17*(1), 143–153. https://doi.org/10.1128/CVI.00292-09

Kleihues, P., & Ohgaki, H. (1999). Primary and secondary glioblastomas: From concept to clinical diagnosis. *Neuro-Oncology*, *1*(1), 44–51. https://doi.org/10.1093/neuonc/1.1.44

Kondo, Y., Kanzawa, T., Sawaya, R., & Kondo, S. (2005). The role of autophagy in cancer development and response to therapy. *Nature Reviews Cancer*, *5*(9), 726–734. https://doi.org/10.1038/nrc1692

Krebs, H. A., Hems, R., & Lund, P. (1973). Accumulation of amino acids by the perfused rat liver in the presence of ethanol. *Biochemical Journal*, *134*(3), 697–705. https://doi.org/10.1042/bj1340697

Lang, F. F., Miller, D. C., Pisharody, S., Koslow, M., & Newcomb, E. W. (1994). High frequency of p53 protein accumulation without p53 gene mutation in human juvenile pilocytic, low grade and anaplastic astrocytomas. *Oncogene*, *9*(3), 949–954.

Lemasters, J. J. (2005). Selective Mitochondrial Autophagy, or Mitophagy, as a Targeted Defense Against Oxidative Stress, Mitochondrial Dysfunction, and Aging. *Rejuvenation Research*, *8*(1), 3–5. https://doi.org/10.1089/rej.2005.8.3

Levine, B., & Kroemer, G. (2008). Autophagy in the Pathogenesis of Disease. *Cell*, *132*(1), 27–42. https://doi.org/10.1016/j.cell.2007.12.018

Levy, J. M. M., Towers, C. G., & Thorburn, A. (2017). Targeting autophagy in cancer. *Nature Reviews Cancer*, *17*(9), 528–542. https://doi.org/10.1038/nrc.2017.53

Li, L., Tan, J., Miao, Y., Lei, P., & Zhang, Q. (2015). ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms. Cellular and Molecular Neurobiology, 35(5), 615–621. https://doi.org/10.1007/s10571-015-0166-x

Li, Z., Guo, J., Ma, Y., Zhang, L., & Lin, Z. (2018). Oncogenic Role of MicroRNA-30b-5p in Glioblastoma Through Targeting Proline-Rich Transmembrane Protein 2. *Oncology Research*, *26*(2), 219–230. https://doi.org/10.3727/096504017X14944585873659

Lian, J., Wu, X., He, F., Karnak, D., Tang, W., Meng, Y., Xiang, D., Ji, M., Lawrence, T. S., & Xu, L. (2011). A natural BH3 mimetic induces autophagy in apoptosis-resistant prostate cancer via modulating Bcl-2–Beclin1 interaction at endoplasmic reticulum. *Cell Death & Differentiation*, *18*(1), 60–71. https://doi.org/10.1038/cdd.2010.74

Liu, E. K., Sulman, E. P., Wen, P. Y., & Kurz, S. C. (2020). Novel Therapies for Glioblastoma. *Current Neurology and Neuroscience Reports*, *20*(7), 19. https://doi.org/10.1007/s11910-020-01042-6

Liu, J., Albrecht, A. M., Ni, X., Yang, J., & Li, M. (2013). Glioblastoma tumor initiating cells: Therapeutic strategies targeting apoptosis and microRNA pathways. *Current Molecular Medicine*, *13*(3), 352–357.

Liu, J., Ma, J., Wu, Z., Li, W., Zhang, D., Han, L., Wang, F., Reindl, K. M., Wu, E., & Ma, Q. (2014). Arginine deiminase augments the chemosensitivity of argininosuccinate synthetase-deficient pancreatic cancer cells to gemcitabine via inhibition of NF-κB signaling. *BMC Cancer*, *14*(1), 686. https://doi.org/10.1186/1471-2407-14-686

Long, Y., Tsai, W.-B., Wang, D., Hawke, D. H., Savaraj, N., Feun, L. G., Hung, M.-C., Chen, H. H. W., & Kuo, M. T. (2017). Argininosuccinate synthetase 1 (ASS1) is a common metabolic marker of chemosensitivity for targeted arginine- and glutamine-starvation therapy. *Cancer Letters*, *388*, 54–63. https://doi.org/10.1016/j.canlet.2016.11.028

