Quercetin therapy for glioblastoma multiforme and its mechanism of action

Jason Choi

Department of Pharmacology and Therapeutics

Faculty of Medicine

McGill University

Montreal, QC, Canada

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science (MSc)

Dedicated to my family,

Jai, Jasmine, and Jun Choi

for their endless love and support.

Thank you all for your guidance

and encouragement

throughout my time at McGill.

Table of Contents

Abstract

Glioblastoma is the most common and malignant type of brain cancer with an average survival time of 12-15 months after diagnosis using current medical interventions. These medical approaches include surgery, radiation, chemotherapy, and their combinations, but there are many side-effects due to the sensitivity of brain tissue surrounding the tumour. Temozolomide (TMZ) is clinically used as an alkylating therapeutic for glioblastoma, but its effectiveness is limited because of cancer heterogeneity, the inability to reach the target cells in sufficient concentrations, and the development of drug resistance.

Quercetin is a plant-derived pleiotropic flavonoid, which induces cell death of breast, liver, and brain cancer cells. Its mechanism of action involves several signal transduction pathways implicated in tumor growth, spreading and cell death. The studies described in this thesis tested the hypothesis that quercetin alone or in combination with other therapeutics (TMZ and avasimibe) eliminates glioblastoma and brain tumor stem cells more effectively than currently used TMZ monotherapy. To test this hypothesis, studies were conducted with the following aims: (1) to determine concentration and time course of quercetin cell death induction with or without other selected therapeutics (TMZ and avasimibe) in U251 glioblastoma 3D cultures (spheroids) and brain tumor stem neurospheres; (2) to determine if the increased cell death with combination therapy consisting of quercetin and avasimibe (an acetyl-CoA acetyltransferase inhibitor) can be explained by avasimibe dysregulation of lipid droplet

biogenesis; and (3) to establish if the reduction in lipid droplets number or size will increase effects of quercetin by preventing drug sequestration in this organelle, thereby facilitating lipid peroxidation.

Our results show that quercetin induced U251 glioblastoma cell death at a concentration 25% of the IC_{50} of TMZ (400μM), and prevented the formation of brain tumor stem cell neurospheres. When combined with avasimibe, there was an increase in cell death accompanied with a decrease in number of lipid droplets. Avasimibe inhibited the accumulation of lipid droplets via ACAT regulation and enhanced cell death induced by quercetin. These studies show that quercetin combined with avasimibe treatment leads to an enhanced loss of glioblastoma U251 cells in 3D spheroids and brain tumor stem cells and that reduction in neutral lipid biogenesis contributes to this enhancement.

Collectively, our studies show the potential for quercetin as an anti-cancer agent against glioblastoma, and suggest that the reduction of lipid droplets using avasimibe can increase the anti-cancer effects of quercetin. *In vivo* studies employing combined therapeutics discussed in this thesis and an extended lipidomic analysis merit further investigations.

Resumé

Le glioblastome est le type de cancer du cerveau le plus prévalent et malin. La moyenne de survie est de 12-15 mois après le diagnostic et les interventions médicales actuelles. Les démarches médicales incluent la chirurgie, la radiothérapie, la chimiothérapie ainsi que leurs combinaisons. Cependant, il y a beaucoup d'effets secondaires en raison de la sensitivité des tissus cérébraux autour de la tumeur. Témozolomide (TMZ) est un agent d'alkylation qui est présentement utilisé chez les patients atteints de glioblastome, mais est très inefficace à cause de l'hétérogénéité des tumeurs, l'inhabilité du médicament à atteindre son objectif en concentrations suffisantes et le développement de la résistance aux médicaments.

La quercétine est un flavonol pléiotrope d'origine végétale qui induit la mort cellulaire du cancer du sein, du foie et du cerveau. Son mécanisme d'action implique plusieurs voies de transduction du signal associées à la croissance, la propagation et la mort cellulaire des tumeurs. Les études dans cette thèse testent l'hypothèse que la quercétine seule ou en combinaison avec d'autres agents thérapeutiques (TMZ et avasimibe) éliminent le glioblastome et les cellules souches du cancer du cerveau (BTSC) plus efficacement que TMZ, utilisé présentement en monothérapie. Pour tester cette hypothèse, les études ont été menées avec les objectifs suivant : (1) déterminer les effets de la quercétine, avec ou sans autres agents thérapeutiques (TMZ et avasimibe), en fonction de la concentration et du temps, sur la mort cellulaire des sphéroïdes de glioblastome U251 et les neurosphères de BTSC; (2) déterminer si la mort cellulaire accrue résultant de la combinaison de la quercétine et de l'avasimibe (un inhibiteur de l'acétyl-CoA

acétyltransférase) est due au dérèglement de la biogenèse des gouttelettes lipidiques causé par l'avasimibe; (3) établir si la diminution du nombre ou de la taille des gouttelettes lipidiques augmente les effets de la quercétine en empêchant la séquestration du médicament dans ces organelles, facilitant ainsi la peroxydation lipidique.

Nos résultats montrent que la quercétine cause la mort cellulaire du glioblastome U251 à une concentration CI_{50} équivalente à 25% de celle de TMZ (400 µM) et empêche la formation des neurophères de BTSC. En combinaison avec l'avasimibe, la mort cellulaire augmente et le nombre de gouttelettes lipidiques est réduit. L'avasimibe inhibe l'accumulation de gouttelettes lipidiques par régulation de l'ACAT et accroît la mort cellulaire induite par la quercétine. Ces études montrent que la quercétine en combinaison avec l'avasimibe augmente la perte des sphéroïdes de glioblastome U251N et les neurosphères de BTSC. Une diminution de la biogenèse des lipides neutres contribue à cette perte.

En résumé, notre étude montre le potentiel de la quercétine en tant qu'agent anticancéreux contre le glioblastome. Elle montre aussi que réduire les gouttelettes lipidiques avec l'avasimibe augmente les effets anticancéreux de la quercétine. Des études *in vivo* utilisant la combinaison d'agents thérapeutiques discutée dans cette thèse, ainsi qu'une analyse lipidomique poussée méritent d'être investiguées.

Acknowledgements

My studies and work in the Department of Pharmacology would not have been possible without Dr. Dusica Maysinger. I would like to thank her for taking me into the lab and believing in me. She has pushed me to try my hardest since Day 1, and since then, I have improved significantly as a student, scientist, and a thinker. I am truly very fortunate to have her as a teacher and a mentor, and I will forever be grateful for her guidance throughout my time in her lab. All her stories and comments were very entertaining and insightful, and I have truly learned so much from her.

A special thanks to Dr. Bastien Castagner, my advisor, and to my committee members Dr. John Gillard and Dr. Guillermina Almazan. They have provided great criticism and feedback on my research, and their time and effort is truly invaluable to me. I would also like to thank Tina Tremblay and Chantal Grignon for always having my back and helping me out whenever I needed them.

Next, I would like to give very special recognition to my lab mates Issan Zhang, Jeff Ji, and Alex Moquin. They were never hesitant to answer any of my questions, and lent a helping hand whenever they could. Issan truly established a great lab environment for all new members, Jeff was the go-to guy whenever I had questions regarding my research, and Alex provided me with the chemicals for my experiments whenever I

needed them. My time in the lab would not have been as enjoyable, nor as efficient, if I did not have them by my side.

Finally, none of this could have been possible without the constant support from my family and friends. I do not directly depend on others for emotional and physical support, but simply being surrounded by motivating and inspiring people have allowed me to elevate my goals in life. I am very fortunate to have the people around me, and I will continue to be the best I can be to motivate them as well.

My project was funded by the Canadian Institute of Health Research (CIHR), the Natural Sciences and Engineering Research Council (NSERC), and the Graduate Excellence Fellowship provided the Department of Pharmacology and Therapeutics.

List of Figures and Tables

List of Abbreviations

ACAT: Acetyl-CoA acetyltransferase

Ava: Avasimibe

BTSC: Brain tumor stem cell

DNA: deoxyribonucleic acid

GBM: Glioblastoma Multiforme

IκK: Iκ Kinase

JNK: c-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinase

NFκB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

PEG: Polyethylene glycol

PI: Propidium Iodide

PI3K: Phosphoinositide 3 kinase

PLGA: Poly (lactic-co-glycolic acid)

Q: Quercetin

ROS: Reactive Oxygen Species

TMZ: Temozolomide

WHO: World Health Organization

Contribution of Authors

The work presented in this thesis has been completed with the help from the following collaborators:

Dr. Alexandre Moquin synthesized the PEG-PLGA micelles and encapsulated quercetin into the micelles. He also characterized the micelles and determined the concentration of quercetin.

Dr. Phuong Uyen Le cultured the brain tumor stem cells and treated them with quercetin.

All of the experiments were done under the supervision of Dr. Dusica Maysinger. I have performed all experiments and drafted the thesis. All versions were discussed, improved and approved by my supervisor.

Rationale and Objectives

GBM is the most common and deadly form of brain cancer with no effective treatment. The current treatment consists of surgical removal of the tumor, radiotherapy, and chemotherapy, and there are many side effects that arises from the procedures. With the rise in interest in the use of phytochemicals for cancer treatment, it is important to understand their mechanism of action. Additionally, it is essential to research methods to increase their bioavailability to enhance their effects *in vivo.*

This thesis focuses the treatment of glioblastoma multiforme with quercetin, a plant-derived flavonoid. To increase the cytotoxic effects of quercetin, avasimibe, an inhibitor of lipid droplet biogenesis, was used in combination. Additionally, PEG-PLGA micelles were synthesized as a nanocarrier to encapsulate quercetin and increase its solubility in aqueous media.

Hypothesis: Quercetin in combination with other therapeutics (TMZ and avasimibe) eliminates glioblastoma and brain tumor stem cells more effectively than currently used TMZ monotherapy.

Objectives:

(1) To determine the concentration and time dependent effects of monotherapies and drugs in combination for quercetin, TMZ and avasimibe in U251 glioblastoma 3D cultures (spheroids) and brain tumor stem cell neurospheres.

(2) To determine if the increased cell death with combination therapy consisting of quercetin and avasimibe can be explained by avasimibe-induced dysregulation of lipid droplet biogenesis, and

(3) To establish if the reduction in lipid droplets number or size will increase effects of quercetin by preventing drug sequestration in this organelle, thereby facilitating lipid peroxidation.

1. General Introduction

1.1 Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the most common, malignant primary brain tumour in humans.[1] GBM can be classified as a primary tumor, with no precursor, or as a secondary tumor where a low-grade tumor transforms into GBM.[2,3] Previously, GBM was thought to have solely originated from glial cells, but recent research has suggested that it may originate from neural cells with stem-cell properties.[4]

Most of glioblastomas are diagnosed in patients in their sixties .[1] Despite the advances in GBM treatment, the median survival rate of patients after diagnosis is 15 months with the combination of surgery, radiation, and chemotherapy.[5, 6] One main factor that contributes to the fatal course of GBM is its high heterogeneity.[7] Four GBM subtypes have been characterized: classical, pro-neural, neural, and mesenchymal, each with different genetic mutations, and thus different disease progression and survival rates.[8] The goal for current GBM treatment research is to move away from single gene-based treatments and gain a greater understanding of the different pathways that promote GBM proliferation, progression, and drug resistance.

The current treatment for GBM involves: surgical removal of the tumor mass, radiation therapy with temozolomide (TMZ), and subsequent chemotherapy with TMZ without radiation for 4 weeks (Figure 1).[5, 9] A surgical GBM removal is invasive (and sometimes not even possible) and can induce further neurological deficits resulting in impaired

speech, motor functions, and senses.[10] Additionally, in 70% of the cases, there is a tumor recurrence, usually due to drug-resistance.[2]

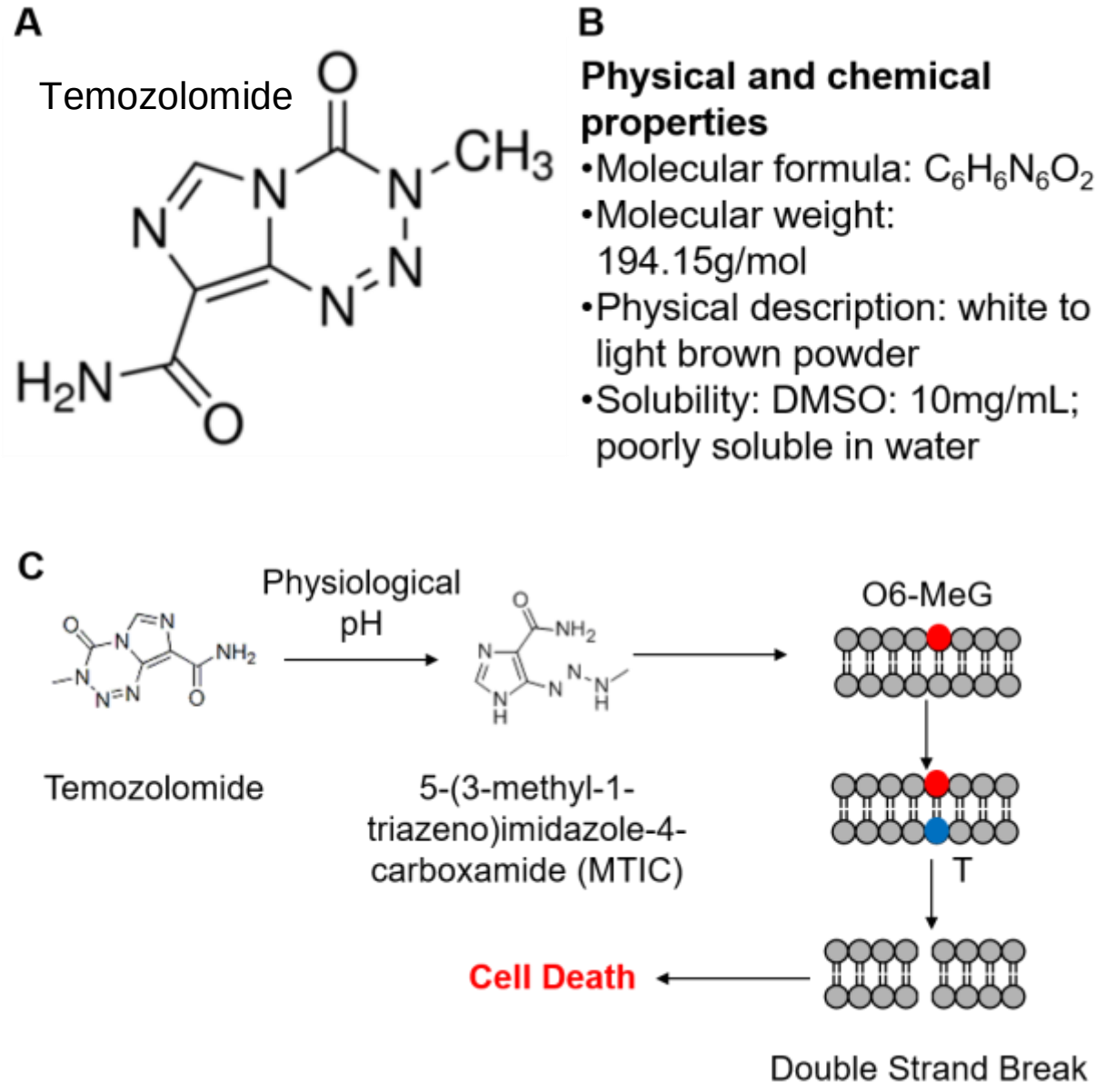


Figure 1. Structure and mechanism of temozolomide

Structure of temozolomide (A). Physical and chemical properties of temozolomide (B). Temozolomide mechanism of action (C).

1.2 GBM Heterogeneity and Microenvironment

The tumor mass of GBM is composed of not just the proliferative cancer cells, but contains also tumor associated macrophages (TAMs) and brain tumor stem cells (Figure 2).[4, 7, 11] The resident macrophages in the brain (microglia) and peripheral macrophages are in the GBM microenvironment. Microglia under physiological conditions constantly survey the brain,[12] but when hyperactive under pathological conditions they can contribute to the propagation of tumor growth and metastases.[13, 14] Immune cells from the periphery (i.e. mononuclear hematopoietic phagocytes) are recruited to GBM and contribute to the neuroinflammation in the GBM microenvironment.[11, 13]

The two classical types of macrophage states are the pro-inflammatory M1 state and the anti-inflammatory M2 state.[14, 15] M1 and M2 states are considered the extreme states within a spectrum of macrophage activation. The activation state of macrophages will determine their contribution to tumor cell death and growth. TAMs produce both pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines (TGF-β and IL-10),[13, 14, 16, 17] thereby promoting or inhibiting cancer cell growth, respectively.[13,14] TAMs facilitate GBM migration and spread of metastasis suggesting that therapeutic interventions should not be targeting only the primary tumor but also the microenvironment of GBM.[11]

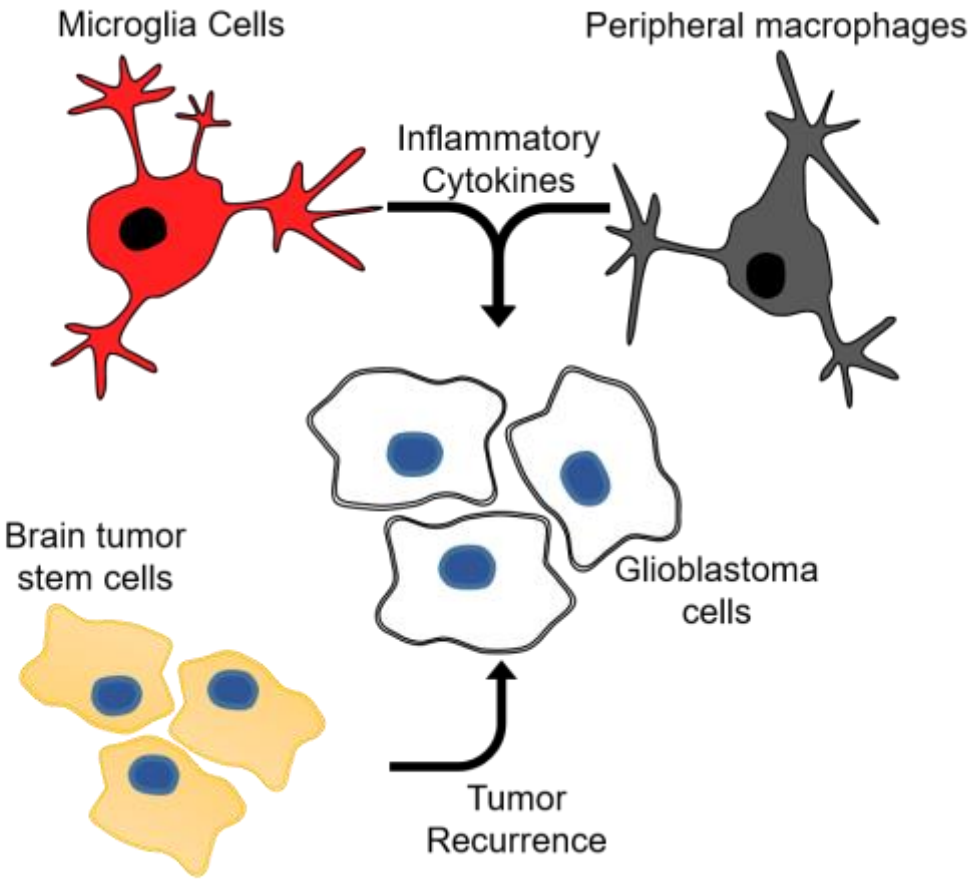


Figure 2. Role of the tumor microenvironment

The tumor microenvironment is composed of tumor associated microglia/macrophages (TAMs) and brain tumor stem cells. The TAMs release inflammatory cytokines that can potentially induce tumor cell death or tumor cell proliferation and migration. Brain tumor stem cells are drug resistant cells that are senescent. They are often the cause of tumor recurrence.

Aside from TAMs, brain tumor stem cells contribute to the glioblastoma heterogeneity. Brain tumor stem cells are present in tumor niches and they are highly resistant to radiation and chemotherapy, which mainly affect actively proliferating cells. Brain tumor

stem cells are often the sources of tumor recurrences. It has also been shown that brain tumor stem cells release a chemoattractant called periostin, which recruits TAMs to the tumor site, thereby enhancing the invasiveness of brain tumor stem cells.[18] Thus, the goal of new therapeutic interventions is to target these cells .[2, 4] Ideally, new therapeutic intervention should eliminate brain tumor stem cells without harmful effects on the healthy brain cells.[4, 19] Thus, experiments in this thesis were designed to test the effectiveness of selected therapeutics both in U251 spheroids and in brain tumor stem cell 3D cultures.

Although the full relationship between the tumor cells, TAMs, and brain tumor stem cells is not known, it is important to acknowledge that we must target all the components of the tumor and its microenvironment, and not only the proliferating cancer cells.[7] To achieve this goal, a combination therapy aimed at different signal transduction pathways in different cell types warrants further investigations.

