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Investigating the effects of the ketone body ß-hydroxybutyrate and the anti-cancer compound MHP88 on glioblastoma multiforme cells in vitro

Master thesis

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Abstract

Introduction: Glioblastoma multiforme (GBM) is the most aggressive human brain tumor with a mean survival of 15 months. It is therefore necessary to explore new treatment strategies, and to study how different hallmarks of cancer can be utilized in this regard. The marine natural product mimic MHP88 can attack the cancer cells' ability to evade immune destruction by inducing immunogenic cell death and hence work as immunotherapy. The cancer cells' reprogrammed energy metabolism, including the Warburg effect, can possibly be utilized simultaneously. This hallmark makes the cancer cells highly dependent on glucose for energy production. A ketogenic diet can target this feature by reducing the blood levels of glucose and increasing the blood levels of ketone bodies and may work as an adjuvant treatment for GBM.

Aims: Investigate if the ketone body ß-hydroxybutyrate (BHB) had an adjuvant effect to MHP88 on GBM cells. Examine if the expression of *luc2* in the murine glioblastoma cell line GL261-luc2 was constant to prepare for future mouse studies with the ketogenic diet and MHP88.

Method: Five cell lines (two murine glioblastoma, one human glioblastoma, one human fibroblast, one murine cardiac muscle) and primary murine hepatocytes were used. Live-cell imaging, MTS assay, Western blot and bioluminescence measurements were performed.

Results: GL261-luc2 cells died of a glucose deprivation but survived in 3.5 mmol/l glucose. The cell cytotoxicity of MHP88 was either unaffected or decreased when the three glioblastoma cell lines and the fibroblast cell line were cultured in 3.5 mmol/l glucose with 5 or 10 mmol/l BHB. The presence of BHB in the cell media lowered the pH and hence reduced the number of active MHP88 molecules. Expression of the rate-limiting ketolytic enzyme, SCOT, appeared to be low in the GBM cells. The luminescence signal from GL261-luc2 cells varied after selection with G418.

Conclusion: The reduced cell cytotoxicity of MHP88 in the presence of BHB could be attributed to a pH reduction in the cell media. However, additional research is needed. Expression of *luc2* in the cell line GL261-luc2 is dependent on selection with G418.

Abstrakt

Introduksjon: Glioblastoma multiforme (GBM) er den hyppigst forekommende hjernesvulsten blant mennesker og har en gjennomsnittlig overlevelse på 15 måneder. Det er derfor nødvendig å utforske nye behandlingsformer, noe som potensielt kan gjøres gjennom å studere ulike kjennetegn ved kreft. Den marine naturprodukt-etterligningen MHP88 kan angripe kreftcellenes evne til å unngå ødeleggelse av immunforsvaret ved å indusere immunogen celledød og slik fungere som immunterapi. Kreftcellene har også en metabolsk reprogrammering, inkludert Warburg effekten, som potensielt kan utnyttes samtidig. Dette kjennetegnet gjør kreftcellene svært avhengige av glukose for energiproduksjon. En ketogen diett kan angripe dette ved å senke blodglukosen og øke konsentrasjonen av ketonlegemer i blodet, og kan muligens fungere som en adjuvant behandling for GBM.

Formål: Undersøke om ketonlegemet ß-hydroksybutyrat (BHB) hadde en adjuvant effekt til MHP88 på GBM-celler. Teste om uttrykkingen av *luc2* i glioblastoma-cellelinjen GL261-luc2 var konstant, for å forberede fremtidige forsøk i mus med MHP88 og ketogen diett.

Metode: Fem cellelinjer (to fra GBM i mus, en fra human GBM, en fra humane fibroblaster, en fra hjertemuskulatur i mus) og primære hepatocytter fra mus ble brukt. Live-cell imaging, MTS assay, Western blot og måling av bioluminescens ble gjennomført.

Resultat: GL261-luc2 celler døde i fravær av glukose, men overlevde i 3.5 mmol/l glukose. Cytotoksisiteten til MHP88 på cellene var enten upåvirket eller redusert når de tre glioblastoma-cellelinjene og fibroblast-cellelinjen ble dyrket i 3.5 mmol/l glukose med 5 eller 10 mmol/l BHB. Tilstedeværelsen av BHB i cellemediet senket pHen og reduserte dermed antallet aktive MHP88-molekyler. Uttrykking av det hastighetsbegrensende ketolytiske enzymet, SCOT, fremstod å være lav i GBM-cellene. Luminescenssignalet fra GL261-luc2 cellene varierte etter seleksjon med G418.