Louis, D. N., Ohgaki, H., Wiestler, O. D., Cavenee, W. K., Burger, P. C., Jouvet, A., Scheithauer, B. W., & Kleihues, P. (2007). The 2007 WHO Classification of Tumours of the Central Nervous System. *Acta Neuropathologica*, *114*(2), 97–109. https://doi.org/10.1007/s00401-007-0243-4

Lukashchuk, N., & Vousden, K. H. (2007). Ubiquitination and Degradation of Mutant p53. *Molecular and Cellular Biology*, *27*(23), 8284–8295. https://doi.org/10.1128/MCB.00050-07

Marin, J. J. G., Lozano, E., & Perez, M. J. (2016). Lack of mitochondrial DNA impairs chemical hypoxia-induced autophagy in liver tumor cells through ROS-AMPK-ULK1 signaling dysregulation independently of HIF-1α. *Free Radical Biology and Medicine*, *101*, 71–84. https://doi.org/10.1016/j.freeradbiomed.2016.09.025

Maugeri-Saccà, M., di Martino, S., & De Maria, R. (2013). Biological and clinical implications of cancer stem cells in primary brain tumors. *Frontiers in Oncology*, *3*. https://doi.org/10.3389/fonc.2013.00006

Mauldin, J. P., Zeinali, I., Kleypas, K., Woo, J. H., Blackwood, R. S., Jo, C.-H., Stone, E. M., Georgiou, G., & Frankel, A. E. (2012). Recombinant Human Arginase Toxicity in Mice Is Reduced by Citrulline Supplementation. *Translational Oncology*, *5*(1), 26–31.

Mauthe, M., Orhon, I., Rocchi, C., Zhou, X., Luhr, M., Hijlkema, K.-J., Coppes, R. P., Engedal, N., Mari, M., & Reggiori, F. (2018). Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy*, *14*(8), 1435–1455. https://doi.org/10.1080/15548627.2018.1474314

McLendon, R., Friedman, A., Bigner, D., Van Meir, E. G., Brat, D. J., M. Mastrogianakis, G., Olson, J. J., Mikkelsen, T., Lehman, N., Aldape, K., Alfred Yung, W. K., Bogler, O., VandenBerg, S., Berger, M., Prados, M., Muzny, D., Morgan, M., Scherer, S., Sabo, A., … National Human Genome Research Institute. (2008). Comprehensive

genomic characterization defines human glioblastoma genes and core pathways. *Nature*, *455*(7216), 1061–1068. https://doi.org/10.1038/nature07385

Messali, A., Villacorta, R., & Hay, J. W. (2014). A Review of the Economic Burden of Glioblastoma and the Cost Effectiveness of Pharmacologic Treatments. *PharmacoEconomics*, *32*(12), 1201–1212. https://doi.org/10.1007/s40273-014-0198-y

Meyer, M., Reimand, J., Lan, X., Head, R., Zhu, X., Kushida, M., Bayani, J., Pressey, J. C., Lionel, A. C., Clarke, I. D., Cusimano, M., Squire, J. A., Scherer, S. W., Bernstein, M., Woodin, M. A., Bader, G. D., & Dirks, P. B. (2015). Single cell-derived clonal analysis of human glioblastoma links functional and genomic heterogeneity. *Proceedings of the National Academy of Sciences*, *112*(3), 851–856. https://doi.org/10.1073/pnas.1320611111

Miraki-Moud, F., Ghazaly, E., Ariza-McNaughton, L., Hodby, K. A., Clear, A., Anjos-Afonso, F., Liapis, K., Grantham, M., Sohrabi, F., Cavenagh, J., Bomalaski, J. S., Gribben, J. G., Szlosarek, P. W., Bonnet, D., & Taussig, D. C. (2015). Arginine deprivation using pegylated arginine deiminase has activity against primary acute myeloid leukemia cells in vivo. *Blood*, *125*(26), 4060–4068. https://doi.org/10.1182/blood-2014-10-608133

Mischel, P. S., & Cloughesy, T. F. (2003). Targeted Molecular Therapy of GBM. *Brain Pathology*, *13*(1), 52–61. https://doi.org/10.1111/j.1750-3639.2003.tb00006.x

Mizushima, N., & Klionsky, D. J. (2007). Protein Turnover Via Autophagy: Implications for Metabolism. *Annual Review of Nutrition*, *27*(1), 19–40. https://doi.org/10.1146/annurev.nutr.27.061406.093749