1.3 Flavonoids

Flavonoids are a class of polyphenols classified by their chemical structure (Figure 3).[20] They consist of two benzene rings joined by a linear three-carbon chain, forming an oxygenated heterocycle (Figure 3A). There are up to 4000 different flavonoids with

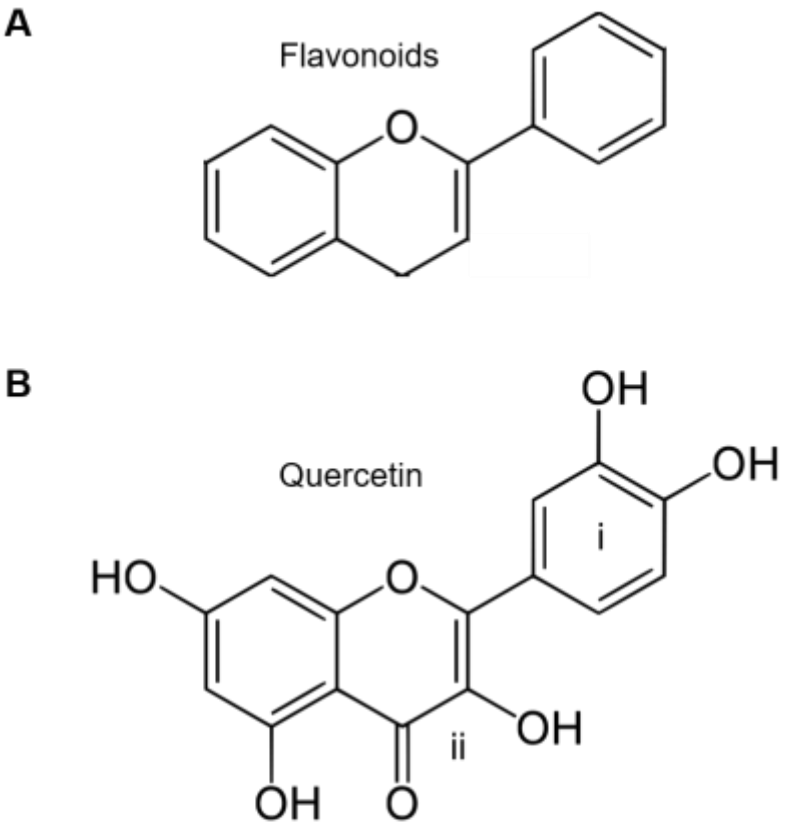


Figure 3. Structure of Quercetin and its properties

Core structure of flavonoids (A). Chemical structure of quercetin (B). Key functional groups include: the catechol group (i), 3-hydroxy 4-keto group (ii). Physical and chemical properties of quercetin (C).

different functional groups determining their biological activities. Flavonoids can be commonly found in the human diet as they are present in many fruits, vegetables and seeds. They are predominantly present as O-glycosides with sugars bound at the C3 position.[4]

Flavonoids have been studied to have many biological activities such as: antioxidant effects, induction of apoptosis, inhibition of proliferation, and binding to DNA.[21] Epidemiologic studies also show that flavonoid intake, especially quercetin, reduced the risk of lung, stomach, and colorectal cancer.[22, 23, 24, 25, 26,27]

1.4 Quercetin as an anti-cancer agent

Quercetin (Q) is a naturally-occurring flavonoid found in fruits and vegetables such as berries and onions (Figure 3B). In plants, quercetin is a polar auxin transport inhibitor, which regulates plant growth.[27, 28] Quercetin is a powerful antioxidant by scavenging free radicals and binding to transition metal ions.[29, 30] However, in the same process, quercetin can have pro-oxidant effects (Figure 4B). When quercetin is oxidized, the first oxidation product is a semiquinone radical that is very unstable and undergoes a second oxidation to produce another quinone. This oxidation product is bound to glutathione to prevent any toxic effects of the quinone, however, when the cells are exposed to quercetin for a long exposure time or a high concentration, there is low

glutathione levels and quinone is then able to bind to proteins, lipids, and DNA, and induce DNA damage in the cell.[31, 32, 33, 34]

Aside from scavenging reactive oxygen species (ROS), quercetin can inhibit a number of kinases implicated in cancer cell expansion (Figure 4A). As a kinase inhibitor, [35, 36, 37] , quercetin has been shown to induce cell cycle arrest by regulating molecular targets such as p21, cyclin B, p27, and cyclin-dependent kinases in breast, esophageal, lung, and liver cancer cell lines at different stages.[28, 38] Additionally, quercetin blocks the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκ-β) transcription factor, which is an essential regulator in cell survival and cell proliferation.[39, 40, 41, 42] Quercetin has also been shown to directly interact with many mitochondrial proteins such as Bcl-2 and BAD to disrupt the integrity of the organelle, and activate caspase-dependent apoptosis.[43]

1.5. Favorable properties and limitations of quercetin

One of the most attractive chemical features of quercetin molecule is its small size and that it is not prone to enzymatic degradation like many biological compounds. From the biological standpoint, quercetin is an attractive anticancer drug because it does not

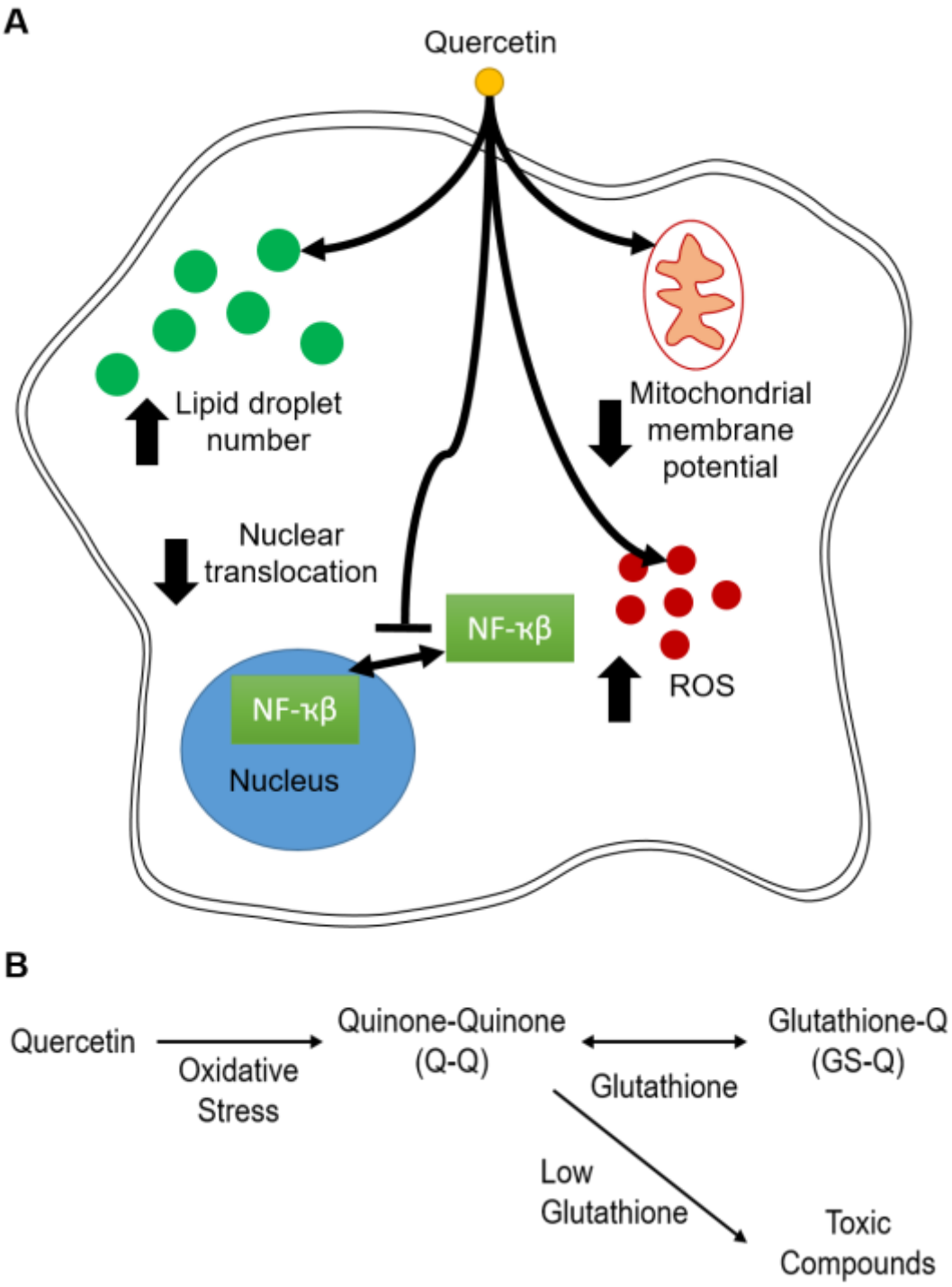


Figure 4. Effect of quercetin in glioblastoma cells

Quercetin mechanism of action (A). Quercetin metabolism under low glutathione conditions (B).

significantly reduce the cell viability of non-cancer cells when used in equimolar concentrations. For example, quercetin was not toxic when tested in non-transformed human lung embryonic fibroblasts, endothelial cells, and peripheral blood lymphocytes.[28] In these cell types, quercetin reduced cell viability only at very high concentrations, much higher than required to induce cell death in the cancer cell lines. These properties of quercetin were the basis for selecting it for investigations in this thesis. Considering the quercetin cell type differential cytotoxic effect, we hypothesized that quercetin will mainly eliminate brain tumor stem cells and U251 cells but not untransformed astrocytes in the surrounding microenvironment.

Aside from the favorable biological properties, quercetin has several limitations as a therapeutic agent. Major limitations are its low bioavailability and low solubility.[44] Especially when studying treatments for GBM. It is important to increase the bioavailability of quercetin at the tumor site and ensure that it can cross the blood brain barrier. When taken orally, most of the quercetin is metabolized in the intestinal tract. Although some of its metabolites like isorhamnetin have anti-oxidant effects, its anti-cancer function is limited by its low absorption.[45] Incorporation of quercetin into nanocarriers can reduce or even eliminate some of quercetin limitations,

For example, to increase the bioavailability, quercetin can be incorporated into polymeric micelles. Micelles are nanostructures particularly suitable for the delivery of poorly water-soluble lipophilic drugs. Several nanocarriers for quercetin have been reported and their advantages and limitations discussed.[94, 95, 96, 101] We have

investigated quercetin micelles to reduce its accumulation in lipid droplets and to enhance its cytosolic concentration. For our studies, we selected PEG-PLGA, a polymer approved by Food and Drug Administration (FDA).

1.6 Lipid droplets and lipophilic cancer agents

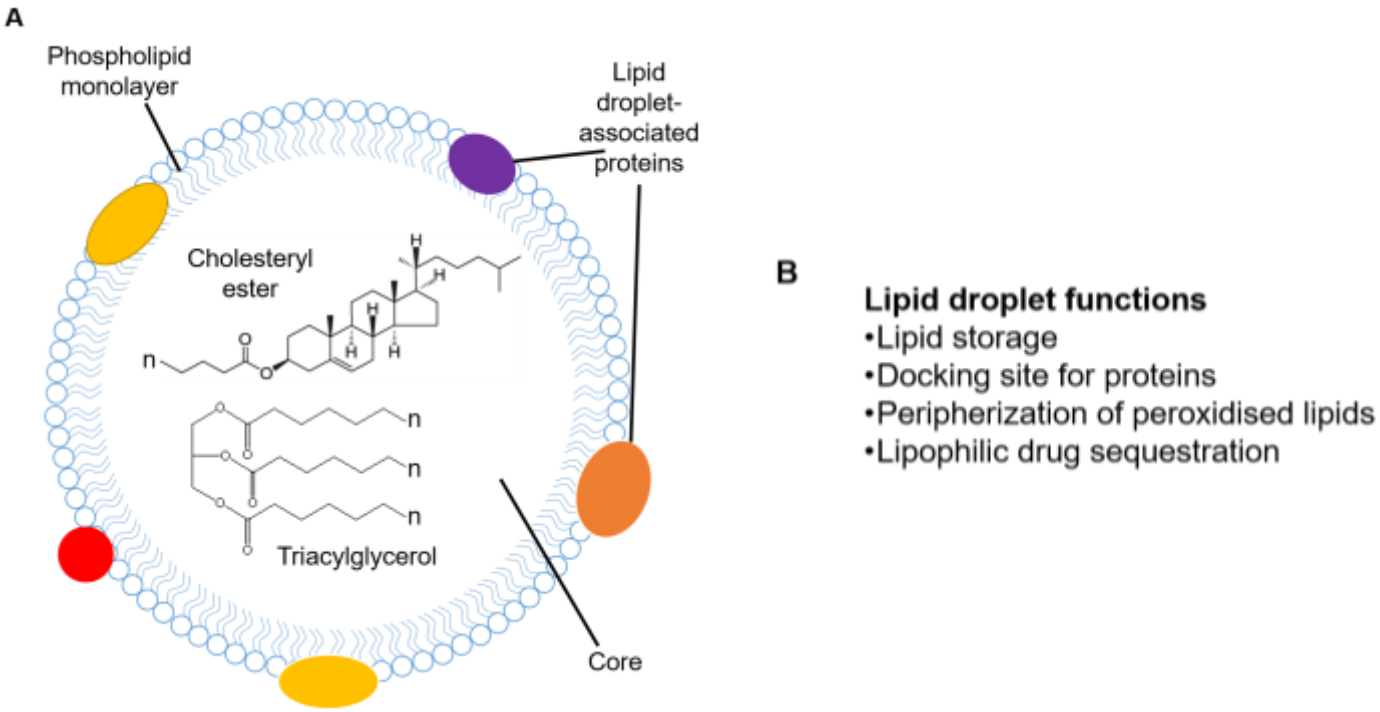


Figure 5. Lipid droplet structure and some essential functions

Lipid droplet structure (A). Functions of lipid droplets (B).

Lipid droplets are organelles composed of a lipid ester core encapsulated by a phospholipid monolayer (Figure 5).[46] Their main cellular function was thought to be lipid storage in adipocytes, however, recent studies have shown that they play many different functions including intracellular signaling, protein docking and sequestration of some lipophilic agents. Interestingly, among the cancer cells resistant to therapeutics, those had higher lipid droplet content than the non-resistant cancer cells. Therefore, we investigated if we can pharmacologically manipulate lipid droplet numbers to make quercetin more effective. It was reported that lipid droplets are induced under hypoxic conditions and that they provide energy in hypoxic cancer environments.[47, 48]

Some chemotherapeutics (including quercetin) induce the formation of reactive oxygen specifies (ROS) leading to apoptosis, DNA damage, and lipid peroxidation.[48] Lipid droplets are not desirable because they will reduce this deleterious effects of ROS in cancer cells. We have tested if lipid droplets act to promote cellular defence by preventing lipid peroxidation and organellar damage induced by reactive oxygen species.[49] To this end, we inhibited lipid droplet biogenesis by employing a drug avasimibe (*vide infra*), and tested if lipid droplets reduction enhances glioblastoma and brain tumor stem cell cell loss.

1.7 Avasimbie – Mechanism of Action

Avasimibe is an orally available Acyl-CoA cholesterol acyltransferase (ACAT) inhibitor that was tested in clinical trials for cardiovascular disease.[50]

Avasimibe did not show significant improvements in Phase III trials in the selected set of patients with cardiovascular complications. Although it failed the clinical trials for cardiovascular disease, clinical trials in cancer patients have been initiated.[53] ACAT catalyzes the chemical reaction that synthesizes cholesterol esters (CE) from cholesterol and fatty acyl-CoA.[51] CEs are a main component of lipid droplets along with triglycerides, and thus by inhibiting CE synthesis, avasimibe will reduce the number of lipid droplets in the cancer cells. It is anticipated that the reduction in lipid droplets will increase the sensitivity of the cells to lipophilic anticancer drugs and decrease glioblastoma cell viability.

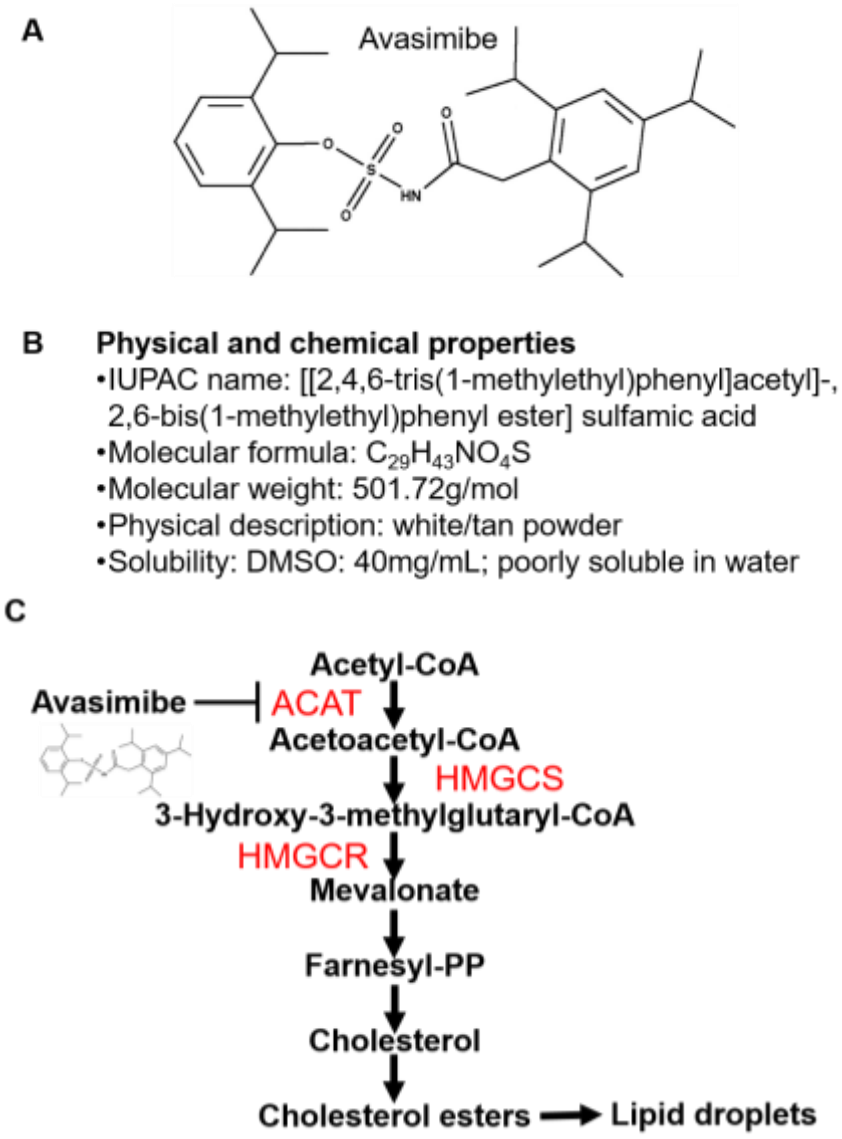


Figure 6. Avasimibe and its mechanism of action

Structure of avasimibe (A). Physical and chemical properties of avasimibe (B). Avasimibe mechanism of action (C). Avasimibe is an ACAT inhibitor. ACAT is an enzyme that converts acetyl-CoA to aceteoacetyl-CoA in the mevalonate pathway to produce cholesterol esters. Avasimibe blocks the initial step in the pathway and inhibits the formations of cholesterol esters.[52]

1.8 Nanodelivery of Quercetin

Due to the low bioavailability and poor solubility of quercetin in aqueous media, we tested polyethylene glycol-poly lactic acid-co-glycolic acid (PEG-PLGA) micelles as a nanocarrier for quercetin. PEG-PLGA are a family of biodegradable polymers that are commonly used as drug carriers due to their biocompatibility.[53] The micelles are formed through hydrophobic interactions between the hydrophobic chains of the polymer, constructing a hydrophilic outer surface and a hydrophobic inner core for drug encapsulation. PEG-PLGA micelles are very stable and can pass through the blood brain barrier effectively, making it an ideal nanocarrier for GBM therapy.[54] Quercetin encapsulated in PEG-PLGA micelles were tested on U251 spheroids and brain tumor stem cell.

Collectively, the introduction in this M. Sc. thesis provides background for the proposed experiments indicating that: (1) GBM is the most malignant primary brain cancer and there is currently no effective treatment, resulting in a very low survival rate; (2) the current therapy is very invasive to the normal and sensitive brain tissue, and the chemotherapeutic agents used are very toxic to surrounding brain cells; (3) drug resistance develops quickly and one of the contributors to this effect is a large lipid droplet content which sequesters the chemotherapeutics. Thus, the overall goal of this study is to reduce glioblastoma drug resistance by treating U251N glioblastoma cells with quercetin, a pleiotropic drug that affects many different cancer signalling pathways. Additionally, a combination of quercetin and avasimibe, an acetyl-CoA: Cholesterol O-

acyltransferase (ACAT) inhibitor, is tested to increase the effect of quercetin by preventing its sequestration in the lipid droplets abundant in poorly responding glioblastoma.