Konklusjon: Den reduserte cytotoksisiteten til MHP88 på cellene i nærvær av BHB kan tilskrives en pH-reduksjon i cellemediet. Likevel trengs ytterligere forskning. Uttrykking av *luc2* i cellelinjen GL261-luc2 er avhengig av seleksjon med G418.

Abbreviations

Abbreviation	Description
ATP	Adenosine triphosphate
BHB	ß-hydroxybutyrate
BSA	Albumine from bovine serum
CNS	Central nervous system
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithoioreitol
ESPEN	The European Society for Clinical Nutrition and Metabolism
FBS	Fetal bovine serum
GBM	Glioblastoma multiforme
G-CIMP	Cytosine-phosphate-guanine island methylator phenotype
GDH1	Glutamate dehydrogenase 1
HMG-CoA	ß-Hydroxy-3-methylglutaryl-CoA
HRP	Horse radish peroxidase
ICD	Immunogenic cell death
IC_{50}	Half-maximal (50%) inhibitory concentration
IDH	Isocitrate dehydrogenase
KD	Ketogenic diet
kDa	Kilodalton
MCT	Monocarboxylate transporters
MEM	Minimum essential media Eagle
MGMT	O^6-methylguanine-DNA methyltransferase
MRI	Magnetic resonance imaging
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced

NOS	Not otherwise specified
PBS	Phosphate buffered saline
PES	Phenazine ethosulfate
RIPA	Radioimmunoprecipitation assay
RLU	Relative light units
ROS	Reactive oxygen species
rpm	Revolutions per minute
SCOT	Succinyl-CoA:3-keto acid (3-oxoacid) CoA transferase
SDS	Sodium dodecyl sulfate
TBRG	Tumor Biology Research Group
TBS-T	Tris-buffered saline – Tween
TCA	Tricarboxylic acid
UiT	UiT The Arctic University of Norway

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1.1 Glioblastoma multiforme

1.1.1 Classification of brain tumors

Less than 2% of all cases of human cancers are classified as brain tumors, making it a relatively small group of neoplasms (1). However, as one of the deadliest cancer types is located to the brain, it is considered a relevant field of research (2).

Brain tumors are primary, intracranial tumors that originate from the meninges, the pituitary gland, tissue or nerves, or secondary tumors metastasized to the central nervous system (CNS) (3). Primary tumors are a diverse group of neoplasms regarding aggressiveness, prognosis and development. They are categorized after «The 2016 World Health Organization Classification of Tumors of the Central Nervous System» (4). This system categorizes the tumors based on histology and molecular patterns. In terms of malignancy, the tumors are graded from I to IV, depending on mitotic activity, cytological atypia, microvascular proliferation and necrosis. Grade I tumors hold none of these features and are nonmalignant. In comparison, grade IV tumors hold three to four and are highly malignant.

Gliomas, with histological and molecular similarities to the glial cells (astrocytes, oligodendrocytes and ependymal cells), constitute the majority of the primary brain tumors (4). They are named after their putative cell of origin and divided into subcategories. Astrocytic tumors, also known as astrocytomas, occur most frequently of the gliomas. They originate from astrocytes and can arise with a malignancy of I to IV. The grade IV astrocytomas are known as glioblastoma multiforme (GBM). These tumors can be separated based on mutations in the isocitrate dehydrogenase (IDH) gene, into IDH-wildtype and IDH-mutant. In lack of genetic testing, or if testing is inconclusive, the tumor is referred to with a designation of «not otherwise specified» (NOS). A schematic illustration of the GBM classification is presented in figure 1.