Mizushima, N., & Komatsu, M. (2011). Autophagy: Renovation of Cells and Tissues. *Cell*, *147*(4), 728–741. https://doi.org/10.1016/j.cell.2011.10.026

Morris, S. M., Bhamidipati, D., & Kepka-Lenhart, D. (1997). Human type II arginase: Sequence analysis and tissue-specific expression. *Gene*, *193*(2), 157–161. https://doi.org/10.1016/S0378-1119(97)00099-1

Morris, S. M., Jr. (2016). Arginine Metabolism Revisited. *The Journal of Nutrition*, *146*(12), 2579S-2586S. https://doi.org/10.3945/jn.115.226621

Müller, S., Kohanbash, G., Liu, S. J., Alvarado, B., Carrera, D., Bhaduri, A., Watchmaker, P. B., Yagnik, G., Di Lullo, E., Malatesta, M., Amankulor, N. M., Kriegstein, A. R., Lim, D. A., Aghi, M., Okada, H., & Diaz, A. (2017). Single-cell profiling of human gliomas reveals macrophage ontogeny as a basis for regional differences in macrophage activation in the tumor microenvironment. *Genome Biology*, *18*(1), 234. https://doi.org/10.1186/s13059-017-1362-4

Mussai, F., Egan, S., Higginbotham-Jones, J., Perry, T., Beggs, A., Odintsova, E., Loke, J., Pratt, G., U, K. P., Lo, A., Ng, M., Kearns, P., Cheng, P., & De Santo, C. (2015). Arginine dependence of acute myeloid leukemia blast proliferation: A novel therapeutic target. *Blood*, *125*(15), 2386–2396. https://doi.org/10.1182/blood-2014-09-600643

Nakamura, S., & Yoshimori, T. (2017). New insights into autophagosome-lysosome fusion. *Journal of Cell Science*, *130*(7), 1209–1216. https://doi.org/10.1242/jcs.196352

Nasreddine, G., El-Sibai, M., & Abi-Habib, R. J. (2020). Cytotoxicity of [HuArgI (co)-PEG5000]-induced arginine deprivation to ovarian Cancer cells is autophagy dependent. *Investigational New Drugs*, *38*(1), 10–19. https://doi.org/10.1007/s10637-019-00756-w

Nieder, C., Grosu, A. L., Astner, S., & Molls, M. (2005). Treatment of Unresectable Glioblastoma Multiforme. *Anticancer Research*, *25*(6C), 4605–4610.

Ohgaki, H., & Kleihues, P. (2013). The Definition of Primary and Secondary Glioblastoma. *Clinical Cancer Research*, *19*(4), 764–772. https://doi.org/10.1158/1078-0432.CCR-12-3002

Onorati, A. V., Dyczynski, M., Ojha, R., & Amaravadi, R. K. (2018). Targeting autophagy in cancer. *Cancer*, *124*(16), 3307–3318. https://doi.org/10.1002/cncr.31335

Ostrom, Q. T., Gittleman, H., Liao, P., Vecchione-Koval, T., Wolinsky, Y., Kruchko, C., & Barnholtz-Sloan, J. S. (2017). CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010–2014. *Neuro-Oncology*, *19*(suppl_5), v1–v88. https://doi.org/10.1093/neuonc/nox158

Ostrom, Q. T., Gittleman, H., Stetson, L., Virk, S. M., & Barnholtz-Sloan, J. S. (2015). Epidemiology of Gliomas. In J. Raizer & A. Parsa (Eds.), *Current Understanding and Treatment of Gliomas* (pp. 1–14). Springer International Publishing. https://doi.org/10.1007/978-3-319-12048-5_1

P, W., & D, C. (2018). WHO 2016 Classification of gliomas. *Neuropathology and Applied Neurobiology*, *44*(2), 139–150. https://doi.org/10.1111/nan.12432

Palikaras, K., Lionaki, E., & Tavernarakis, N. (2018). Mechanisms of mitophagy in cellular homeostasis, physiology and pathology. *Nature Cell Biology*, *20*(9), 1013–1022. https://doi.org/10.1038/s41556-018-0176-2