2. Materials and Methods

2.1 Materials

Quercetin, Hoechst 33342, propidium Iodide, temozolomide, buthionine-sulfoximine, avasimibe, and Bodipy 493/503 were purchased from Sigma (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) and penicillin/streptomycin were purchased from Invitrogen (Burlington, ON), and fetal bovine serum (FBS) were purchased from Wisent (Saint-Jean-Baptiste, QC). U251N glioblastoma cell line was acquired in October 2010 from Dr. Josephine Nalbantoglu (MNI, Neuroimmunology Unit, Webster Pavillion; rmW010K). U251N cell line was originally obtained from the American Type Culture Collection. The 6-well, 24-well, and 96-well culture plates were purchased from Sarstedt (Princetone, NJ). The 100mm tissue culture plates, TMRE, and CellROX were purchased from Thermofisher (Waltham, MA).

2.2 Cell culture

U251 human glioblastoma monolayers were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life Technologies Inc., Burlington, ON, Canada) supplemented with 10% (v/v) FBS (Gibco) and 1% (v/v) penicillin–streptomycin (Gibco). They were maintained at 37 °C with 5% CO2 and 95% relative humidity. The cells were maintained in T75cm^2 flasks at 90% confluency and split every two to three days. The cells were washed three times with PBS and 2mL of trypsin was added for 2 minutes to detach the cells. After the detachment, 3mL of culture media was added to the flask to deactivation the trypsin and the cells were then collected in a 15mL tube. The cell concentration was

determined using a hemocytometer. The cells were seeded 24 h before treatment to the appropriate cell density for the indicated assay (described in detail below).

2.3 Cell treatment

Confluent monolayer cell cultures were detached using 0.05% trypsin-EDTA, and seeded in 10cm culture plates (1 x 10^6 cells/plate), 6-well plates (2 x 10^5 cells/well), 24-well-plates (5 x 10^4 cells/well), or 96-well plates (5 x 10^3 cells/well). The cells were cultured for 24 hours before treatment. The cells were treated with quercetin (0.1-500μM) for 24-72 hours. Spheroids were treated with the same drug combinations as monolayers for 1-7 days. Stock solution of quercetin (100mM) and avasimibe (1mM) were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich), and were added to cells for a final DMSO concentration of <0.5%. The control cells were treated with DMSO at 0.5%.

2.4 Spheroid formation

Spheroid cultures were prepared using a protocol adapted from the liquid overlay system previously established by Dhanikula et al. Flat bottomed 96-well plates were coated with 2% agarose dissolved in serum-free DMEM solution. The agarose was dissolved by adding it to the media and autoclaving the mixture in the liquid setting. After the autoclaving process, 70μL the warm solution of agar in media was added to each well. The coated plates were sealed with parafilm and stored at 4°C upside down to prevent condensation on the agar.

Confluent U251 monolayer cell cultures were detached using 0.05% trypsin-EDTA (Gibco), and seeded at 5,000 cells per well in 96-well plates pre-coated with 2% agarose (Invitrogen) in serum-free DMEM solution. Spheroids were seeded and grown in DMEM medium supplemented with 10% FBS for four days before drug treatment. For treatments, half of the culture media (100µL) was carefully removed from the wells and replaced with another 100µL of fresh media with the drug dilution.

2.5 Hoechst 33342 and Propidium Iodide labeling in monolayer and spheroid cultures

In monolayer cultures, Hoechst 33342 (10µM) diluted in serum free media was added to the culture medium following the treatments and incubated at 37 °C for 10 minutes. The cells were then washed three times with PBS, and serum free media was added to the wells during the imaging. Analysis of the plates was immediately followed. Cell imaging was conducted using an automated microscopy platform (Operetta High Content Imaging System; Perkin Elmer). Image analysis and cell counting was performed using the Columbus Image Data Storage and Analysis platform (Perkin Elmer) and Cell Profiler. In spheroid cultures, Hoechst 33342 (10µM) and PI (1.5 µM) fluorescent dyes were added 4h prior to measurements. Following treatment, individual spheroids were carefully transferred onto a microscope slide using a pipette, and flattened under a coverslip. Imaging was conducted using fluorescence microscope (Leica), and fluorescence intensity was quantified using ImageJ software.

2.6 Organelle staining

Lipid droplets were stained using Bodipy (4,4-difluoro-3a,4adiaza-s-indacene), which is a lipophilic dye that mimics the property of natural lipids and sequesters inside the lipid droplets. The cells were stained with Bodipy (20µM) for 10 minutes. Lysosomes were stained using Lysotracker Red DND-99, a acidotropic dye that stains acidic compartments of the cell. The cells were stained with Lysotracker Red (500nM) for 3 minutes. Mitochondria were stained with tetramethylrhodamine, ethyl ester (TMRE), a positive-charged dye that accumulates in the negative-charged mitochondria. The cells were stained with TMRE (200nM) for 20 minutes.

2.7 CellRox Reactive Oxygen Species Detection

The cells were culture in 96-well plates and incubated for 24 h prior to treatments. After the treatments, CellROX (Ex 485nm/ Em 520nm) was added to the culture media at a final concentration of 5µM for 30 minutes at 37ºC. The cells were then washed fixed with 4% paraformaldehyde for 10 minutes and imaged using an automated microscopy platform (Operetta High Content Imaging System; Perkin Elmer). Image analysis and was performed using ImageJ and Microsoft Office Powerpoint.

2.8 Western blot

U251 cells were harvested in PBS and separated by nuclear and cytoplasmic fractions. The extracts were then separated by SDS-PAGE and transferred to a PVDF membrane.

After the transfer, the membrane was blocked with 5% milk in TBST (10mM Tris pH 8.0, 150nM NaCl, 0.5% Tween 20) for 1 hour. The membrane was incubated with goat antibody against NF-kB p65 (1:1000) (Santa Cruz Tech) at 4ºC overnight. The membranes were then washed with TBST three times, followed by incubated with secondary antibody (rabbit anti-goat 1:3000) for 1 hour at room temperature. Blots were washed three times with TBST and developed with the Clarity ECL solution (Bio-Rad). The blots were quantified using ImageJ by comparing the intensity of signal from the immunoreactive bands corresponding to the analyzed protein of interest and the loading control bands.

2.9 PEG-PLGA micelle preparation

PEG-PLGA micelles were prepared by the co-solvent evaporation method. PEG-PLGA co-polymer (10 mg) was mixed with quercetin (1 mg) and solubilized in tetrahydrofuran (THF, 1 mL). The solution was added dropwise into Milli-Q Ultrapure water (2 mL) under constant agitation using a magnetic stirrer. The vial was left open to let the THF evaporate slowly. After 24 h a stream of nitrogen was placed in the solution and left to bubble for 30 min. The vial was placed under vacuum overnight to remove traces of THF. Excess unencapsulated quercetin was removed by a first pass through a PDVF 0.45 µm syringe filter. The solution was concentrated to the required stock concentration using 10 kDa cut-off Amicon® filters and filtered through a 0.45 µm PVDF filter to remove any contaminants/dust. Aliquots were diluted (20 times) in DMSO and sonicated for 15 min before taking a UV-absorbance spectrum. Quercetin concentration was determined by comparing the absorbance at 379 nm with the calibration curve of

free quercetin dissolved in DMSO spiked with empty micelles. The dry weight of an aliquot of the stock solution was determined after freeze-drying the aliquot.

2.10 Statistical analysis

Data was graphed and tabulated using Microsoft Excel®. Each experiment was performed in triplicates at least three times, except for Figure 9, which was performed twice. All data are expressed as mean ± S.E.M. The student's t-test with Bonferroni correction was used to analyze significant differences between two group means (p values < 0.05 were considered significant) and one-way ANOVA with post hoc Tukey's test was used to compare three or more conditions.

3. Results

3.1 Anticancer effects of Quercetin on U251 Glioblastoma cells

To study the anti-cancer effects of quercetin on U251 glioblastoma cells, we established the dose- and time-dependent effects of quercetin with a cell viability assay using the Hoechst 33342 nuclei stain. This fluorescent dye was used to stain the nuclei of the cells to quantify the number of living cells in the field. The U251 cells were treated with quercetin (0.1-500μM) for 1-3 days. In addition to quercetin, the cells were also treated with TMZ (0.1-400μM) to compare our drug to the current clinical chemotherapeutic agent for GBM. After the treatments, there was a significant decrease in glioblastoma cell viability after 24 h at a concentration of 100μM, with 55% cell viability (Figure 7A). There was also a significant decrease in cell viability after 48 h and 72 h treatments at 40%, and 30% cell viability, respectively (Figure 7A). When compared to TMZ, quercetin induced greater cell death with significantly fewer Hoechst stained nuclei when treated at the same concentration (100μM) (Figure 7), suggesting that for the comparable cell killing effect, TMZ is required in approximately four-fold higher concentration than Q: 400μM for TMZ vs 100μM for quercetin (Figure 7A). In addition to the decreased cell viability, we observed the presence of pyknotic nuclei when treated with quercetin (Figure 7B). Pyknosis is the irreversible condensation of chromatin that occurs during apoptotic or necrotic cell death.[55] This result suggests that quercetin does not simply prevent the cancer cells proliferation, but also induces cell death. The data from these studies suggested that the further experiments are warranted in a more complex system such as spheroids. Thus, the next step was to establish the optimal conditions for U251

spheroid formation and test the effectiveness of the selected compounds in such a model.

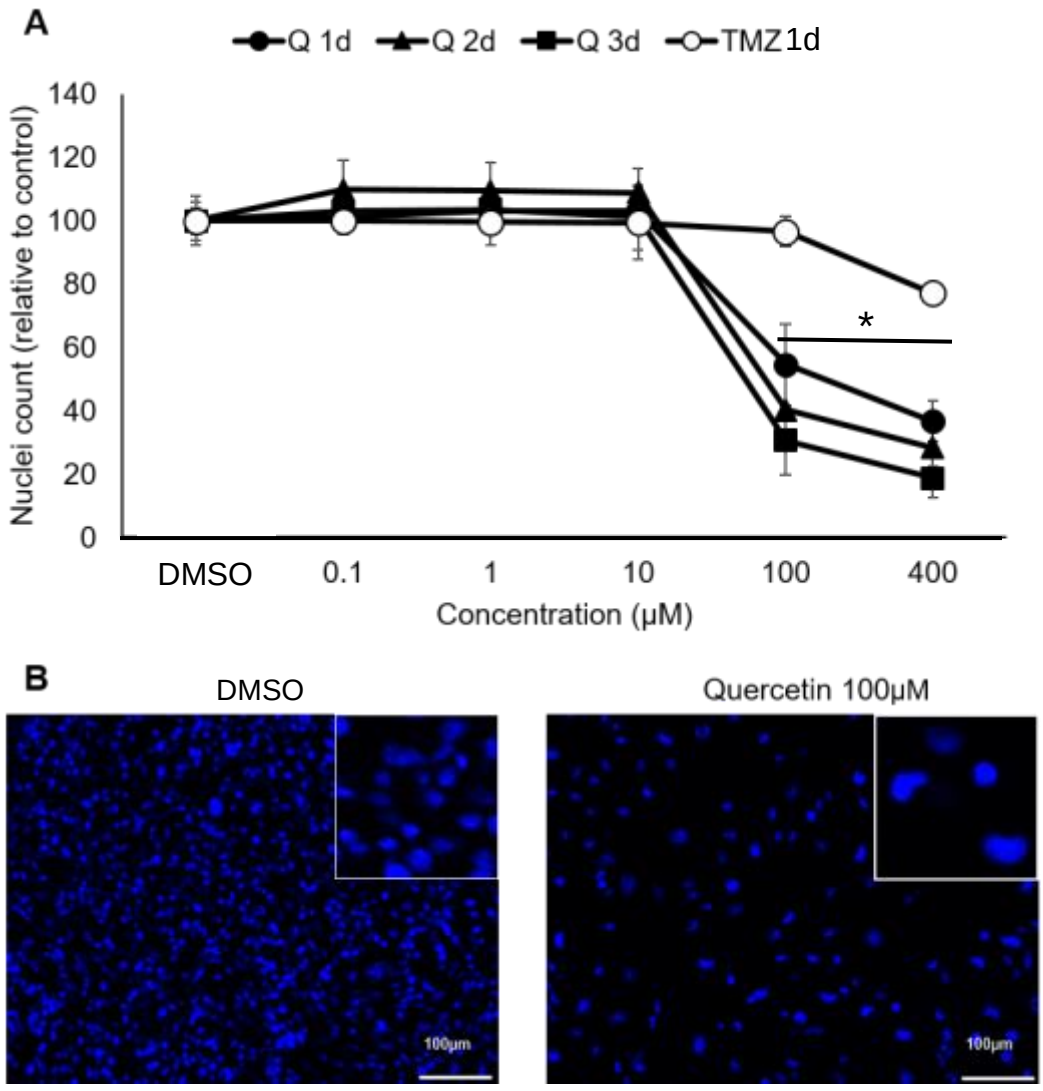


Figure 7. Quercetin as anticancer agents for glioblastoma.

A. Dose and time dependent effect of quercetin on U251 cell viability measured by nuclei count stained with Hoechst 33342. B. Micrographs of Hoechst 33342 stained nuclei under control and quercetin treatment. Statistically significant differences were calculated using the one-way ANOVA and post hoc Tukey's test to detect significant cell death. * = $P<0.05$

3.2 Effect of Quercetin on 3-D Spheroid cultures

The major limitation with monolayer cell cultures is that they do not adequately represent a tumor mass. In an actual tumor mass, which is 3D, there is a hypoxic and necrotic core, and a proliferative outer layer zone.[56] Additionally, long-term drug treatments cannot be done in monolayer cultures because the cells in the wells grow to confluency and eventually die due to cell contact inhibition.[57] This is important when studying anti-cancer drugs because often the chemotherapy is given to the patient for many consecutive weeks at a time. Hence, 3-D spheroid cultures can be formed by growing the cells in agar-coated 96-well plate and allowing the cells to clump together and form a sphere. These 3-D cultures can be treated for more than 3 days, and they are a better model to study the anti-cancer effects of a potential therapeutic agent.

3-D spheroid cultures of primary mouse astrocytes and U251 glioblastoma cells were formed and treated with TMZ (100μM) and quercetin (100μM) for 7 days. After the treatments, the spheroids were stained with propidium iodide to detect for cell death induced by TMZ and quercetin. Propidium iodide stains for cells with leaky plasma membranes undergoing cell death such as apoptosis or necrosis.[58] The astrocyte spheroid cultures showed virtually no relative fluorescence of PI, meaning that TMZ and quercetin were not cytotoxic to the normal, slow dividing astrocytes (Figure 8). Alternatively, both drugs induced cell death in the U251 spheroids, indicated by the PI staining (Figure 8). This shows that quercetin induces cell death in 3-D spheroid cultures, and further experiments need to done to understand its mechanism of action.

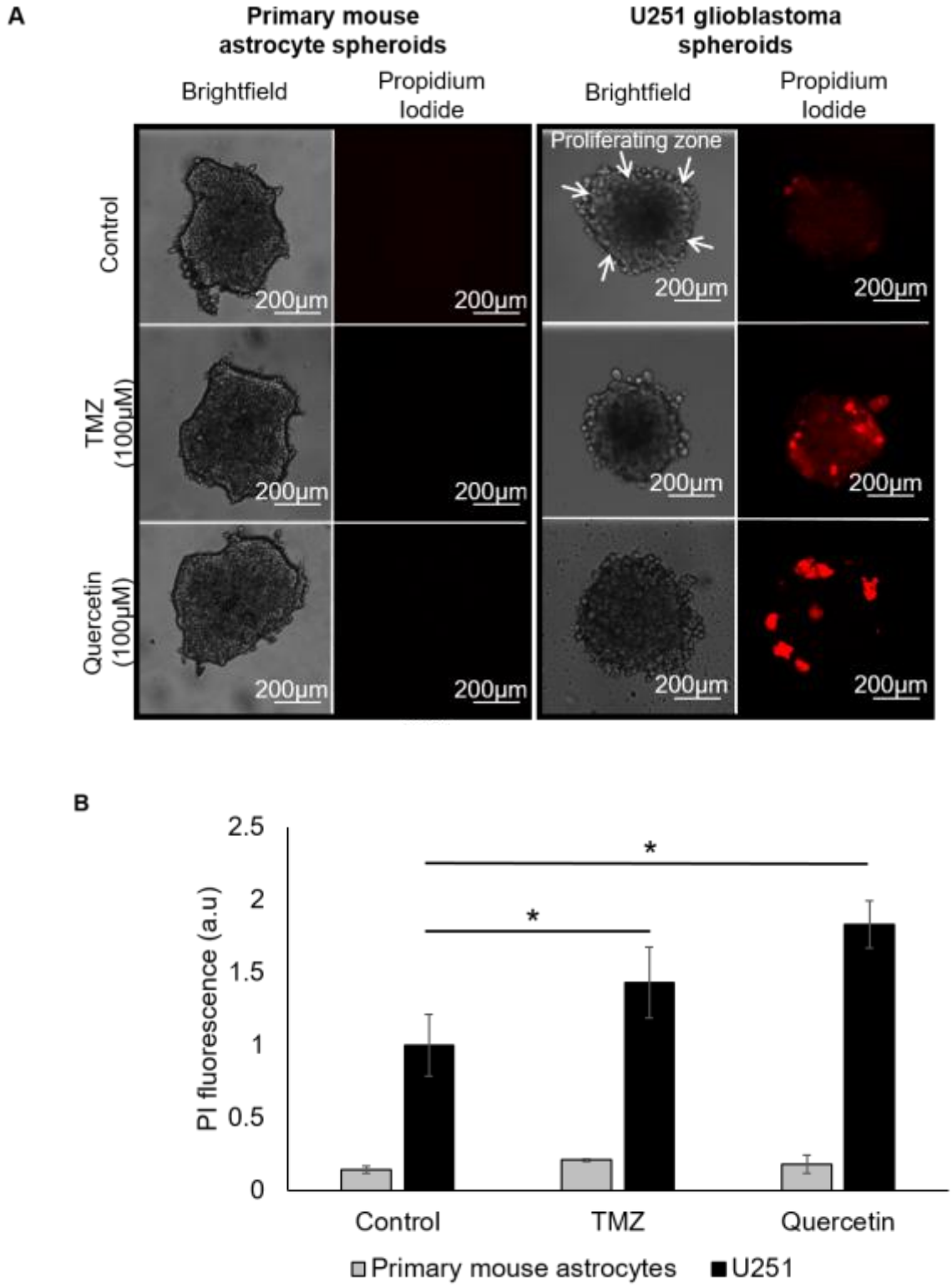

A
Primary mouse astrocyte spheroids
U251 glioblastoma spheroids
Brightfield
Propidium Iodide
Brightfield
Propidium Iodide
Proliferating zone
Control
TMZ (100μM)
Quercetin (100μM)
200μm
B
PI fluorescence (a.u)
2.5
2
1.5
1
0.5
0
*
*
Control
TMZ
Quercetin
Primary mouse astrocytes
U251

Fig 8. Cytotoxic effects of quercetin and temozolomide on 3-D spheroids

Primary astrocyte and U251 spheroids were grown for four days before exposure to quercetin. The spheroids were treated with TMZ (100μM) and quercetin (100μM) for 7 days. The spheroids were then stained with propidium iodide (1.5μM) and fluorescence micrographs of the spheroids were taken (A). The fluorescence intensity was measured using ImageJ (B). Statistically significant differences were calculated using one-way ANOVA with post hoc Tukey's test. * = $P<0.05$

3.3 Effects of Quercetin on Brain Tumor Stem Cell growth

Brain tumor stem cells are the main cause of tumor recurrence in patients, and these cells are typically known to be unaffected by chemotherapeutic agents. The brain tumor stem cells were treated with quercetin (100μM) for 7 days. These cultures are non-adherent cultures that form floating neurospheres. In the untreated and vehicle conditions, the BTSCs formed round neurospheres (Figure 9). When treated with quercetin, the cells did not form neurospheres, but appeared to be dissociated (Figure 9). Therefore, this indicates that quercetin prevents the normal growth of brain tumor stem cells and it has an effect on not just the actively proliferating glioblastoma cells, but the stem cells as well. This does not show that quercetin induces stem cell death, but shows that it prevents normal proliferation and growth of the brain tumor stem cells.