Pang, B., Xu, J., Hu, J., Guo, F., Wan, L., Cheng, M., & Pang, L. (2019). Single-cell RNA-seq reveals the invasive trajectory and molecular cascades underlying glioblastoma progression. *Molecular Oncology*, *13*(12), 2588–2603. https://doi.org/10.1002/1878-0261.12569

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. https://doi.org/10.1186/s12885-017-3148-1

Park, S.-H., Won, J., Kim, S.-I., Lee, Y., Park, C.-K., Kim, S.-K., & Choi, S.-H. (2017). Molecular Testing of Brain Tumor. *Journal of Pathology and Translational Medicine*, *51*(3), 205–223. https://doi.org/10.4132/jptm.2017.03.08

Pavlova, N. N., & Thompson, C. B. (2016). The Emerging Hallmarks of Cancer Metabolism. *Cell Metabolism*, *23*(1), 27–47. https://doi.org/10.1016/j.cmet.2015.12.006

Pavlyk, I., Rzhepetskyy, Y., Jagielski, A. K., Drozak, J., Wasik, A., Pereverzieva, G., Olchowik, M., Kunz-Schugart, L. A., Stasyk, O., & 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. https://doi.org/10.1007/s00726-014-1857-1

Plunkett, W. (2015). Arginine addiction in AML. *Blood*, *125*(26), 3971–3972. https://doi.org/10.1182/blood-2015-05-643411

Preusser, M., Ribaupierre, S. de, Wöhrer, A., Erridge, S. C., Hegi, M., Weller, M., & Stupp, R. (2011). Current concepts and management of glioblastoma. *Annals of Neurology*, *70*(1), 9–21. https://doi.org/10.1002/ana.22425

Prognosis | CERN Foundation. (n.d.). Retrieved 27 May 2021, from https://www.cern-foundation.org/education/diagnosis/prognosis

Przystal, J. M., Hajji, N., Khozoie, C., Renziehausen, A., Zeng, Q., Abaitua, F., Hajitou, A., Suwan, K., Want, E., Bomalaski, J., Szlosarek, P., O'Neill, K., Crook, T., & Syed, N. (2018). Efficacy of arginine depletion by ADI-PEG20 in an intracranial model of GBM. *Cell Death and Disease*, *9*(12). https://ora.ox.ac.uk/objects/uuid:b9ffe66a-dbec-4317-8100-bcdbef1b70e3

Puram, S. V., Tirosh, I., Parikh, A. S., Patel, A. P., Yizhak, K., Gillespie, S., Rodman, C., Luo, C. L., Mroz, E. A., Emerick, K. S., Deschler, D. G., Varvares, M. A., Mylvaganam, R., Rozenblatt-Rosen, O., Rocco, J. W., Faquin, W. C., Lin, D. T., Regev, A., & Bernstein, B. E. (2017). Single-Cell Transcriptomic Analysis of Primary and Metastatic Tumor Ecosystems in Head and Neck Cancer. *Cell*, *171*(7), 1611-1624.e24. https://doi.org/10.1016/j.cell.2017.10.044

Quick Brain Tumor Facts. (n.d.). National Brain Tumor Society. Retrieved 27 May 2021, from https://braintumor.org/brain-tumor-information/brain-tumor-facts/

Ravanan, P., Srikumar, I. F., & Talwar, P. (2017). Autophagy: The spotlight for cellular stress responses. *Life Sciences*, *188*, 53–67. https://doi.org/10.1016/j.lfs.2017.08.029

Reifenberger, G., & Collins, V. P. (2004). Pathology and molecular genetics of astrocytic gliomas. *Journal of Molecular Medicine (Berlin, Germany)*, *82*(10), 656–670. https://doi.org/10.1007/s00109-004-0564-x

Sanchez-Perez, L. A., Choi, B. D., Archer, G. E., Cui, X., Flores, C., Johnson, L. A., Schmittling, R. J., Snyder, D., Ii, J. E. H., Bigner, D. D., Mitchell, D. A., & Sampson, J. H. (2013). Myeloablative Temozolomide Enhances CD8+ T-Cell Responses to Vaccine and Is Required for Efficacy against Brain Tumors in Mice. *PLOS ONE*, *8*(3), e59082. https://doi.org/10.1371/journal.pone.0059082

Sarkar, C., Jain, A., & Suri, V. (2009). Current concepts in the pathology and genetics of gliomas. *Indian Journal of Cancer*, *46*(2), 108. https://doi.org/10.4103/0019-509X.49148