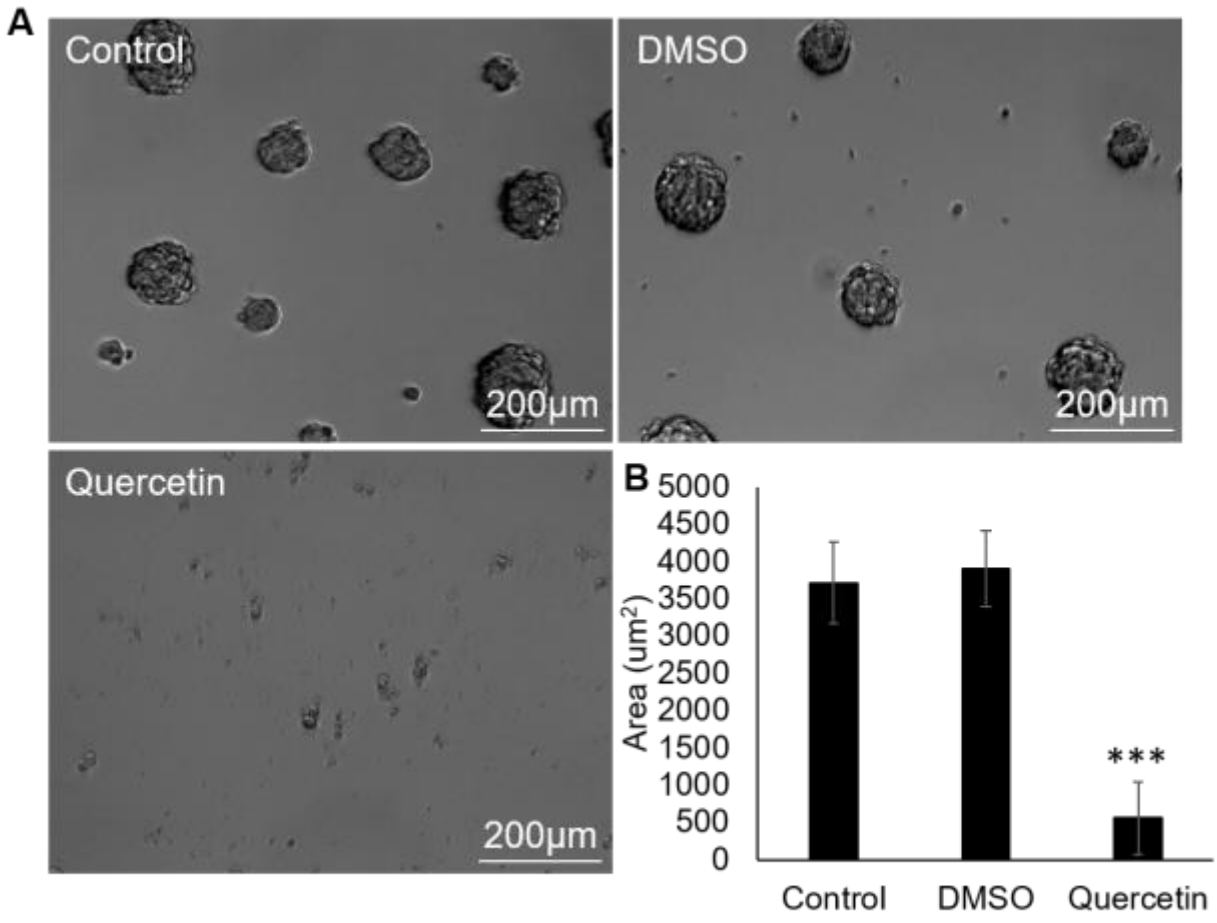
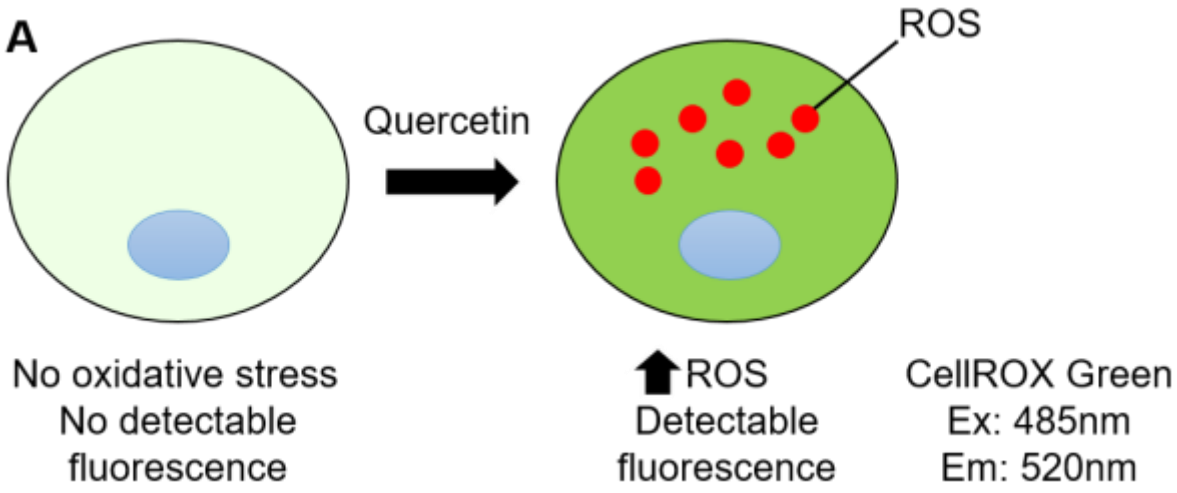


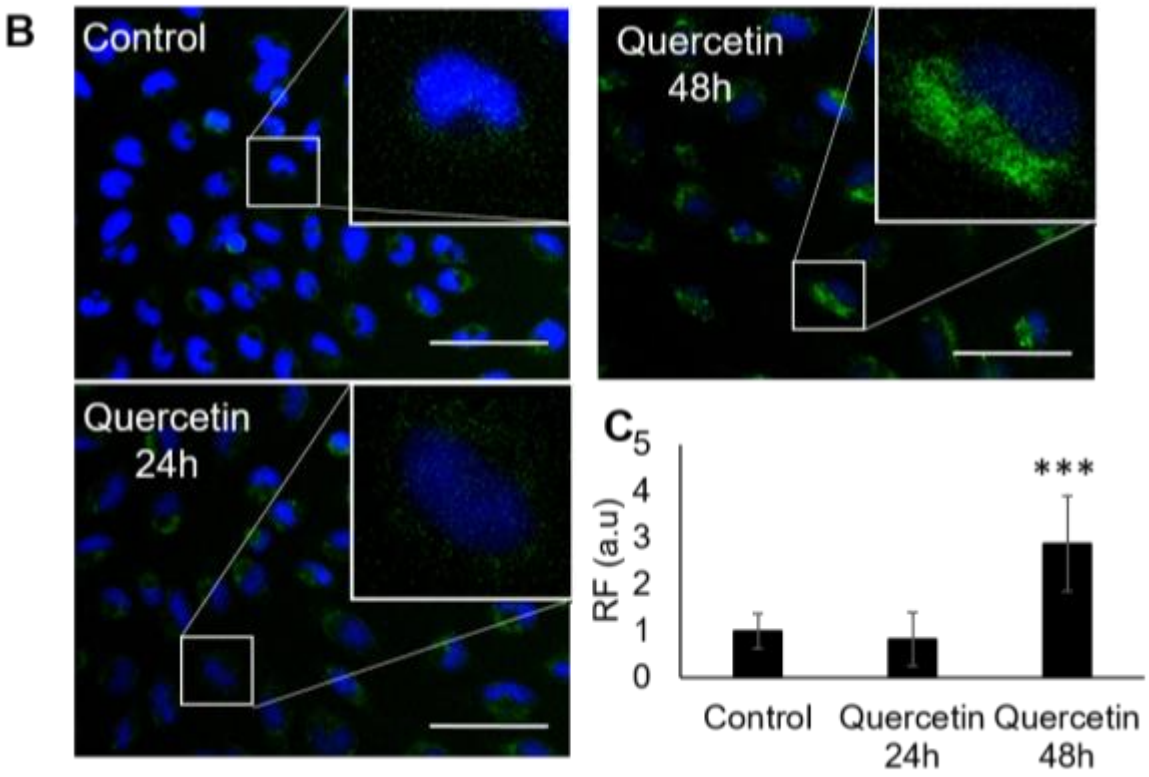
Figure 9. Quercetin prevents the formation of brain tumor stem cell neurospheres

Brain tumor stem cells were cultured and treated with vehicle (DMSO) and quercetin (100uM) for 7 days. The areas of the cultures were measured after the 7-day treatment to determine whether quercetin treatment prevents brain tumor stem cell neurosphere formation. Micrographs were taken using a light microscope at 100X (A). The area of the neurosphere were quantified using ImageJ (B). Statistically significant differences were calculated using the t-test with Bonferroni corrections. *** = $P<0.001$

3.4 Quercetin and ROS formation and mitochondria dysfunction

Although quercetin is known to be an anti-inflammatory agent, it can also induce ROS production at high concentrations. We investigated the intracellular ROS production in cells treated with quercetin after 24 and 48 hours. We observed that there was a significant intracellular production of ROS after 48 hours of quercetin treatment (Figure 10B and C). This increase in oxidative stress induced by quercetin can in turn cause mitochondrial damage through peroxidation of the mitochondrial membrane. We then subsequently studied the effect of quercetin treatment on mitochondrial membrane potential. After the treatment, the mitochondria were labelled with TMRE, a functional dye for the mitochondria. Similar to the production of ROS, we observed a decrease in TMRE relative fluorescence after the 48-hour treatment with quercetin (Figure 10 D and E). In figure 7, we showed that quercetin is able to induce cell death after 24 hours. However, we only see a change in ROS production and mitochondrial potential after 48 hours. This could be due to the sensitivity of the assays that cannot detect the small changes in redox and mitochondrial potential that induce cell death. To confirm that this production of ROS induces cell death, we inhibited the formation of intracellular antioxidants in combination with quercetin treatment.


A
ROS
Quercetin
No oxidative stress
No detectable
fluorescence
ROS
Detectable
fluorescence
CellROX Green
Ex: 485nm
Em: 520nm


B
Control
Quercetin
48h
Quercetin
24h
C
RF (a.u)
0
1
2
3
4
5
***
Control
Quercetin
24h
Quercetin
48h

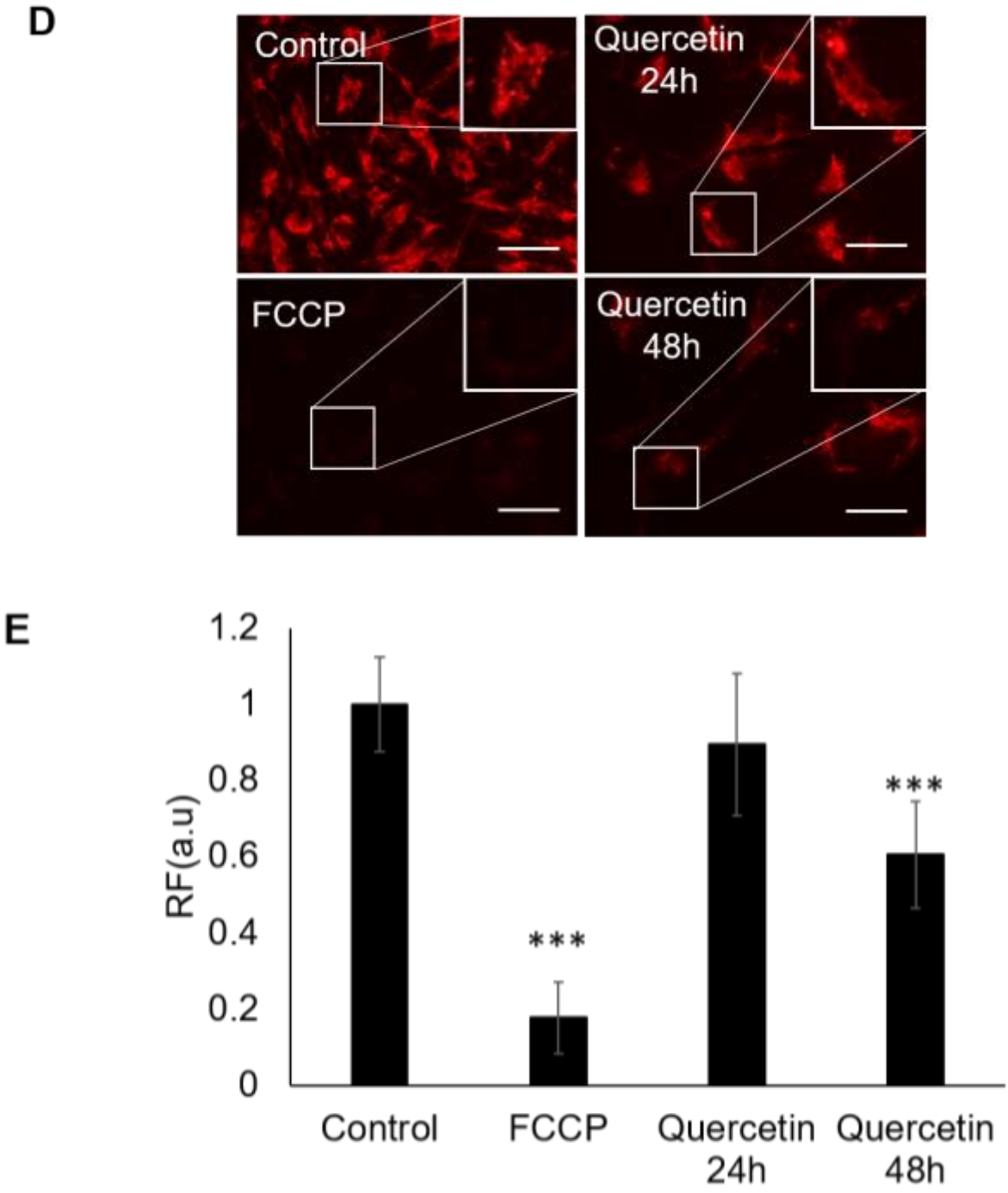


Figure 10. Quercetin induces ROS production and mitochondria dysfunction

Schematic of the CellROX ROS assay (A). U251 cells were treated with quercetin (100μM) for 24 and 48 hours. After treatment, CellRox (5μM) and Hoechst (10μM) were added to the cell

medium (B). The fluorescence was quantified using ImageJ (C). The changes in mitochondrial membrane potential were measured using TMRE (200nM) (D). FCCP (10µM) was used as a negative control for mitochondrial membrane potential. The fluorescent micrographs were quantified with ImageJ to measure the relative fluorescence (E). Scale bar = 100µm. Statistically significant differences were calculated using one-way ANOVA with post hoc Tukey's test. *** = $P<0.001$

3.5 Effect of quercetin under reduced glutathione

Given that quercetin induces ROS formation in glioblastoma cells, as shown in the previous section, we have used buthionine sulfoximine (BSO) in combination with quercetin. BSO inhibits gamma-glutamylcysteine synthetase, the enzyme required for the initial step in glutathione formation.[59] Glutathione is an important anti-oxidant that reduces ROS damage and maintains redox homeostasis.[60] A lower concentration of quercetin was used to test whether BSO increase the cytotoxicity of quercetin. Additionally, serum-deprived media was used to ensure antioxidants in the serum did not interfere with the measurements. A reduction of levels of glutathione with BSO lead to an enhanced glioblastoma cell death compared with a lower concentration of quercetin alone (50µM) (Figure 11B). This showed that the cell killing effect of quercetin is in part due to quercetin-induced ROS production and unopposed oxidative stress. We have previously shown that oxidative stress leads to the formation of lipid droplets, which sequester quercetin. The following section (3.6) will address deal with this subject.

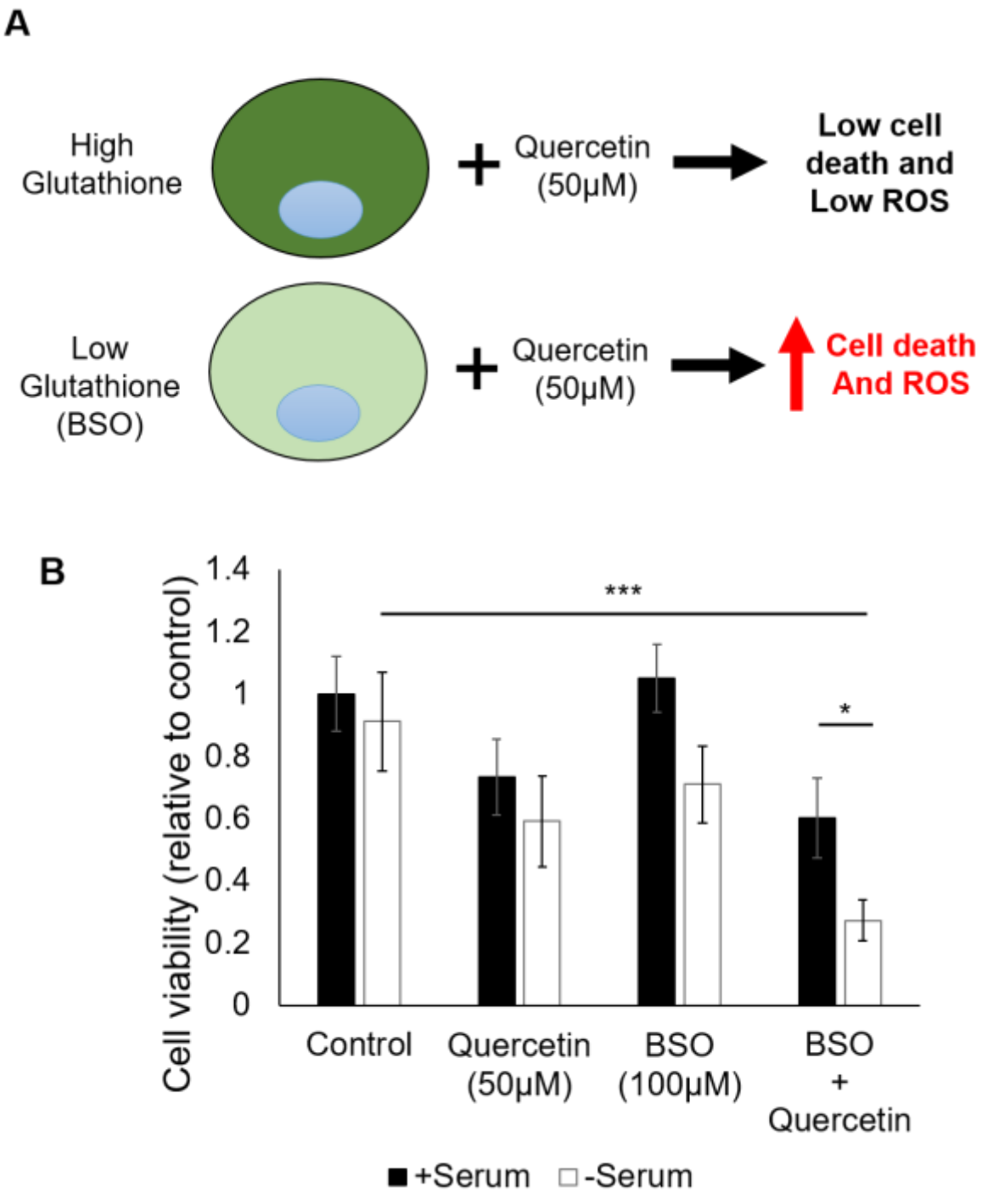


Figure 11. BSO enhances the effect of quercetin on glioblastoma cells

BSO reduces glutathione levels in glioblastoma cells and increase the cell killing effect of quercetin (A). U251 glioblastoma cells were treated with quercetin (50μM), buthionine sulfoximine (100μM), and their combination for 24 hours in both serum free and serum containing media. The nuclei were then stained with Hoechst 33342 and the number of nuclei were quantified using Cell Profiler. Statistically significant differences between conditions with and without serum were calculated using the t-test with Bonferroni corrections. Statistically significant differences between the different treatments were calculated using one-way ANOVA with post hoc Tukey's test. * = $P<0.05$ ***= $P<0.0001$

3.6 Quercetin and lipid droplets

Among the limitations of hydrophobic drugs for cancer treatment is their sequestration in the lipid droplets. The cells use lipid droplets as a defence mechanism to re-direct the location of therapeutic agents. They sequester toxic hydrophobic drugs and reduce their availability in the cytosol and nucleus, locations of cellular target structures. Additionally, an increase in lipogenesis to facilitate the high rate of cancer cell proliferation (which requires phospholipids for membranes, cholesterols for signalling, and triglycerides for energy), has been reported.[61] Thus, a decrease of cytoplasmic lipid droplet content could render glioblastoma cells more responsive to quercetin.