Scheurer, M. E., Amirian, E. S., Davlin, S. L., Rice, T., Wrensch, M., & Bondy, M. L. (2011). Effects of antihistamine and anti-inflammatory medication use on risk of specific glioma histologies. *International Journal of Cancer*, *129*(9), 2290–2296. https://doi.org/10.1002/ijc.25883

Schwartzbaum, J. A., Fisher, J. L., Aldape, K. D., & Wrensch, M. (2006). Epidemiology and molecular pathology of glioma. *Nature Clinical Practice. Neurology*, *2*(9), 494–503; quiz 1 p following 516. https://doi.org/10.1038/ncpneuro0289

Seliger, C., Meier, C. R., Becker, C., Jick, S. S., Bogdahn, U., Hau, P., & Leitzmann, M. F. (2016). Use of Selective Cyclooxygenase-2 Inhibitors, Other Analgesics, and Risk of Glioma. *PLoS ONE*, *11*(2). https://doi.org/10.1371/journal.pone.0149293

Seyed, H. S., Venant, T.-N., Marzieh, L., Malihe, L., Ahmad, G., & Hamid, R. S. (2020). Wnt/beta-catenin and PI3K/Akt/mTOR Signaling Pathways in Glioblastoma: Two Main Targets for Drug Design: A Review. *Current Pharmaceutical Design*, *26*(15), 1729–1741.

Shaid, S., Brandts, C. H., Serve, H., & Dikic, I. (2013). Ubiquitination and selective autophagy. *Cell Death & Differentiation*, *20*(1), 21–30. https://doi.org/10.1038/cdd.2012.72

Shefa, U., Jeong, N. Y., Song, I. O., Chung, H.-J., Kim, D., Jung, J., & Huh, Y. (2019). Mitophagy links oxidative stress conditions and neurodegenerative diseases. Neural Regeneration Research, 14(5), 749–756. https://doi.org/10.4103/1673-5374.249218

Stasyk, O. V., Boretsky, Y. R., Gonchar, M. V., & Sibirny, A. A. (2015). Recombinant arginine-degrading enzymes in metabolic anticancer therapy and bioanalytics. *Cell Biology International*, *39*(3), 246–252. https://doi.org/10.1002/cbin.10383

Stegle, O., Teichmann, S. A., & Marioni, J. C. (2015). Computational and analytical challenges in single-cell transcriptomics. *Nature Reviews Genetics*, *16*(3), 133–145. https://doi.org/10.1038/nrg3833

Stone, E. M., Chantranupong, L., & Georgiou, G. (2010). The Second-Shell Metal Ligands of Human Arginase Affect Coordination of the Nucleophile and Substrate. *Biochemistry*, *49*(49), 10582–10588. https://doi.org/10.1021/bi101542t

Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J. B., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E., Mirimanoff, R. O., … National Cancer Institute of Canada Clinical Trials Group. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England Journal of Medicine*, *352*(10), 987–996. https://doi.org/10.1056/NEJMoa043330

Szefel, J., Danielak, A., & Kruszewski, W. J. (2019). Metabolic pathways of L-arginine and therapeutic consequences in tumors. *Advances in Medical Sciences*, *64*(1), 104–110. https://doi.org/10.1016/j.advms.2018.08.018

Szlosarek, P. W., Lee, S., & Pollard, P. J. (2014). Rewiring Mitochondrial Pyruvate Metabolism: Switching Off the Light in Cancer Cells? *Molecular Cell*, *56*(3), 343–344. https://doi.org/10.1016/j.molcel.2014.10.018

Tabe, Y., Lorenzi, P. L., & Konopleva, M. (2019). Amino acid metabolism in hematologic malignancies and the era of targeted therapy. *Blood*, *134*(13), 1014–1023. https://doi.org/10.1182/blood.2019001034

Takano, S., Kimu, H., Tsuda, K., Osuka, S., Nakai, K., Yamamoto, T., Ishikawa, E., Akutsu, H., Matsuda, M., & Matsumura, A. (2013). Decrease in the apparent diffusion coefficient in peritumoral edema for the assessment of recurrent glioblastoma treated by bevacizumab. *Acta Neurochirurgica. Supplement*, *118*, 185–189. https://doi.org/10.1007/978-3-7091-1434-6_34