U251 cells were cultured in high-glucose DMEM (glucose 25mM), or in low-glucose DMEM (glucose 5mM). This was done to lower the concentration of glucose available,

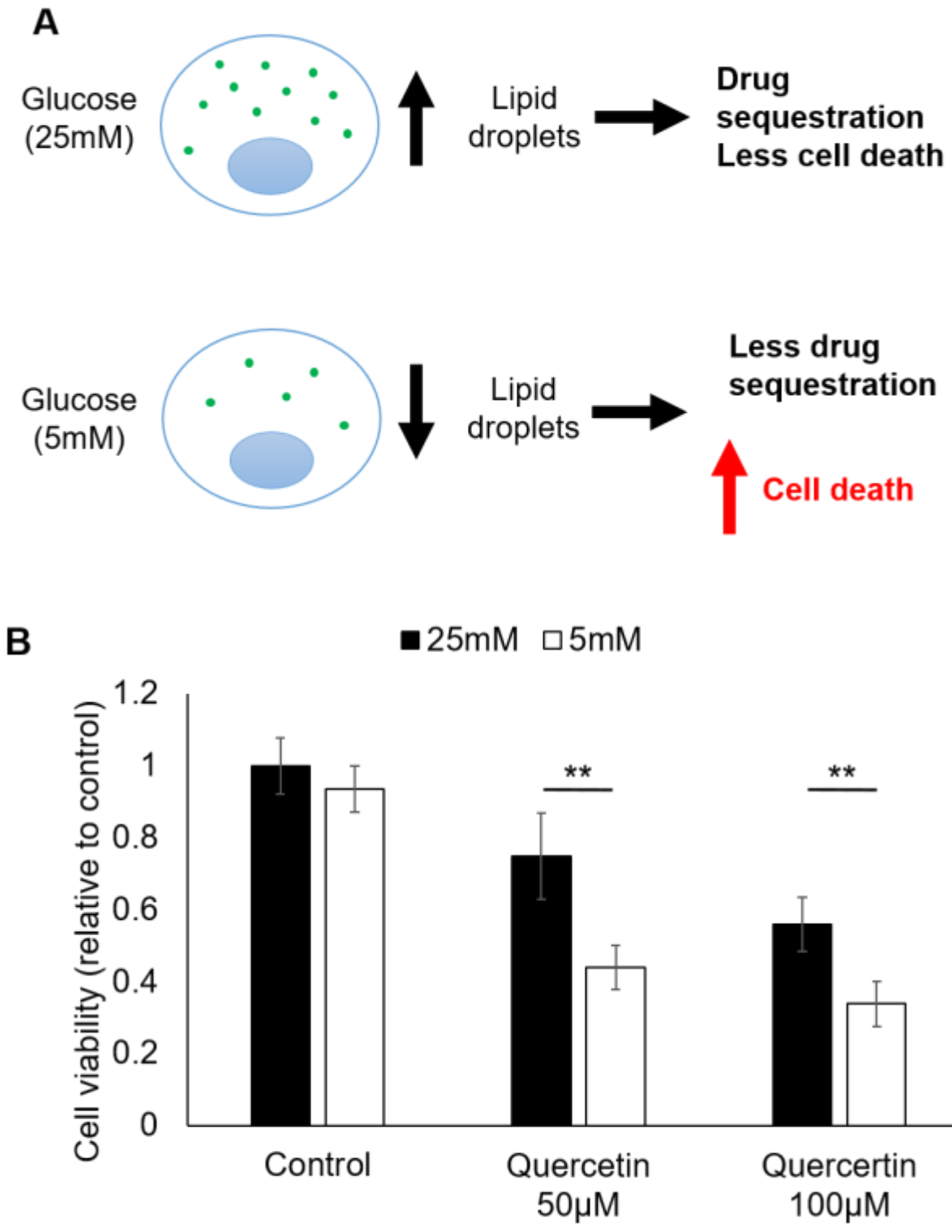


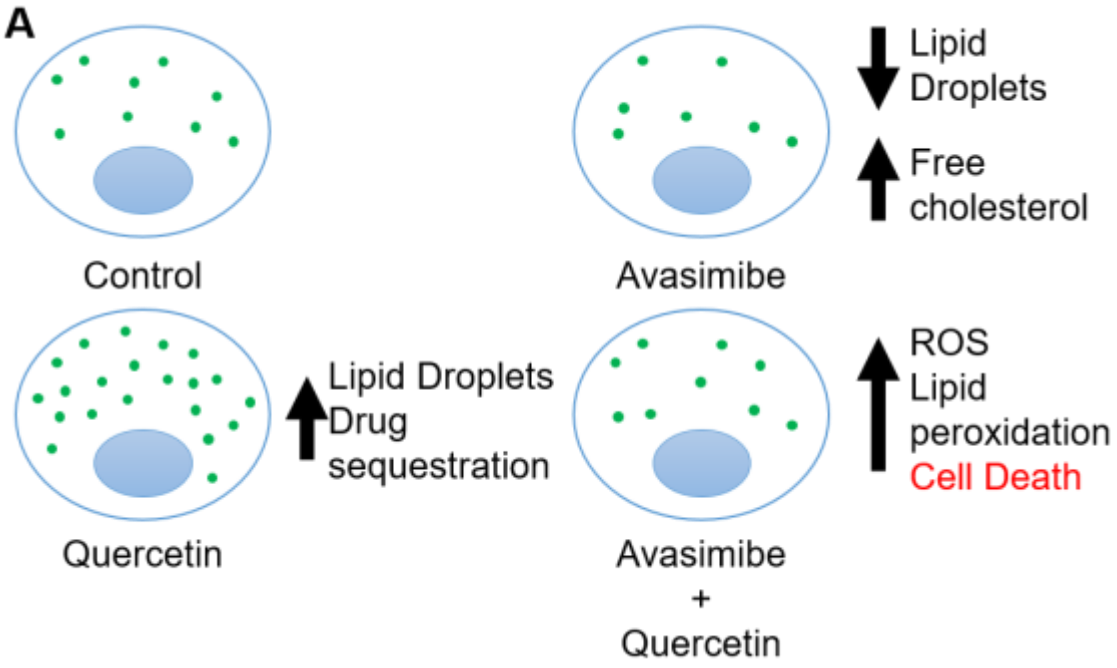
Figure 12. Lower glucose media increases cytotoxicity of quercetin on U251 cells

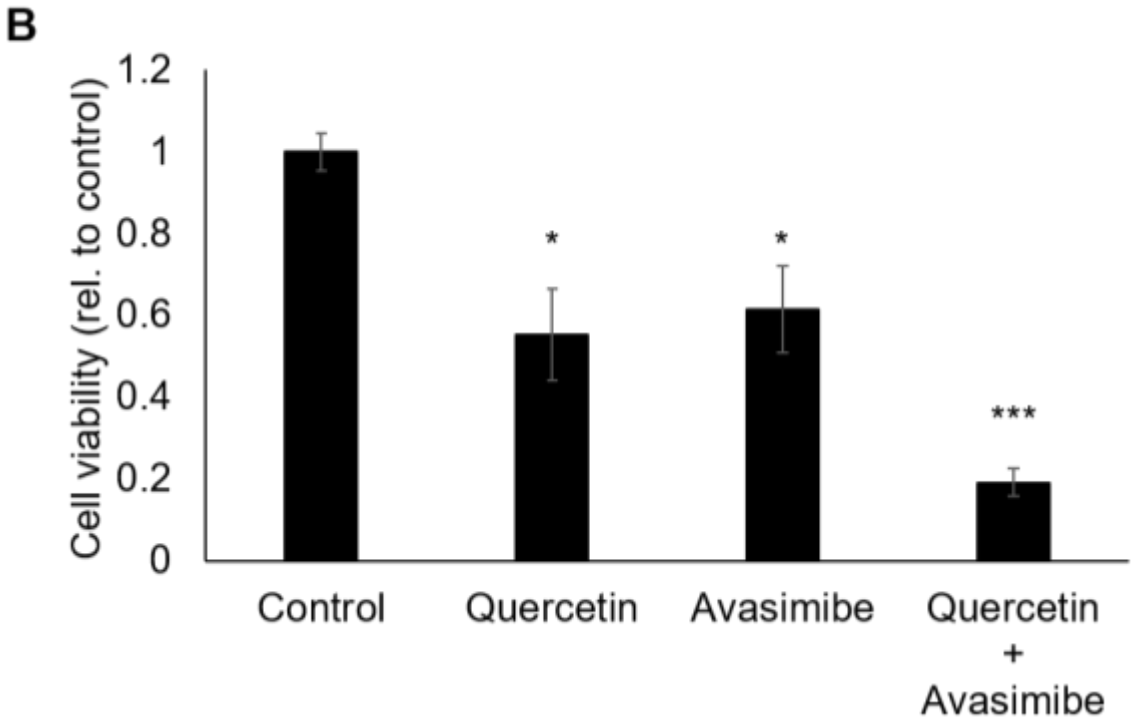
Schematic of U251 cells in high glucose (25mM) and low glucose (5mM) concentrations (A). U251 cells were treated with quercetin (50μM and 100μM) in media with 25mM and 5mM of glucose for 24 hours. After the treatment, the nuclei were stained with Hoechst 33342 (10μM) and were quantified to detect cell viability (B). Statistically significant differences between the different treatments were calculated using t-test with Bonferroni corrections. * = $P<0.05$ **= $P<0.001$

and promote lipid metabolism, gradually leading to the decline of lipid droplet content. Blood glucose is higher in type 2 diabetic and obese patients, and these patients have been shown to have poorer prognosis.[62] The cells were treated with quercetin (50μM and 100μM) for 24 hours with normal media and low glucose media. After the treatment, there was a significant cell loss in low (5mM, normoglycemic) glucose media for both concentrations (Figure 12B). There was no significant difference between the untreated cells in either media in the absence of quercetin. This data suggest that quercetin could be more effective in killing glioblastoma cells in patients with normoglycemia (corresponding to 5mM glucose) than in diabetic patients with hyperglycemia.

Subsequently, we tested whether an ACAT inhibitor, avasimibe, could be used to decrease the number of lipid droplets and increase the effect of quercetin. U251 cells were treated with quercetin (100μM) and avasimibe (10μM) for 24 hours. Avasimibe decreased the cell viability to 61% (Figure 13B). When treated in combination with quercetin, this further decreased the cell viability to 19% (Figure 13C).

To determine whether this decrease in cell viability was caused by the decrease in lipid droplets, we labelled the organelles with Bodipy Alexa-fluor 488. The cells were treated in both normal DMEM and DMEM 5mM glucose to investigate if the increased cytotoxicity of quercetin in DMEM 5mM glucose was related to a decrease in lipid droplets. When the cells were treated with Q, there was an increase in the average number of lipid droplets to 49 lipid droplets per cell from 16 lipid droplets per cell (Figure 13C and D). Moreover, when grown in 5mM glucose, there were fewer lipid droplets than when the cells were grown in DMEM 25mM glucose (Figure 13C and D). Avasimibe alone was able to slightly reduce the number of lipid droplets per cell, but the differences were not significant. However, when the cells were treated with both quercetin and avasimibe, the number of lipid droplets were comparable to the number of lipid droplets at the basal level (Figure 13C and D).


A
Control
Avasimibe
Lipid Droplets
Free cholesterol
Quercetin
Lipid Droplets
Drug sequestration
Avasimibe + Quercetin
ROS
Lipid peroxidation
Cell Death


B
Cell viability (rel. to control)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
***
Control
Quercetin
Avasimibe
Quercetin + Avasimibe

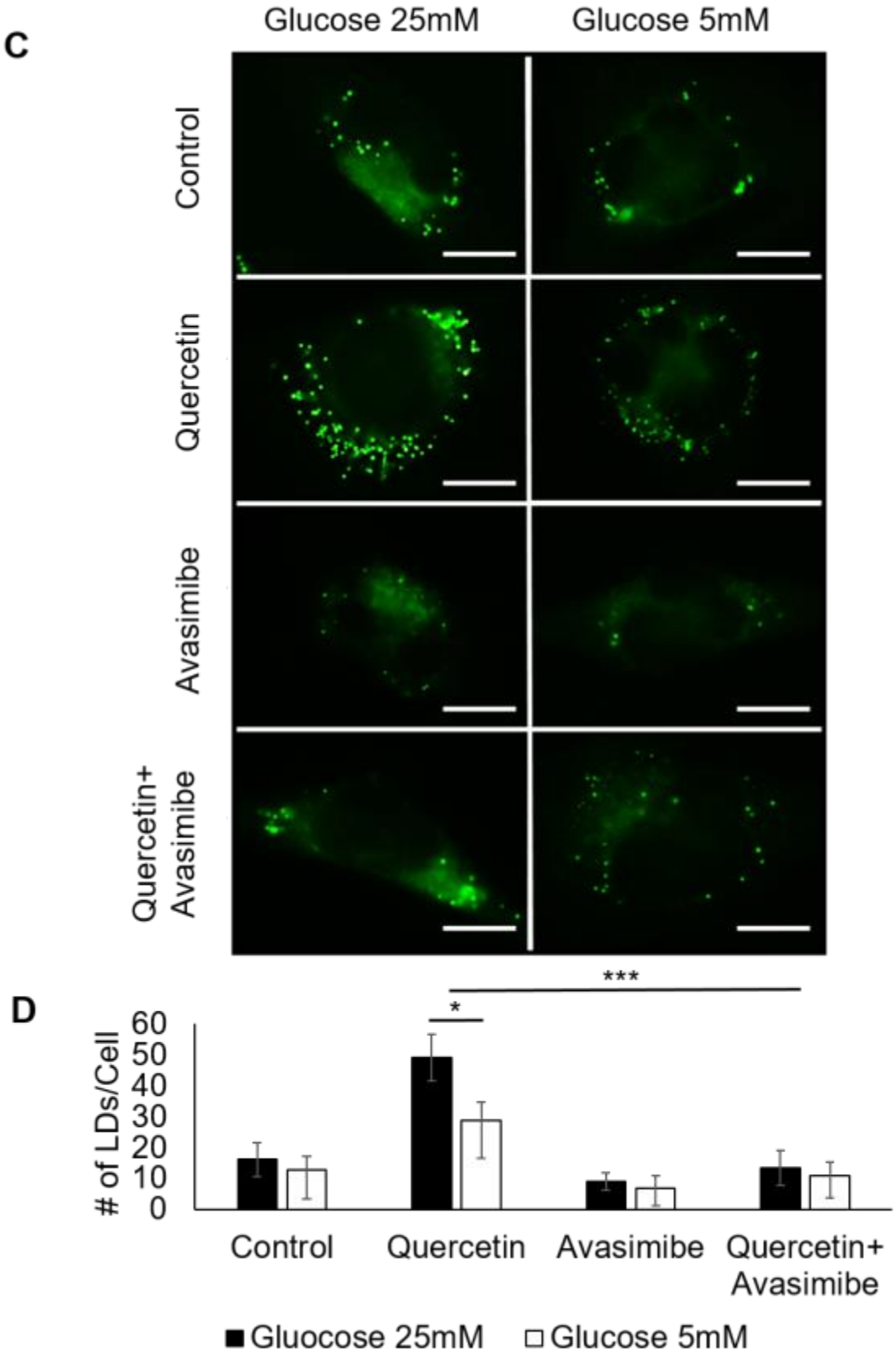

C
Glucose 25mM
Glucose 5mM
Control
Quercetin
Avasimibe
Quercetin+
Avasimibe
D
***
*
# of LDs/Cell
60
50
40
30
20
10
0
Control
Quercetin
Avasimibe
Quercetin+
Avasimibe
Gluocose 25mM
Glucose 5mM

Figure 13. Quercetin induces the formation of lipid droplets in glioblastoma cells

Schematic of quercetin and avasimibe combination therapy (A). Avasimibe sensitizes U251 glioblastoma cells to the anticancer effects of quercetin by reducing drug sequestration in the lipid droplets. Avasimibe also increases the concentration of toxic free cholesterol inside the cell. U251 cells were treated with quercetin (100μM), avasimibe (10μM), and their combinations for 24 hours in 25mM glucose DMEM. After the treatment, the nuclei were stained with Hoechst 33342 (10μM) and the nuclei were quantified to detect cell viability (B). U251 cells were treated with quercetin (100μM), avasimibe (10μM), and their combination for 24 hours in normal DMEM (25mM) and lower glucose (5mM). The lipid droplets were then labelled with Bodipy (20μM) and quantified per cell using ImageJ (C and D). Scale bar = 20μm. Statistically significant differences between the drug treatments were calculated using one-way ANOVA with post hoc Tukey's test. * = $P<0.05$ *** = $P<0.001$

Furthermore, we treated U251 glioblastoma spheroids with quercetin (100μM) and avasimibe (10μM) for 7 days. The combination treatment, however, did not induce greater cell death than the single drug treatments in comparison (Figure 14 A and B). Lipid droplet staining of the spheroids were not conducted in this experiment, but will be performed in the future to determine whether lipid droplet had an effect in the outcome of the combination treatment in the spheroids. Furthermore, we investigated other signaling pathways regulated by quercetin.

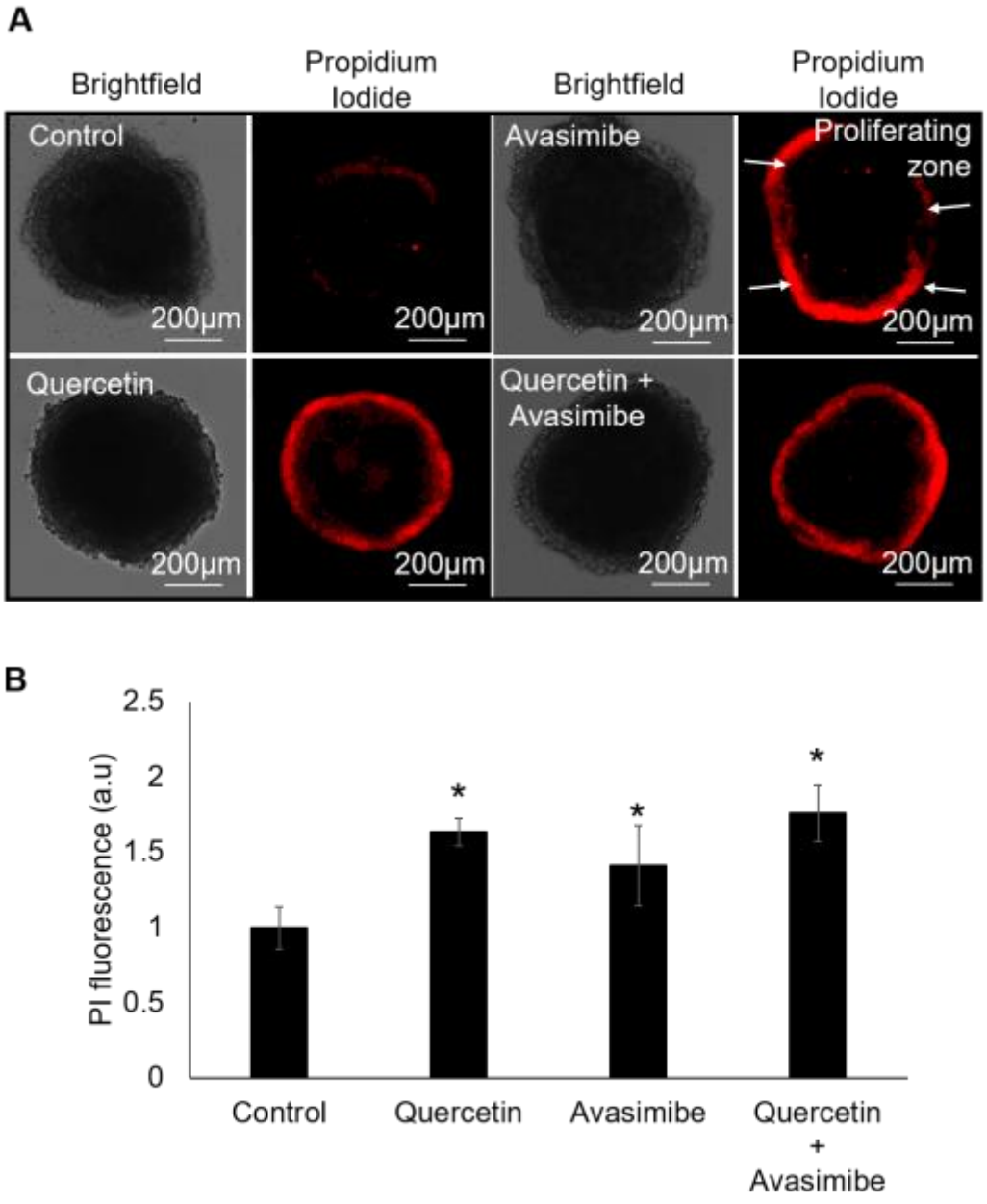


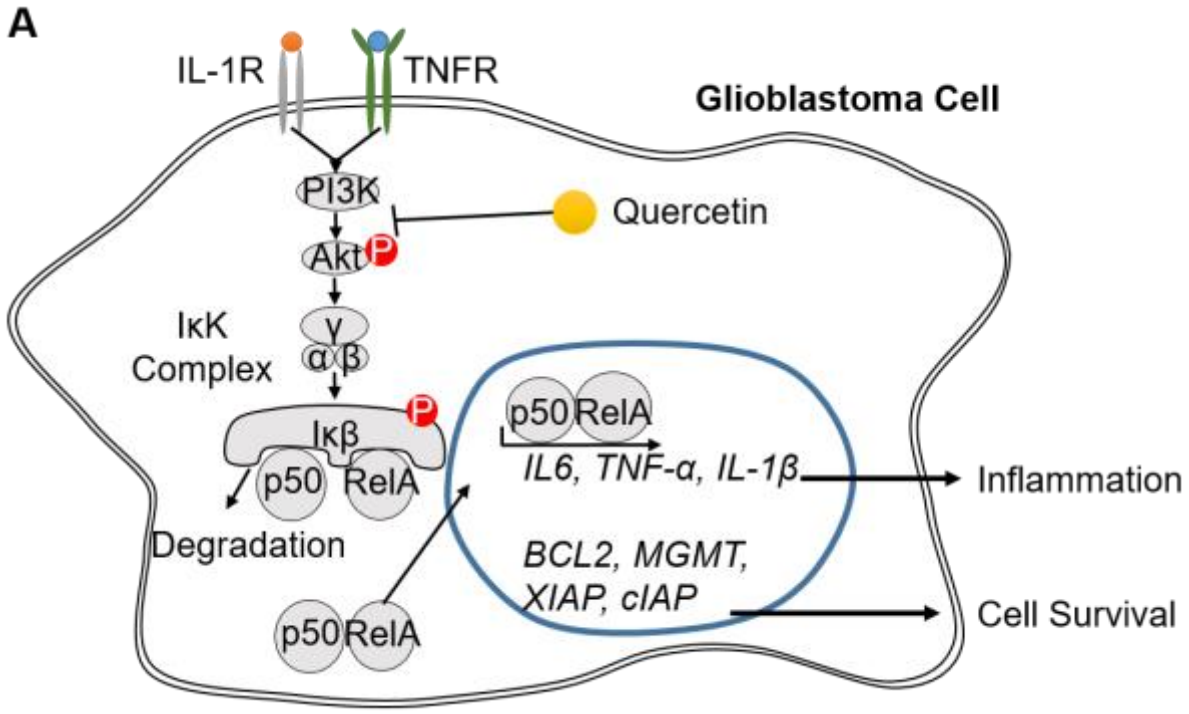
Figure 14. Quercetin and Avasimibe induces cell death in U251 spheroids

U251 spheroids were treated with quercetin (100µM), avasimibe (10µM), and their combination for 7 days. The spheroids were then stained with Propidium Iodide (1.5µM) (E). The relative fluorescence intensity was analyzed using ImageJ (F). Statistically significant differences

between the drug treatments were calculated using one-way ANOVA with post hoc Tukey's test. * = P<0.05 *** = P<0.001

3.7 Quercetin and NF-κβ nuclear translocation

One of the key transcription factors implicated in quercetin mechanism of action is NF-κβ. NF-κβ is downstream from the PI3K/Akt pathway. This pathway is the main survival pathway in many cancer cells including glioblastoma.[62] NF-κβ nuclear translocation and activity promotes cell survival and tumor invasiveness.[63] Quercetin is a pleiotropic kinase inhibitor, and it has been previously shown to inhibit PI3K and its downstream effects.[39, 42] Thus, the inhibition of PI3K and its downstream effectors promote cell death



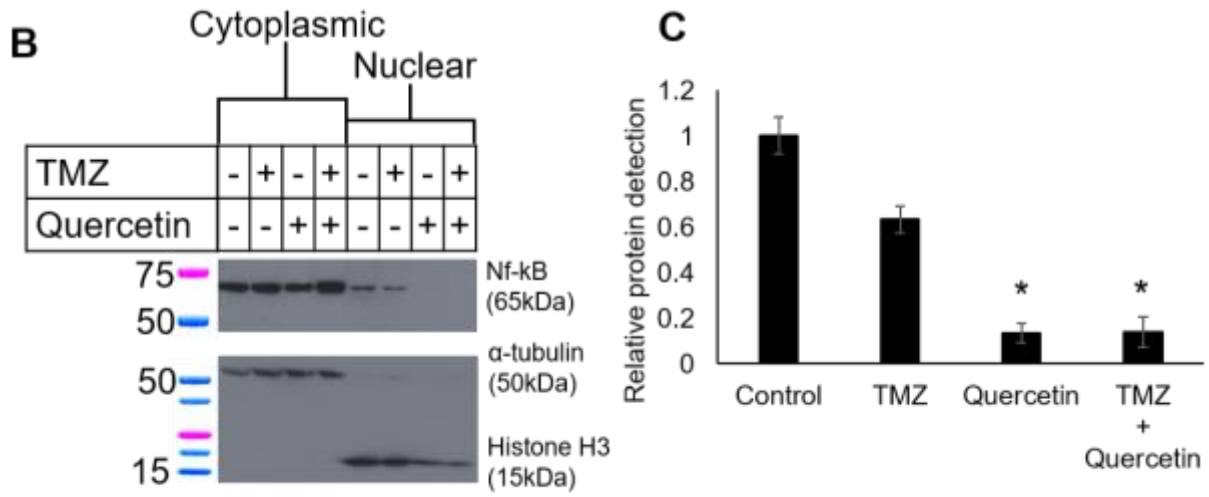


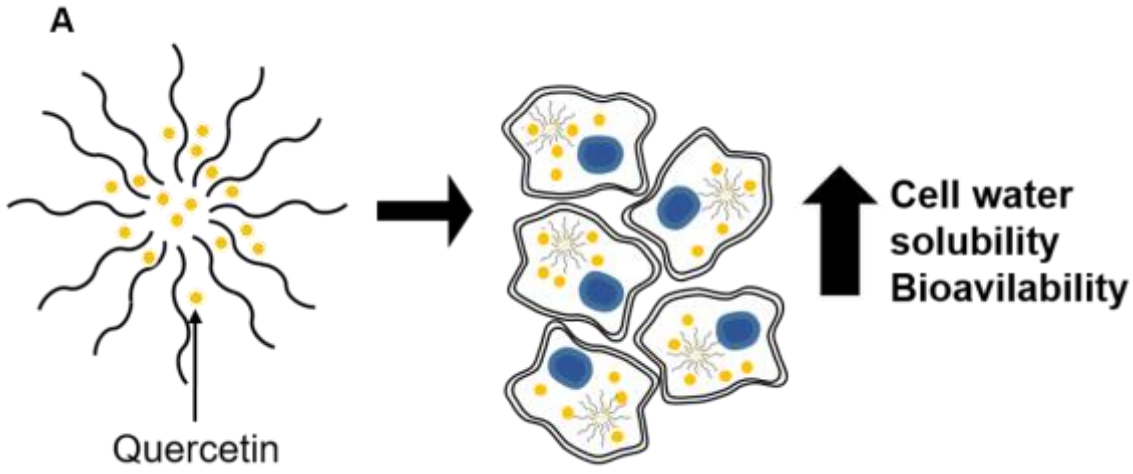
Figure 15. Mechanism of quercetin action through the inhibition of NF-κβ nuclear translocation

Upon stimulation by cytokines, the PI3K/Akt pathway is activated in glioblastoma cells (A). Downstream of this signaling pathway is the NF-κβ transcription factor. NF-κβ is normally bound to Iκβ and sequestered in the cytosol. However, upon phosphorylation by IκK, Iκβ releases NF-κβ, and NF-κβ translocates to the nucleus to facilitate inflammation and cell survival. Nuclear fractionation western blots were done to detect the nuclear translocation of NF-κβ upon quercetin treatment after 24 hours (B). The nuclear detection was quantified using ImageJ (C). Statistically significant differences were calculated using the t-test with Bonferroni corrections. * = $P<0.05$.

and reduces drug resistance. Translocation of NF-κβ is significantly inhibited by quercetin treatment after 24 hours (Figure 14B and C) suggesting that genes regulated by NF-κβ will be downregulated (Figure 14A).

3.8 Quercetin Nanodelivery using PEG-PLGA Micelles

One of the limitations of quercetin as a therapeutic agent is its poor water solubility and low bioavailability. To overcome this problem, quercetin was incorporated into nanocarriers. Quercetin is a highly lipophilic drug, and it cannot be administered intravenously. When ingested, quercetin can be metabolized in the gastrointestinal tract.[27] In our study, we have chosen to encapsulate quercetin in PEG-PLGA micelles to increase its solubility in media. PEG-PLGA micelles were prepared by the co-solvent evaporation method in THF/water mixture in the presence of Q and characterized using AF_4/UV/DLS for size and size distribution analysis (Figure 15A and B). UV-absorbance spectrometry was used to measure quercetin loading and determine encapsulation efficiency which was equal to 41% for the linear PEG-PLGA copolymer based micelles (Figure 15C and D). Stock solutions of 1.2mM of quercetin in water were reached after encapsulation into the PEG-PLGA micelles and removal of the non-encapsulated quercetin, which corresponds to a 115-fold solubility enhancement.



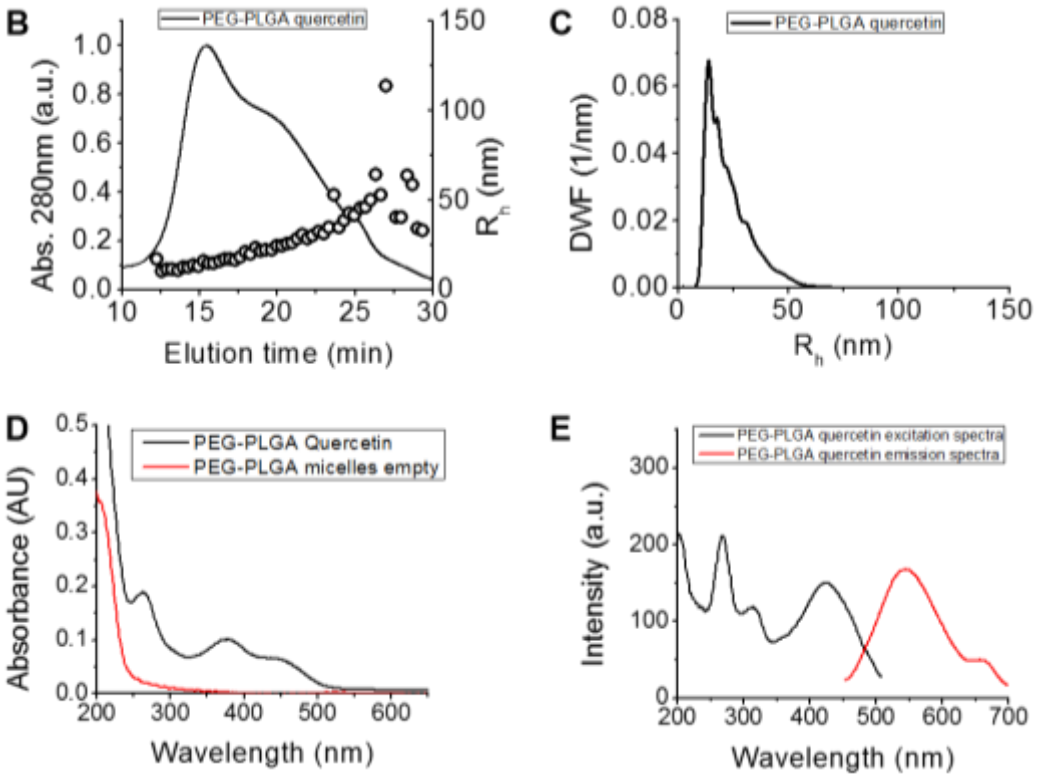
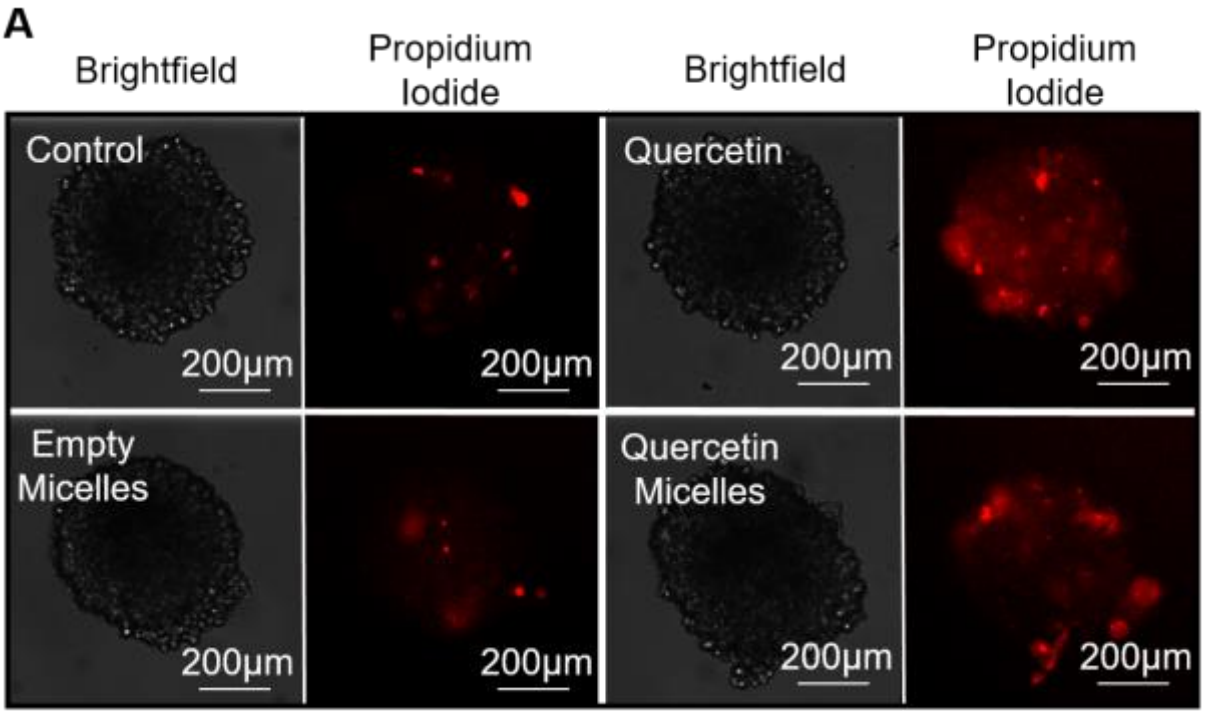


Figure 16. Micelles preparation for quercetin encapsulation

The PEG-PLGA micelles have been characterized using free flow fractionation (FFF) (A). Size characterization of PEG-PLGA based micelles loaded with Quercetin. 50 µL of a solution containing micelles was injected in an AF4/UV/DLS system. Elution of the micelles was monitored using UV absorbance at 280 nm and the size of the micelles was measured by dynamic light scattering (DLS). Fractogram of the micelles eluting out of the AF4 system. Size distribution of the micelles reported as the differential weight fraction of the micelles as a function of their hydrodynamic radii(B). Spectral characterization of Quercetin containing micelles (C). UV absorbance spectra of empty PEG-PLGA micelles and quercetin-containing micelles measured in water. (D) Fluorescence excitation and emission spectra of quercetin containing micelles (λex = 421nm; λem = 541nm).

U251 spheroids were treated with empty micelles, quercetin (100μM), and quercetin micelles (100μM) for 7 days. The propidium iodide relative fluorescence was used to detect the cytotoxic effect of quercetin encapsulated in the PEG-PLGA micelles. The empty micelles did not induce cytotoxicity after 7 days (Figure 12). Both the free drug quercetin and quercetin incorporated micelles induced cell death as shown by PI fluorescence in the U251 spheroids (Figure 16A and B). Although there was no significant difference between the free drug and the nanodelivered drug, we show that quercetin encapsulated in micelles retains its anti-cancer effects.



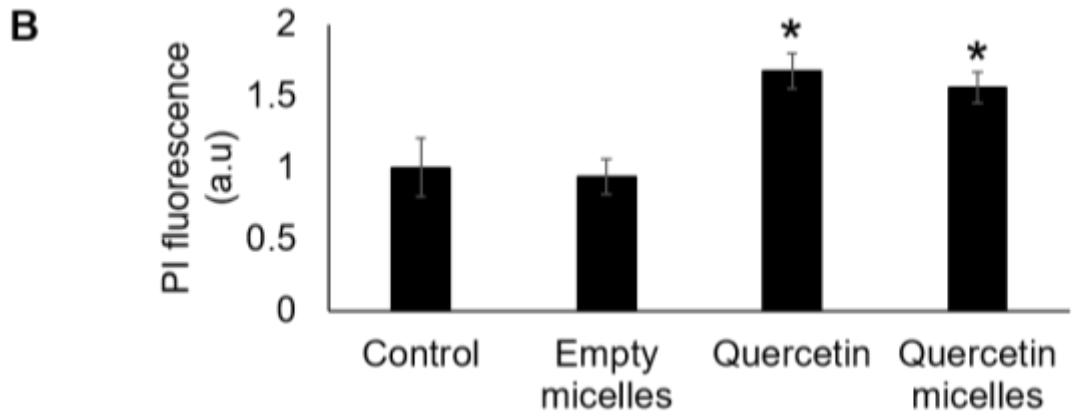


Figure 16. Glioblastoma cell death induced by quercetin released from micelles

U251 spheroids were treated with quercetin (100μM) as a free drug and encapsulated in PEG-PLGA micelles with equivalent concentrations (100μM) for 7 days (A). The spheroids were then stained with propidium Iodide (1.5μM). The fluorescence intensity was measured using ImageJ (B). Statistically significant differences were calculated using one-way ANOVA with post hoc Tukey's test. * = $P<0.05$

4. General Discussion

The studies presented in this thesis address several questions related to the limitations of current treatment of glioblastoma multiforme and provide novel findings with combination therapy. Due to the poor outcome with temozolomide therapy, we tested a phytochemical, pleiotropic compound quercetin which has not been previously tested for glioblastoma multiforme and associated brain tumor stem cells. We also investigated drugs in combination: quercetin plus avasimibe. We selected avasimibe because it is currently in clinical trials as an anticancer agent.[50] It effectively reduces lipid droplet formation through the inhibition of cholesterol esterification. Lipid droplets are unique organelles which accumulate lipophilic compounds and can reduce effectiveness of anticancer agents by redirecting them away from their intracellular targets.

The study of phytochemicals for cancer therapy has been increasingly more popular due to the pleiotropic effects of these biological compounds.[64] Cancer heterogeneity is a limiting factor for many monotherapies, which only effectively kills the actively dividing cancer cells. Phytochemical compounds are attractive oncotherapeutics because they are usually well tolerated and their side effects are less frequent and milder than those induced by temozolomide. In addition, phytochemicals often attenuate or block several signal transduction pathways implicated in tumor progression. The main limitation of these biological compounds is the low bioavailability due to their low water solubility and instability when administered orally.[65] We addressed this issue by employing a nanodelivery system made of biodegradable polymer.

The experiments in this thesis were designed to: (1) establish the time and concentration dependent anti-cancer effects of quercetin; (2) increase the cell killing effect of quercetin by inhibiting lipid droplet formation; and (3) utilize PEG-PLGA micelles to deliver quercetin in a glioblastoma spheroid model.

We show that quercetin decreases U251 glioblastoma cell viability in monolayer and 3-D spheroids. When compared to the current clinical drug, TMZ, quercetin was more effective in both 2D and 3D models (Section 3.1-3.2). We have also shown that quercetin inhibits the growth of brain tumor stem cells. (Section 3.3). Then, we revealed the functional impairments induced by quercetin i.e. loss of mitochondrial potential and significant ROS production, particularly after the pharmacological depletion of glutathione. (Section 3.4).

Clinical studies suggest that obesity and cachexia play negative role in patients treated with anticancer agents.[66] This observation has not been studied in detail in such studies. We therefore investigated lipid droplets, organelles which are abundant in cells of obese patients or in experiments where cells were exposed to high amounts of nutrients. Lipid droplets are ubiquitous organelles which have multiple functions including drug sequestration and communication with other organelles.[52] The results from our experiments indicate that quercetin increases lipid droplet formation in the glioblastoma cells (Section 3.5), most likely as the cell's defense mechanism from quercetin-induced ROS formation and lipid peroxidation. To weaken the defense through lipid droplet accumulation, we used avasimibe, an ACAT inhibitor, to reduce

cholesterol esterification and lipid droplet number. This resulted with greater glioblastoma cell death by quercetin in monolayer and but not in 3-D spheroids (Section 3.5-3.6). Finally, we explored the use of quercetin encapsulated in PEG-PLGA micelles as a nanocarrier for glioblastoma treatment. The use of PEG-PLGA increased the solubility of quercetin in water by ~250 fold and lead to a significantly higher glioblastoma cell death than following quercetin treatment without nanocarrier.

In summary, results from this thesis show that: (1) quercetin induces cell death in both glioblastoma cells and brain tumor stem cells; (2) the anticancer effect of quercetin is enhanced when the number of lipid droplets are decreased using avasimibe; and (3) PEG-PLGA micelles are suitable nanocarriers for quercetin which overcome quercetin poor water solubility. Further implications of the results and limitations of our studies are elaborated in the following sections.