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

Tanida, I., Minematsu-Ikeguchi, N., Ueno, T., & Kominami, E. (2005). Lysosomal Turnover, but Not a Cellular Level, of Endogenous LC3 is a Marker for Autophagy. *Autophagy*, *1*(2), 84–91. https://doi.org/10.4161/auto.1.2.1697

Tanida, I., Ueno, T., & Kominami, E. (2008). LC3 and Autophagy. In V. Deretic (Ed.), *Autophagosome and Phagosome* (pp. 77–88). Humana Press. https://doi.org/10.1007/978-1-59745-157-4_4

Tanios, R., Bekdash, A., Kassab, E., Stone, E., Georgiou, G., Frankel, A. E., & Abi-Habib, R. J. (2013). Human recombinant arginase I(Co)-PEG5000 [HuArgI(Co)-PEG5000]-induced arginine depletion is selectively cytotoxic to human acute myeloid leukemia cells. *Leukemia Research*, *37*(11), 1565–1571. https://doi.org/10.1016/j.leukres.2013.08.007

The Human Protein Atlas. (n.d.). Retrieved 31 May 2021, from http://www.proteinatlas.org/

Tsun, Z.-Y., & Possemato, R. (2015). Amino acid management in cancer. *Seminars in Cell & Developmental Biology*, *43*, 22–32. https://doi.org/10.1016/j.semcdb.2015.08.002

Um, J.-H., & Yun, and J. (2017). Emerging role of mitophagy in human diseases and physiology. *BMB Reports*, *50*(6), 299–307. https://doi.org/10.5483/BMBRep.2017.50.6.056

Verhaak, R. G. W., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., Miller, C. R., Ding, L., Golub, T., Mesirov, J. P., Alexe, G., Lawrence, M., O'Kelly, M., Tamayo, P., Weir, B. A., Gabrie, S., Winckler, W., Gupta, S., Jakkula, L., … Hayes, D. N. (2010). An integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR and NF1. *Cancer Cell*, *17*(1), 98. https://doi.org/10.1016/j.ccr.2009.12.020

Veronese, F. M., & Pasut, G. (2005). PEGylation, successful approach to drug delivery. *Drug Discovery Today*, *10*(21), 1451–1458. https://doi.org/10.1016/S1359-6446(05)03575-0

Vigneswaran, K., Neill, S., & Hadjipanayis, C. G. (2015). Beyond the World Health Organization grading of infiltrating gliomas: Advances in the molecular genetics of glioma classification. *Annals of Translational Medicine*, *3*(7), 10–10. https://doi.org/10.3978/j.issn.2305-5839.2015.03.57

Voss, V., Senft, C., Lang, V., Ronellenfitsch, M. W., Steinbach, J. P., Seifert, V., & Kögel, D. (2010). The Pan-Bcl-2 Inhibitor (−)-Gossypol Triggers Autophagic Cell Death in Malignant Glioma. *Molecular Cancer Research*, *8*(7), 1002–1016. https://doi.org/10.1158/1541-7786.MCR-09-0562

Vousden, K. H., & Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. *Cell*, *137*(3), 413–431. https://doi.org/10.1016/j.cell.2009.04.037

Wang, Z., Shi, X., Li, Y., Fan, J., Zeng, X., Xian, Z., Wang, Z., Sun, Y., Wang, S., Song, P., Zhao, S., Hu, H., & Ju, D. (2014). Blocking autophagy enhanced cytotoxicity induced by recombinant human arginase in triple-negative breast cancer cells. *Cell Death & Disease*, *5*(12), e1563. https://doi.org/10.1038/cddis.2014.503

Wei, Z., Liu, X., Cheng, C., Yu, W., & Yi, P. (2021). Metabolism of Amino Acids in Cancer. *Frontiers in Cell and Developmental Biology*, *8*. https://doi.org/10.3389/fcell.2020.603837

Wheatley, D. N., Kilfeather, R., Stitt, A., & Campbell, E. (2005). Integrity and stability of the citrulline–arginine pathway in normal and tumour cell lines. *Cancer Letters*, *227*(2), 141–152. https://doi.org/10.1016/j.canlet.2005.01.004