4.1 Quercetin – Cytotoxicity

Quercetin is a widely consumed flavonoid in the western diet. It has been studied extensively for its anti-inflammatory, anti-diabetic, anti-biotic, anti-viral, and anti-cancer effects.[67, 68, 69] However, due to its pleiotropic effects, its mechanisms of action are not well understood. The anti-cancer effects of quercetin have been studied in many different models such as: breast cancer, lung cancer, liver cancer, stomach cancer, and brain cancer cells.[70, 71, 72] The main commonality between all these models is that

quercetin induces cytoprotective effects on normal cells and cytotoxic effects on cancer cells. These opposing effects of quercetin depend on cell type and major signal transduction pathways turned on by this drug. Importantly, quercetin inhibits cancer cells proliferation, kills senescent stem cells, and enhances the effect of other chemotherapeutics.[36, 37, 38]

Our study has shown that quercetin has a cytotoxic effect on U251 glioblastoma cells and brain tumor stem cells, and it is non-toxic to primary astrocytes. Many different mechanisms of quercetin action have been studied to prevent the proliferation of cancer cells. Quercetin functions as a pleiotropic kinase inhibitor; it inactivates the PI3K-Akt pathway, which regulates nuclear factor κB (NF-κB).[39, 42] NF-κB is a master regulator of cell survival, inflammation, and immunity.[73, 74, 75, 76] In the canonical pathway, NF-κB homodimers or heterodimers are retained in the cytoplasm by IκB through a noncovalent interaction. In the case of an external or internal stimuli such as ROS, growth factors, cytokines, or DNA damage, IκB is phosphorylated by IκK, which separates it from NF-κB, allowing it to translocate to the nucleus. NF-κB regulates the transcription of many cytokines, growth factors, and regulators of apoptosis. The inhibition of NF-κB nuclear translocation induces tumor cell apoptosis. We (Figure 14) and others have shown that quercetin inhibits nuclear translocation of NF-κB by acting as a kinase inhibitor and prevents phosphorylation of Akt by PI3K.[39,42] Additionally, quercetin regulates the mTOR, p38-MAPK, and JNK pathways to induce cell death; these mechanisms are not fully understood.[77, 78, 79, 80]

Most chemotherapeutics target actively dividing cells which have greater proliferative capacity than the corresponding non-transformed cells. Similarly, cancer stem cells are poorly responding to anticancer therapeutics due to their senescence.[77, 78] Quercetin has been shown to kill senescent cells and has an anti-aging effect in worms.[81, 82].[83, 84] In glioblastoma patients, brain tumor stem cells contribute to the recurrence of cancer after therapy and occurrence in other brain regions.[85] Since quercetin does not have much of the killing effect in non-transformed, not-rapidly dividing cells, it is leaving the "normal" cells relatively undamaged. It is an interesting observation which requires careful analysis and comparison of signal transduction pathways in tumor versus healthy tissues.

4.2 Quercetin – Inflammation

In addition to the stem cells, the glioblastoma tumor mass is composed of tumor-associated macrophages (TAMs), which facilitate tumor growth and metastasis.[11] The relationship between inflammation and cancer has been a controversial discussion for many years. Many studies show that a pro-inflammatory environment provides a cancer killing effect, while an anti-inflammatory environment induces the proliferation and migration of the cancer cells.[12, 13, 14] However, prolonged pro-inflammatory signals are also detrimental to the brain tissue and cause neurodegeneration.[86, 87] Therefore, these two inflammatory states are not dichotomous, and need to be further investigated. Quercetin is a powerful anti-inflammatory agent, which increases the expression and activity of Nuclear factor (erythroid-derived)-like 2 (Nrf2) in microglia. Increased Nrf2

upregulation has been observed in cancer patients in correlation with the WHO grade for glioblastoma.[88] Nrf2 regulates the expression of anti-inflammatory proteins such as heme oxygenase-1, glutathione S-transferases, glutathione peroxidase, superoxide dismutase, and catalase.[82] Thus, future studies are required to investigate whether quercetin increases the activation of Nrf2 in the glioblastoma and potentially increases the cancer's defense mechanism against inflammation.

One limitation of our studies is lack of human primary microglia, astrocytes, endothelial cells and TAMs to perform critical co-culture experiments in 3-D. Developing a 3-D model with glioblastoma spheroids with microglia would have been an ideal model to study the relationship with glioblastoma and inflammation. Lipopolysaccharide (LPS) could be used as a stimulus to induce signal transduction pathways and activate microglia. However, in a separate study,[89] we have employed similar experimental paradigm where microglia were hyperactivated and quercetin was able to partly reduce microglia hyperactivity. Ongoing experiments are set to investigate the role of brain tumor stem cells in glioblastoma progression in a 3-D models in the presence of U251 cells and glia. So far most of the reports use monolayers and some co-cultures. However, better models resembling complex tumor microenvironment (e.g. organoids or 3D co-cultures and in vivo animal models) are required to study glioblastoma progression (without treatments) and regression (with chemotherapeutic interventions).

4.3 Quercetin – Mitochondria and ROS

Our studies showed that quercetin induces ROS production and mitochondrial potential disruption in U251 glioblastoma cells. Redox therapy has been explored as a possible mode of cancer treatment by enhancing ROS production and consequently, ROS-mediated apoptosis.[90] ROS production and mitochondrial membrane potential go hand in hand because mitochondria are the largest sources of ROS in the cell.[91] Quercetin has been previously shown to bind to mitochondrial membrane Bcl-2 family proteins.[92] Bcl-2 is an anti-apoptotic protein, which is upregulated in cancer cells and prevents apoptosis by stabilizing the mitochondrial membrane and preventing cytochrome c release.[93, 94] Bcl-2 inhibition by quercetin will destabilize of the mitochondrial membrane, which will cause the loss of mitochondrial membrane potential. Thus, when the mitochondrial membrane becomes leaky, mitochondrial ROS then diffuses into the cytoplasm and induces cell damage via DNA oxidation, protein oxidation, and lipid peroxidation. Therefore, aside from producing ROS directly, quercetin can indirectly increase the concentration of intracellular ROS by inhibiting mitochondrial membrane proteins.

4.4 Quercetin – Lipid Droplets

Recent studies on lipid droplets have shown that lipid droplets have a protective role in cancer cells thereby promoting drug resistance.[95] This protective role of the lipid droplets are especially highlighted in obese patients undergoing treatment for cancer.[93] They are prescribed ketogenic diets to reduce carbohydrate intake, forcing the cancer

cells to metabolize fat as a source of energy.[96] Since lipid droplets sequester lipophilic anticancer drugs, an increased number or size of lipid droplets will greatly reduce the effectiveness of chemotherapeutics.[97] For example, the entrapment of TMZ in the lipid droplet prevents the drug from reaching its target site of action (nucleus) to cause DNA damage. Similarly, quercetin is a lipophilic drug, and lipid droplets act as physical compartments sequestering the drug and restricting its access to the target proteins. In our study, we employed conditions which reflect large or low content of lipid droplets by using low and high glucose media and pharmacological agents. Under normoglycemic conditions (5mM glucose in the media), lipid droplets can serve as a source of energy for glioblastoma growth and proliferation. However, a recent study has shown that blood glucose levels are not indicative of the prognosis of the patient.[62] The authors propose that in type 2 diabetic patients, the insulin-like growth factor-1 (IGF-1) receptor pathway is activated and it regulates the cell cycle in various cells, including astrocytes. Patients with diabetes have high amounts of circulating insulin caused by insulin resistance. This increased insulin is likely to activate IGF-1 receptors and regulate cell metabolism. Therefore, when serum deprived media was used in our experiment, the lack of IGF-1 receptor activation could have been dysregulated in the glioblastoma cells, resulting in cell cycle and growth impairments.

Another protective role of lipid droplets is the prevention of lipid peroxidation by ROS.[98] In glial cells, mitochondrial defects promote ROS production and lipid droplet formation.[99] Similar findings showing ROS-promoting lipid droplet formation was

reported in lymphoma cells under stressful conditions.[100] This study suggests that the accumulation of lipid droplets is caused by the inhibition of fat metabolism, rather than an increase in fat biogenesis.[92] The authors proposed that the lipid droplet accumulation was due to the inhibition of mitochondrial fatty acid β-oxidation and the re-direction of fatty acids from oxidation to accumulation in lipid organelles. Thus, a potential explanation for lipid droplet accumulation in glioblastoma cells treated with quercetin is that the intracellular ROS produced by the disrupted mitochondria inhibited fatty acid oxidation and caused the accumulation of lipid droplets. This accumulation of fatty acids in lipid droplets can be considered as a defense mechanism of glioblastoma cell against quercetin ROS-induced lipid peroxidation by accumulating the cholesterol esters and fatty acid esters within the lipid droplets. Hence, this makes the fat not easily accessible to ROS from the cytosol. If so, pharmacological intervention which reduces cholesterol esterification could result with a more extensive cell death due to lipotoxicity caused by lipid peroxidation. To achieve this goal we used avasimibe which significantly reduced a number of lipid droplets. In turn, this reduction reduced glioblastoma cell viability with quercetin treatment more effectively than with either of the individual drugs.

4.5 Quercetin Nanodelivery

As previously discussed, the bioavailability of quercetin is very low due to its insolubility in aqueous solutions and it is quickly metabolized by the digestive tract. Thus, much effort has been made to develop and test nanocarriers for quercetin.[101, 102, 103, 104, 105] The most common are PLGA- and PLA-based nanoparticles as carriers for quercetin.[106]

These polymers form micelles, which accommodate lipophilic drugs in their cores.[107] In our study, we used PEG-PLGA polymers because the PEG provides a corona which is miscible with aqueous medium and contributes to the stability of the micelles.[108] Our laboratory has used micelles made of several polymeric materials but only those ones employed in this thesis are FDA approved. Other studies have used PEG-PLGA polymers for quercetin delivery in HEPG2 cancer cells,[109] however, these studies were done in monolayer cultures, and do not show the effect of quercetin nanodelivery in a 3-D model system. Pilot experiments were conducted *in vivo* with PEG PLGA micelles as drug carriers but they were not performed with incorporated quercetin.[110, 111]

In our study, we used PEG-PLGA micelles encapsulating quercetin, and treated them to glioblastoma spheroids. The encapsulated quercetin resulted in comparable cell killing effect to free quercetin. Micelles are avidly internalized by glioblastoma cells turning on intracellular signaling pathways and promoting cell death. Such a statement is only valid when the data are obtained by cell counting and not by measurement of fluorescence intensities because we noticed a small but measurable energy transfer between quercetin and fluorescent dyes.

Aside from non-specific internalization of nanocarriers by rapidly dividing cells, efforts were made to modify nanocarrier surfaces with targeting moieties, e.g. ligands and antibodies. Two most commonly studied targeting moieties for cancer cells are folic acid and epidermal growth factor (EGF).[112, 113] Folic acid receptors (FR) and EGF receptors

(EGFR) are highly expressed in cancer cells, including glioblastoma cells, and since these receptors are expressed on the surface of the cells, they can be used to target the nanocarriers directly to the cancer cells. FRs are expressed in normal cells, but less than in transformed cells in the lungs, kidneys, placenta, and choroid plexus.[114] Folate is a water-soluble B vitamin that is essential for DNA synthesis, methylation, and repair.[106] It is inexpensive, easily produced, and very stable. Since glioblastoma express higher number of folic acid receptors than normal astrocytes and quercetin does not significantly reduce the viability of astrocytes, micelles with folic acid modified corona could be of interest in a limited number patients with strong enhanced and permeability (EPR) effect.[115] However, considering that these are more complex and more expensive delivery systems with restricted applicability to the glioblastoma patients, we did not investigate ligand-modified nanocarriers.

In summary, significant elimination of glioblastoma cells with quercetin alone, incorporated into micelles combined with another therapeutic merit further investigations in experimental animals with intracranial tumors such as astrocytomas.

Conclusions

This thesis work demonstrates the use of quercetin as an anti-cancer agent for the treatment against GBM. Sensitization of GBM cells with avasimibe enhances chemotherapeutic efficacy of quercetin and it is achieved by inhibiting lipid droplet biosynthesis. Additionally, polymeric nanocarriers used to incorporate quercetin can overcome solubility problems common with highly liposoluble drugs. Results from these studies suggest that quercetin combined with avasimibe merit investigations in glioblastoma models in experimental animals and eventually in humans.

Specific findings reported in this thesis are:

(1) Quercetin induces cell death in both glioblastoma cells and brain tumor stem cells;

(2) The anticancer effect of quercetin is increased when the number of lipid droplets are reduced using avasimibe, and;

(3) PEG-PLGA is an FDA approved, biodegradable polymer which can enhance quercetin solubility in biological media and merits testing *in vivo*.

References

1. Omuro, Antonio, and Lisa M. DeAngelis. "Glioblastoma and other malignant gliomas: a clinical review." *Jama* 310.17 (2013): 1842-1850

2. Roy, Sanjoy, Debarshi Lahiri, Tapas Maji, and Jaydip Biswas. "Recurrent glioblastoma: where we stand." *South Asian journal of cancer* 4.4 (2015): 163.

3. Marucci, G., P. V. Fabbri, L. Morandi, D. De Biase, E. Di Oto, G. Tallini, C. Sturiale, E. Franceschi, G. P. Frezza, and M. P. Foschini. "Pathological spectrum in recurrences of glioblastoma multiforme." *pathologica* 107.1 (2015): 1-8.

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

5. Alifieris, Constantinos, and Dimitrios T. Trafalis. "Glioblastoma multiforme: Pathogenesis and treatment." *Pharmacology & therapeutics* 152 (2015): 63-82.

6. Carlsson, Steven K., Shaun P. Brothers, and Claes Wahlestedt. "Emerging treatment strategies for glioblastoma multiforme." *EMBO molecular medicine* 6.11 (2014): 1359-1370.

7. Eder, Katalin, and Bernadette Kalman. "Molecular heterogeneity of glioblastoma and its clinical relevance." *Pathology & Oncology Research* 20.4 (2014): 777-787.

8. Verhaak, Roel GW, Katherine A. Hoadley, Elizabeth Purdom, Victoria Wang, Yuan Qi, Matthew D. Wilkerson, C. Ryan Miller et al. "Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1." *Cancer cell* 17.1 (2010): 98-110.

9. Johansson, Fredrik, Simon Ekman, Erik Blomquist, Roger Henriksson, Stefan Bergström, and Michael Bergqvist. "A review of dose-dense temozolomide alone and in combination with bevacizumab in patients with first relapse of glioblastoma." *Anticancer research* 32.9 (2012): 4001-4006.

10. Yong, Raymund L., and Russell R. Lonser. "Surgery for glioblastoma multiforme: striking a balance." *World neurosurgery* 76.6 (2011): 528.

11. Hambardzumyan, Dolores, David H. Gutmann, and Helmut Kettenmann. "The role of microglia and macrophages in glioma maintenance and progression." *Nature neuroscience* 19.1 (2016): 20-27.

12. Nimmerjahn, Axel, Frank Kirchhoff, and Fritjof Helmchen. "Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo." *Science*308.5726 (2005): 1314-1318.

13. Hussain, S. Farzana, David Yang, Dima Suki, Kenneth Aldape, Elizabeth Grimm, and Amy B. Heimberger. "The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses." *Neuro-oncology* 8.3 (2006): 261-279.

14. Sowers, James L., Kenneth M. Johnson, Charles Conrad, Joel T. Patterson, and Lawrence C. Sowers. "The role of inflammation in brain cancer." In *Inflammation and Cancer*, pp. 75-105. Springer Basel, 2014.

15. Galvão, Rui Pedro, and Hui Zong. "Inflammation and gliomagenesis: bi-directional communication at early and late stages of tumor progression." *Current pathobiology reports* 1.1 (2013): 19-28.

16. Wang, Hongxiang, Tao Xu, Ying Jiang, Hanchong Xu, Yong Yan, Da Fu, and Juxiang Chen. "The challenges and the promise of molecular targeted therapy in malignant gliomas." *Neoplasia* 17.3 (2015): 239-255.

17. Gabrusiewicz, Konrad, Benjamin Rodriguez, Jun Wei, Yuuri Hashimoto, Luke M. Healy, Sourindra N. Maiti, Ginu Thomas et al. "Glioblastoma-infiltrated innate immune cells resemble M0 macrophage phenotype." *JCI insight* 1.2 (2016).

18. Zhou, Wenchao, Susan Q. Ke, Zhi Huang, William Flavahan, Xiaoguang Fang, Jeremy Paul, Ling Wu et al. "Periostin secreted by glioblastoma stem cells recruits M2

tumour-associated macrophages and promotes malignant growth." *Nature cell biology* 17.2 (2015): 170-182.

19. Dean, Michael, Tito Fojo, and Susan Bates. "Tumour stem cells and drug resistance." *Nature Reviews Cancer* 5.4 (2005): 275-284.

20. Ravishankar, Divyashree, Amit Kumar Rajora, Francesca Greco, and Helen MI Osborn. "Flavonoids as prospective compounds for anti-cancer therapy." *The international journal of biochemistry & cell biology* 45.12 (2013): 2821-2831.

21. Chahar, Maheep K., Neelu Sharma, Mahabeer P. Dobhal, and Yogesh C. Joshi. "Flavonoids: a versatile source of anticancer drugs." *Pharmacognosy reviews* 5. 9 (2011): 1.

22. Batra, Priya, and Anil K. Sharma. "Anti-cancer potential of flavonoids: recent trends and future perspectives." *3 Biotech* 3.6 (2013): 439-459.

23. Sak, Katrin. "Cytotoxicity of dietary flavonoids on different human cancer types." *Pharmacognosy reviews* 8.16 (2014): 122.

24. Ravishankar, Divyashree, Amit Kumar Rajora, Francesca Greco, and Helen MI Osborn. "Flavonoids as prospective compounds for anti-cancer therapy." *The international journal of biochemistry & cell biology* 45.12 (2013): 2821-2831.

25. Das, Arabinda, Naren L. Banik, and Swapan K. Ray. "Flavonoids activated caspases for apoptosis in human glioblastoma T98G and U87MG cells but not in human normal astrocytes." *Cancer* 116.1 (2010) : 164-176.

26. Santos, B. L., A. R. Silva, B. P. S. Pitanga, C. S. Sousa, M. S. Grangeiro, B. O. Fragomeni, P. L. C. Coelho et al. "Antiproliferative, proapoptotic and morphogenic effects of the flavonoid rutin on human glioblastoma cells." *Food chemistry* 127. 2 (2011): 404-411.

27. D'Andrea, Gabriele. "Quercetin: a flavonol with multifaceted therapeutic applications?." *Fitoterapia* 106 (2015): 256-271.

28. Srivastava, Shikha, Ranganatha R. Somasagara, Mahesh Hegde, Mayilaadumveettil Nishana, Satish Kumar Tadi, Mrinal Srivastava, Bibha Choudhary, and Sathees C. Raghavan. "Quercetin, a natural flavonoid interacts with DNA, arrests cell cycle and causes tumor regression by activating mitochondrial pathway of apoptosis." *Scientific reports* 6 (2016).

29. Costa, Lucio G., Jacqueline M. Garrick, Pamela J. Roquè, and Claudia Pellacani. "Mechanisms of neuroprotection by quercetin: counteracting oxidative stress and more." *Oxidative medicine and cellular longevity* 2016 (2016).

30. Sun, Grace Y., Zihong Chen, Kimberly J. Jasmer, Dennis Y. Chuang, Zezong Gu, Mark Hannink, and Agnes Simonyi. "Quercetin attenuates inflammatory responses in BV-2 microglial cells: role of MAPKs on the Nrf2 pathway and induction of heme oxygenase-1." *PloS one* 10.10 (2015): e0141509.

31. Metodiewa, Diana, Anil K. Jaiswal, Narimantas Cenas, Egle Dickancaité, and Juan Segura-Aguilar. "Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product." *Free radical biology and medicine* 26.1 (1999): 107-116.

32. Chang, Yuh-Fang, Chin-Wen Chi, and Jane-Jen Wang. "Reactive oxygen species production is involved in quercetin-induced apoptosis in human hepatoma cells." *Nutrition and cancer* 55.2 (2006): 201-209.

33. Fonseca-Silva, Fernanda, Job DF Inacio, Marilene M. Canto-Cavalheiro, and Elmo E. Almeida-Amaral. "Reactive oxygen species production by quercetin causes the death of Leishmania amazonensis intracellular amastigotes." *Journal of natural products* 76.8 (2013): 1505-1508.

34. Bishayee, K., et al. "Quercetin induces cytochrome-c release and ROS accumulation to promote apoptosis and arrest the cell cycle in G2/M, in cervical carcinoma: signal cascade and drug-DNA interaction." *Cell proliferation* 46.2 (2013): 153-163.

35. Gibellini, Lara, Marcello Pinti, Milena Nasi, Jonas P. Montagna, Sara De Biasi, Erika Roat, Linda Bertoncelli, Edwin L. Cooper, and Andrea Cossarizza. "Quercetin and cancer chemoprevention." *Evidence-Based Complementary and Alternative Medicine* 2011 (2011).

36. Russo, Gian Luigi, Maria Russo, Carmela Spagnuolo, Idolo Tedesco, Stefania Bilotto, Roberta Iannitti, and Rosanna Palumbo. "Quercetin: a pleiotropic kinase inhibitor against cancer." In *Advances in nutrition and cancer*, pp. 185-205. Springer Berlin Heidelberg, 2014.

37. Filipa Brito, Ana, Marina Ribeiro, Ana Margarida Abrantes, Ana Salome Pires, Ricardo Jorge Teixo, Jose Guilherme Tralhao, and Maria Filomena Botelho. "Quercetin in cancer treatment, alone or in combination with conventional therapeutics?." *Current medicinal chemistry* 22. 26 (2015): 3025-3039.

38. Atashpour, Shekoufeh, Shamileh Fouladdel, Tahereh Komeili Movahhed, Elmira Barzegar, Mohammad Hossein Ghahremani, Seyed Nasser Ostad, and Ebrahim Azizi. "Quercetin induces cell cycle arrest and apoptosis in CD133+ cancer stem cells of human colorectal HT29 cancer cell line and enhances anticancer effects of doxorubicin." *Iranian journal of basic medical sciences* 18.7 (2015): 635.

39. Zhang, Xiang-An, Shuangxi Zhang, Qing Yin, and Jing Zhang. "Quercetin induces human colon cancer cells apoptosis by inhibiting the nuclear factor-kappa B Pathway." *Pharmacognosy magazine* 11.42 (2015): 404.

40. Friedmann-Morvinski, Dinorah, Rajesh Narasimamurthy, Yifeng Xia, Chad Myskiw, Yasushi Soda, and Inder M. Verma. "Targeting NF-κB in glioblastoma: A therapeutic approach." *Science advances* 2.1 (2016): e1501292.

41. Zhou, Anwu, Shane Scoggin, Richard B. Gaynor, and Noelle Sevilir Williams. "Identification of NF-κB-regulated genes induced by TNFα utilizing expression profiling and RNA interference." *Oncogene* 22.13 (2003): 2054-2064.

42. Xiang, Tao, Yong Fang, and Shi-xuan Wang. "Quercetin suppresses HeLa cells by blocking PI3K/Akt pathway." *Journal of Huazhong University of Science and Technology [Medical Sciences]* 34.5 (2014): 740-744.

43. Primikyri, Alexandra, Maria V. Chatziathanasiadou, Evdoxia Karali, Eleftherios Kostaras, Michalis D. Mantzaris, Eleftheria Hatzimichael, Jae-Sun Shin et al. "Direct binding of Bcl-2 family proteins by quercetin triggers its pro-apoptotic activity." *ACS chemical biology* 9.12 (2014): 2737-2741.

44. Srinivas, Keerthi, Jerry W. King, Luke R. Howard, and Jeana K. Monrad. "Solubility and solution thermodynamic properties of quercetin and quercetin dihydrate in subcritical water." *Journal of Food Engineering* 100.2 (2010): 208-218.

45. Ramachandran, Lalitha, Kanjoormana Aryan Manu, Muthu K. Shanmugam, Feng Li, Kodappully Sivaraman Siveen, Shireen Vali, Shweta Kapoor et al. "Isorhamnetin inhibits proliferation and invasion and induces apoptosis through the modulation of peroxisome proliferator-activated receptor γ activation pathway in gastric cancer." *Journal of Biological Chemistry* 287.45 (2012): 38028-38040.

46. Thiam, Abdou Rachid, Robert V. Farese Jr, and Tobias C. Walther. "The biophysics and cell biology of lipid droplets." *Nature reviews Molecular cell biology* 14.12 (2013): 775-786.

47. Koizume, Shiro, and Yohei Miyagi. "Lipid Droplets: A Key Cellular Organelle Associated with Cancer Cell Survival under Normoxia and Hypoxia." *International Journal of Molecular Sciences* 17.9 (2016): 1430.

48. Welte, Michael A. "Expanding roles for lipid droplets." *Current Biology* 25.11 (2015): R470-R481.

49. Boren, J., and K. M. Brindle. "Apoptosis-induced mitochondrial dysfunction causes cytoplasmic lipid droplet formation." *Cell Death & Differentiation* 19.9 (2012): 1561-1570.

50. Llaverías, Gemma, Juan C. Laguna, and Marta Alegret. "Pharmacology of the AC AT Inhibitor Avasimibe (CI-1011)." *Cardiovascular Therapeutics* 21.1 (2003): 33-50.

51. Chang, Ta-Yuan, Bo-Liang Li, Catherine CY Chang, and Yasuomi Urano. "Acyl-coenzyme A: cholesterol acyltransferases." *American Journal of Physiology-Endocrinology and Metabolism* 297.1 (2009): E1-E9.

52. Baenke, Franziska, Barrie Peck, Heike Miess, and Almut Schulze. "Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development." *Disease models & mechanisms* 6.6 (2013): 1353-1363.

53. Makadia, Hirenkumar K., and Steven J. Siegel. "Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier." *Polymers* 3.3 (2011): 1377-1397.

54. Lü, Jian-Ming, Xinwen Wang, Christian Marin-Muller, Hao Wang, Peter H. Lin, Qizhi Yao, and Changyi Chen. "Current advances in research and clinical applications of PLGA-based nanotechnology." *Expert review of molecular diagnostics* 9.4 (2009): 325-341

55. Burgoyne, L. A. "The mechanisms of pyknosis: hypercondensation and death." *Experimental cell research* 248.1 (1999): 214-222.

56. Baba, Alecsandru Ioan, and Cornel Câtoi. *Comparative oncology*. Bucharest: Publishing House of the Romanian Academy, 2007.

57. Martz, Eric, and Malcolm S. Steinberg. "The role of cell-cell contact in “contact” inhibition of cell division: A review and new evidence." *Journal of cellular physiology* 79.2 (1972): 189-210.

58. Nicoletti, I., G. Migliorati, M. C. Pagliacci, F. Grignani, and C. Riccardi. "A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry." *Journal of immunological methods* 139.2 (1991): 271-279.

59. Drew, Roger, and John O. Miners. "The effects of buthionine sulphoximine (BSO) on glutathione depletion and xenobiotic biotransformation." *Biochemical pharmacology* 33.19 (1984): 2989-2994.

60. Aoyama, Koji, and Toshio Nakaki. "Glutathione in cellular redox homeostasis: association with the excitatory amino acid carrier 1 (EAAC1)." *Molecules* 20.5 (2015): 8742-8758.

61. Santos, Claudio R., and Almut Schulze. "Lipid metabolism in cancer." *The FEBS journal* 279.15 (2012): 2610-2623.

62. Chambless, Lola B., Scott L. Parker, Laila Hassam-Malani, Matthew J. McGirt, and Reid C. Thompson. "Type 2 diabetes mellitus and obesity are independent risk factors for poor outcome in patients with high-grade glioma." *Journal of neuro-oncology* 106.2, (2012): 383-389.

63. Bentires-Alj, Mohamed, Veronique Barbu, Marianne Fillet, Alain Chariot, Biserka Relic, Nathalie Jacobs, Jacques Gielen, Marie-Paule Merville, and Vincent Bours. "NF-[kappa] B transcription factor induces drug resistance through MDR1 expression in cancer cells." *Oncogene* 22.1 (2003): 90.

64. Vidak, Marko, Damjana Rozman, and Radovan Komel. "Effects of flavonoids from food and dietary supplements on glial and glioblastoma multiforme cells." *Molecules* 20.10 (2015): 19406-19432.

65. Thilakarathna, Surangi H., and H. P. Rupasinghe. "Flavonoid bioavailability and attempts for bioavailability enhancement." *Nutrients* 5.9 (2013): 3367-3387.

66. Tsai, V. W. W., S. Lin, D. A. Brown, A. Salis, and S. N. Breit. "Anorexia-cachexia and obesity treatment may be two sides of the same coin: role of the TGF-b superfamily cytokine MIC-1/GDF15." *International Journal of Obesity* 40.2 (2016): 193.

67. Askari, Gholamreza, Reza Ghiasvand, Awat Feizi, Syed Mustafa Ghanadian, and Jahangir Karimian. "The effect of quercetin supplementation on selected markers of inflammation and oxidative stress." *Journal of Research in Medical Sciences* 17.7 (2012).

68. Vessal, Mahmood, Mina Hemmati, and Mohammad Vasei. "Antidiabetic effects of quercetin in streptozocin-induced diabetic rats." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 135.3 (2003): 357-364.

69. Choi, Hwa-Jung, Jin-Hee Kim, Choong-Hwan Lee, Young-Joon Ahn, Jae-Hyoung Song, Seung-Hwa Baek, and Dur-Han Kwon. "Antiviral activity of quercetin 7-rhamnoside against porcine epidemic diarrhea virus." *Antiviral research* 81.1 (2009): 77-81.

70. Xingyu, Zhu, Ma Peijie, Peng Dan, Wang Youg, Wang Daojun, Chen Xinzheng, Zhang Xijun, and Song Yangrong. "Quercetin suppresses lung cancer growth by targeting Aurora B kinase." *Cancer Medicine* 5.11 (2016): 3156-3165.

71. Wang, Guanyu, Jiawei Zhang, Luying Liu, Sherven Sharma, and Qinghua Dong. "Quercetin potentiates doxorubicin mediated antitumor effects against liver cancer through p53/Bcl-xl." *Plos one* 7.12 (2012): e51764.

72. Wang, Kui, Rui Liu, Jingyi Li, Jiali Mao, Yunlong Lei, Jinhua Wu, Jun Zeng et al. "Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR-and hypoxia-induced factor 1α-mediated signaling." *Autophagy* 7.9 (2011): 966-978.

73. Baeuerle, Patrick A., and Vijay R. Baichwal. "NF-kB as a frequent target for immunosuppressive and anti-inflammatory molecules." *Advances in immunology* 65 (1997): 111-138.

74. Oeckinghaus, Andrea, and Sankar Ghosh. "The NF-κB family of transcription factors and its regulation." *Cold Spring Harbor perspectives in biology* 1.4 (2009): a000034.

75. Lawrence, Toby. "The nuclear factor NF-κB pathway in inflammation." *Cold Spring Harbor perspectives in biology* 1.6 (2009): a001651.

76. Friedmann-Morvinski, Dinorah, Rajesh Narasimamurthy, Yifeng Xia, Chad Myskiw, Yasushi Soda, and Inder M. Verma. "Targeting NF-κB in glioblastoma: A therapeutic approach." *Science advances* 2.1 (2016): e1501292.

77. Pozsgai, Eva, Szabolcs Bellyei, Anna Cseh, Arpad Boronkai, Boglarka Racz, Aliz Szabo, Balazs Sumegi, and Eniko Hocsak. "Quercetin increases the efficacy of glioblastoma treatment compared to standard chemoradiotherapy by the suppression of PI-3-kinase-Akt pathway." *Nutrition and cancer* 65.7 (2013): 1059-1066.

78. Wang, Kui, Rui Liu, Jingyi Li, Jiali Mao, Yunlong Lei, Jinhua Wu, Jun Zeng et al. "Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR-and hypoxia-induced factor 1α-mediated signaling." *Autophagy* 7.9 (2011): 966-978.

79. Nam, Tae Wook, Chong Il Yoo, Hui Taek Kim, Chae Hwa Kwon, Ji Yeon Park, and Yong Keun Kim. "The flavonoid quercetin induces apoptosis and inhibits migration through a MAPK-dependent mechanism in osteoblasts." *Journal of bone and mineral metabolism* 26.6 (2008): 551-560.

80. Ishikawa, Yoshihisa, and Masanori Kitamura. "Anti-apoptotic effect of quercetin: intervention in the JNK-and ERK-mediated apoptotic pathways." *Kidney international* 58.3 (2000): 1078-1087

81. Malavolta, Marco, Elisa Pierpaoli, Robertina Giacconi, Laura Costarelli, Francesco Piacenza, Andrea Basso, Maurizio Cardelli, and Mauro Provinciali. "Pleiotropic effects of tocotrienols and quercetin on cellular senescence: introducing the perspective of senolytic effects of phytochemicals." *Current drug targets* 17.4 (2016): 447-459.

82. Koch, Karoline, Susannah Havermann, Christian Büchter, and Wim Wätjen. "Caenorhabditis elegans as model system in pharmacology and toxicology: effects of

flavonoids on redox-sensitive signalling pathways and ageing." *The Scientific World Journal* 2014 (2014).

83. Atashpour, Shekoufeh, Shamileh Fouladdel, Tahereh Komeili Movahhed, Elmira Barzegar, Mohammad Hossein Ghahremani, Seyed Nasser Ostad, and Ebrahim Azizi. "Quercetin induces cell cycle arrest and apoptosis in CD133+ cancer stem cells of human colorectal HT29 cancer cell line and enhances anticancer effects of doxorubicin." *Iranian journal of basic medical sciences* 18.7 (2015): 635.

84. Zhou, Wei, Georgios Kallifatidis, Bernd Baumann, Vanessa Rausch, Jürgen Mattern, Jury Gladkich, Nathalia Giese et al. "Dietary polyphenol quercetin targets pancreatic cancer stem cells." *International journal of oncology* 37.3 (2010): 551-561.

85. Roy, Sanjoy, Debarshi Lahiri, Tapas Maji, and Jaydip Biswas. "Recurrent glioblastoma: where we stand." *South Asian journal of cancer* 4.4 (2015): 163.

86. Amor, Sandra, Fabiola Puentes, David Baker, and Paul Van Der Valk. "Inflammation in neurodegenerative diseases." *Immunology* 129.2 (2010): 154-169.

87. Glass, Christopher K., Kaoru Saijo, Beate Winner, Maria Carolina Marchetto, and Fred H. Gage. "Mechanisms underlying inflammation in neurodegeneration." *Cell* 140.6 (2010): 918-934.

88. Tsai, Wen-Chiuan, Dueng-Yuan Hueng, Chii-Ruey Lin, Thomas CK Yang, and Hong-Wei Gao. "Nrf2 Expressions Correlate with WHO Grades in Gliomas and Meningiomas." *International journal of molecular sciences* 17.5 (2016): 722.

89. Choi, Jason, Alexandre Moquin, Enzo Bomal, Li Na, Dusica Maysinger, and Ashok Kakkar. "Telodendrimers for physical encapsulation and covalent linking of individual or combined therapeutics." *Molecular Pharmaceutics* (2017).

90. Salazar-Ramiro, Aleli, Daniela Ramírez-Ortega, Verónica Pérez de la Cruz, Norma Y. Hérnandez-Pedro, Dinora Fabiola González-Esquivel, Julio Sotelo, and Benjamín Pineda. "Role of redox status in development of glioblastoma." *Frontiers in immunology* 7 (2016).

91. Murphy, Michael P. "How mitochondria produce reactive oxygen species." *Biochemical Journal* 417.1 (2009): 1-13.

92. Oltersdorf, Tilman, Steven W. Elmore, Alexander R. Shoemaker, Robert C. Armstrong, David J. Augeri, Barbara A. Belli, Milan Bruncko et al. "An inhibitor of Bcl-2 family proteins induces regression of solid tumours." *Nature*435.7042 (2005): 677-681.

93. Tsujimoto, Yoshihide. "Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?." *Genes to cells* 3.11 (1998): 697-707.

94. Hardwick, J. Marie, and Lucian Soane. "Multiple functions of BCL-2 family proteins." *Cold Spring Harbor perspectives in biology* 5.2 (2013): a008722.

95. Zhang, Issan, Yiming Cui, Abdolali Amiri, Yidan Ding, Robert E. Campbell, and Dusica Maysinger. "Pharmacological inhibition of lipid droplet formation enhances the effectiveness of curcumin in glioblastoma." *European Journal of Pharmaceutics and Biopharmaceutics* 100 (2016): 66-76.

96. Maroon, Joseph, Jeffrey Bost, Austin Amos, and Giulio Zuccoli. "Restricted calorie ketogenic diet for the treatment of glioblastoma multiforme." *Journal of child neurology* 28.8 (2013): 1002-1008.

97. Singh, Rajat, Susmita Kaushik, Yongjun Wang, Youqing Xiang, Inna Novak, Masaaki Komatsu, Keiji Tanaka, Ana Maria Cuervo, and Mark J. Czaja. "Autophagy regulates lipid metabolism." *Nature* 458.7242 (2009): 1131-1135.

98. Bailey, Andrew P., Grielof Koster, Christelle Guillermier, Elizabeth MA Hirst, James I. MacRae, Claude P. Lechene, Anthony D. Postle, and Alex P. Gould. "Antioxidant role for lipid droplets in a stem cell niche of Drosophila." *Cell* 163,.2 (2015): 340-353.

99. Liu, Lucy, et al. "Glial lipid droplets and ROS induced by mitochondrial defects promote neurodegeneration." *Cell* 160.1 (2015): 177-190.

100. Boren, J., and K. M. Brindle. "Apoptosis-induced mitochondrial dysfunction causes cytoplasmic lipid droplet formation." *Cell Death & Differentiation* 19.9 (2012): 1561-1570.

101. Pool, Hector, David Quintanar, Juan de Dios Figueroa, Camila Marinho Mano, J. Etelvino H. Bechara, Luis A. Godínez, and Sandra Mendoza. "Antioxidant effects of quercetin and catechin encapsulated into PLGA nanoparticles." *Journal of Nanomaterials* 2012 (2012): 86.

102. El-Gogary, Riham I., Noelia Rubio, Julie Tzu-Wen Wang, Wafa'T. Al-Jamal, Maxime Bourgognon, Houmam Kafa, Muniba Naeem et al. "Polyethylene glycol conjugated polymeric nanocapsules for targeted delivery of quercetin to folate-expressing cancer cells in vitro and in vivo." *ACS nano* 8.2 (2014): 1384-1401.

103. Guan, Xin, Meng Gao, Hong Xu, Chenghong Zhang, Hongyan Liu, Li Lv, Sa Deng, Dongyan Gao, and Yan Tian. "Quercetin-loaded poly (lactic-co-glycolic acid)-d-α-tocopheryl polyethylene glycol 1000 succinate nanoparticles for the targeted treatment of liver cancer." *Drug delivery* 23.9 (2016): 3307-3318.

104. Daneshmehr, S. "Carbon Nanotubes for Delivery of Quercetin as Anticancer Drug: Theoretical Study." *Procedia Materials Science* 11 (2015): 131-136.

105. Kumar, Pramod, Gajanand Sharma, Rajendra Kumar, Bhupinder Singh, Ruchi Malik, Om Prakash Katare, and Kaisar Raza. "Promises of a biocompatible nanocarrier in improved brain delivery of quercetin: Biochemical, pharmacokinetic and biodistribution evidences." *International journal of pharmaceutics* 515.1 (2016): 307-314.

106. Zhang, Keru, Xing Tang, Juan Zhang, Wei Lu, Xia Lin, Yu Zhang, Bin Tian, Hua Yang, and Haibing He. "PEG–PLGA copolymers: Their structure and structure-influenced drug delivery applications." *Journal of Controlled Release* 183 (2014): 77-86.

107. Xu, Wei, Peixue Ling, and Tianmin Zhang. "Polymeric micelles, a promising drug delivery system to enhance bioavailability of poorly water-soluble drugs." *Journal of drug delivery* 2013 (2013).

108. Verhoef, Johan JF, and Thomas J. Anchordoquy. "Questioning the use of PEGylation for drug delivery." *Drug delivery and translational research* 3.6 (2013): 499.

109. Guan, Xin, Meng Gao, Hong Xu, Chenghong Zhang, Hongyan Liu, Li Lv, Sa Deng, Dongyan Gao, and Yan Tian. "Quercetin-loaded poly (lactic-co-glycolic acid)-d-α-tocopheryl polyethylene glycol 1000 succinate nanoparticles for the targeted treatment of liver cancer." *Drug delivery* 23.9 (2016): 3307-3318.

110. Cheng, Jianjun, Benjamin A. Teply, Ines Sherifi, Josephine Sung, Gaurav Luther, Frank X. Gu, Etgar Levy-Nissenbaum, Aleksandar F. Radovic-Moreno, Robert Langer, and Omid C. Farokhzad. "Formulation of functionalized PLGA–PEG nanoparticles for in vivo targeted drug delivery." *Biomaterials* 28. 5 (2007): 869-876.

111. Koopaei, Mona Noori, Mohammad Reza Khoshayand, Seyed Hossein Mostafavi, Mohsen Amini, Mohammad Reza Khorramizadeh, Mahmood Jeddi Tehrani, Fatemeh Atyabi, and Rassoul Dinarvand. "Docetaxel loaded PEG-PLGA nanoparticles: optimized drug loading, in-vitro cytotoxicity and in-vivo antitumor effect." *Iranian journal of pharmaceutical research: IJPR* 13.3 (2014): 819.

112. Acharya, Sarbari, Fahima Dilnawaz, and Sanjeeb K. Sahoo. "Targeted epidermal growth factor receptor nanoparticle bioconjugates for breast cancer therapy." *Biomaterials* 30.29 (2009): 5737-5750.

113. E Taylor, T., F. B Furnari, and W. K Cavenee. "Targeting EGFR for treatment of glioblastoma: molecular basis to overcome resistance." *Current cancer drug targets* 12.3 (2012): 197-209.

114. Low, Philip S., Walter A. Henne, and Derek D. Doorneweerd. "Discovery and development of folic-acid-based receptor targeting for imaging and therapy of cancer and inflammatory diseases." *Accounts of chemical research* 41.1 (2007): 120-129.

115. Tietjen, Gregory T., and W. Mark Saltzman. "Nanomedicine gets personal." *Sci. Transl. Med.* 7 (2015): 314fs47.