Xia, X., Li, X., Li, F., Wu, X., Zhang, M., Zhou, H., Huang, N., Yang, X., Xiao, F., Liu, D., Yang, L., & Zhang, N. (2019). A novel tumor suppressor protein encoded by circular AKT3 RNA inhibits glioblastoma tumorigenicity by competing with active phosphoinositide-dependent Kinase-1. *Molecular Cancer*, *18*(1), 131. https://doi.org/10.1186/s12943-019-1056-5

Xiao, S., Yang, Z., Lv, R., Zhao, J., Wu, M., Liao, Y., & Liu, Q. (2014). MiR-135b Contributes to the Radioresistance by Targeting GSK3β in Human Glioblastoma Multiforme Cells. *PLOS ONE*, *9*(9), e108810. https://doi.org/10.1371/journal.pone.0108810

Xu, J., Wu, Y., Lu, G., Xie, S., Ma, Z., Chen, Z., Shen, H.-M., & Xia, D. (2017). Importance of ROS-mediated autophagy in determining apoptotic cell death induced by physapubescin B. Redox Biology, 12, 198–207. https://doi.org/10.1016/j.redox.2017.02.017

Yan, Y., Chen, X., Wang, X., Zhao, Z., Hu, W., Zeng, S., Wei, J., Yang, X., Qian, L., Zhou, S., Sun, L., Gong, Z., & Xu, Z. (2019). The effects and the mechanisms of autophagy on the cancer-associated fibroblasts in cancer. *Journal of Experimental & Clinical Cancer Research : CR*, *38*. https://doi.org/10.1186/s13046-019-1172-5

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. https://doi.org/10.1158/1535-7163.MCT-11-0047

Yoo, S.-M., & Jung, and Y.-K. (2018). A Molecular Approach to Mitophagy and Mitochondrial Dynamics. *Moleucles and Cells*, *41*(1), 18–26. https://doi.org/10.14348/molcells.2018.2277

Yoon, C. Y., Sung, D. J., Lee, J. H., Kim, A. R., Oh, C. W., Je, J. H., Weon, B. M., Seol, S. K., Pyun, A., Hwu, Y., Margaritondo, G., Joo, K. J., & Yoon, D. K. (2007). Imaging of renal and prostate carcinoma with refractive index radiology. *International Journal of Urology*, *14*(2), 96–103. https://doi.org/10.1111/j.1442-2042.2007.01614.x

Yuile, P., Dent, O., Cook, R., Biggs, M., & Little, N. (2006). Survival of glioblastoma patients related to presenting symptoms, brain site and treatment variables. *Journal of Clinical Neuroscience*, *13*(7), 747–751. https://doi.org/10.1016/j.jocn.2005.10.011

Zaffagnini, G., & Martens, S. (2016). Mechanisms of Selective Autophagy. *Journal of Molecular Biology*, *428*(9, Part A), 1714–1724. https://doi.org/10.1016/j.jmb.2016.02.004

Zhang, L., Liu, M., Jamil, S., Han, R., Xu, G., & Ni, Y. (2015). PEGylation and pharmacological characterization of a potential anti-tumor drug, an engineered arginine deiminase originated from Pseudomonas plecoglossicida. *Cancer Letters*, *357*(1), 346–354. https://doi.org/10.1016/j.canlet.2014.11.042

Zhang, L., Wang, H., Xu, J., Zhu, J., & Ding, K. (2014). Inhibition of cathepsin S induces autophagy and apoptosis in human glioblastoma cell lines through ROS-mediated PI3K/AKT/mTOR/p70S6K and JNK signaling pathways. *Toxicology Letters*, *228*(3), 248–259. https://doi.org/10.1016/j.toxlet.2014.05.015

Zhao, X., Gao, S., Wu, Z., Kajigaya, S., Feng, X., Liu, Q., Townsley, D. M., Cooper, J., Chen, J., Keyvanfar, K., Fernandez Ibanez, M. del P., Wang, X., & Young, N. S. (2017). Single-cell RNA-seq reveals a distinct transcriptome signature of aneuploid hematopoietic cells. *Blood*, *130*(25), 2762–2773. https://doi.org/10.1182/blood-2017-08-803353

Zou, S., Wang, X., Liu, P., Ke, C., & Xu, S. (2019). Arginine metabolism and deprivation in cancer therapy. *Biomedicine & Pharmacotherapy*, *118*, 109210. https://doi.org/10.1016/j.biopha.2019.109210