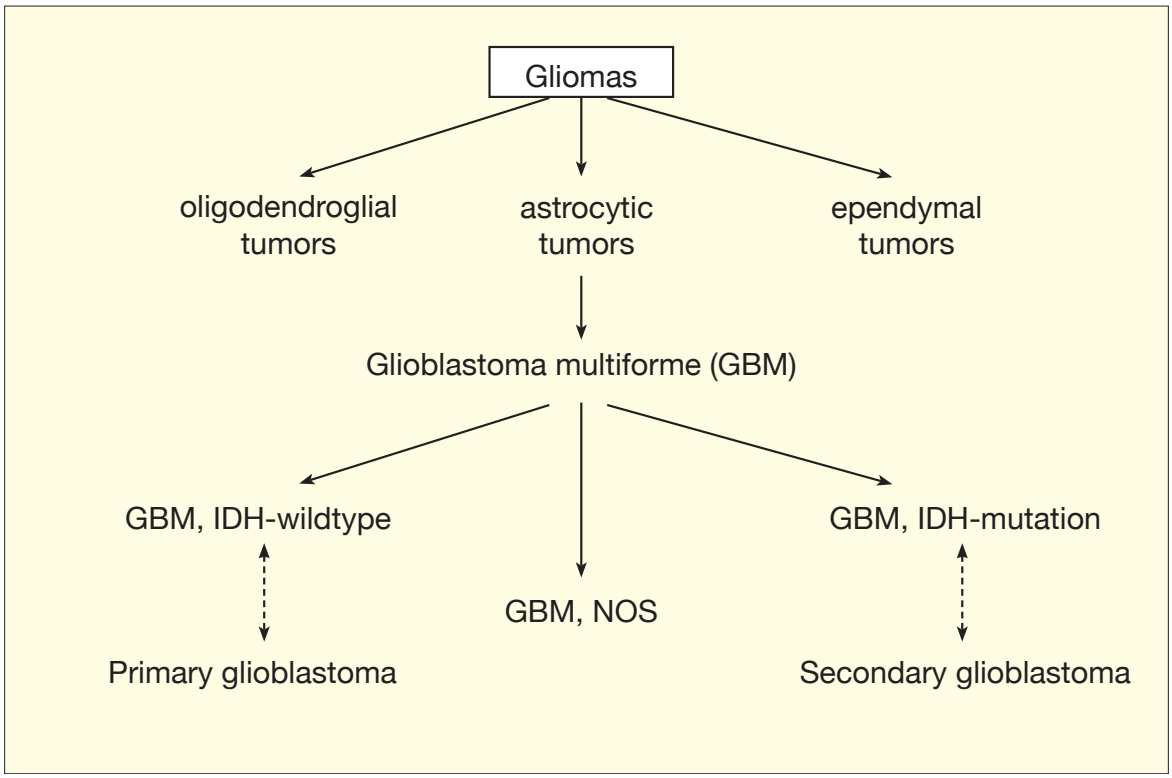

Figure 1. The origin and subtypes of glioblastoma multiforme
The figure depicts a simplified categorization of GBM. Gliomas are a subtype of CNS tumors with their origin in glial cells (4). The tumors can be categorized based on their putative cell of origin into oligodendroglial tumors, astrocytic tumors and ependymal tumors. Further, the tumors can be classified based on their malignancy. The most malignant type of astrocytoma, and also one of the deadliest types of human cancer, is GBM. The GBM tumors are categorized based on the mutation of their IDH gene. IDH-wildtype have no mutation of the gene, IDH-mutation have a mutation of the gene and NOS tumors have an unknown mutation status. The IDH-wildtype tumor corresponds most frequently with primary glioblastoma and the IDH-mutation with secondary glioblastoma. CNS, Central nervous system; GBM, Glioblastoma multiforme; IDH, Isocitrate dehydrogenase; NOS, Not otherwise specified.

IDH are enzymes that catalyze the conversion of isocitrate to α-ketoglutarate, and the production of reduced nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH). This takes place in the tricarboxylic acid (TCA) cycle in the mitochondria and in the cytosol (5). The enzymes are necessarily important for energy production and breakdown of nutrients. If a tumor is classified as IDH-mutant, the function of the enzyme is altered, leading to the production of 2-hydroxyglutarate instead of NADPH. This somehow contributes to an oncogenic transformation. In addition, the IDH-mutation is associated with glioma cytosine-phosphate-guanine island methylator phenotype (G-CIMP) and methylation of the O^6-methylguanine-DNA methyltransferase (MGMT) promoter (6). Both are favorable for the prognosis of GBM. An IDH-wildtype tumor has no mutation of the IDH gene.

1.1.2 Epidemiology and prognosis

GBM is the most malignant, and also the most common, tumor in the CNS as it constitutes over 50% of all cases of astrocytic tumors (1). The incidence is 3-5/100,000 per year, with a median age of 60-65 years at diagnosis and a male to female ratio of 1.6:1 (1, 7, 8). In Norway, about 200 new incidents are registered each year (3.7/100,000) (9). Mean survival time for patients with GBM is 15 months, and only 3-5% live longer than three years after diagnosis, making it one of the deadliest types of human cancers (2, 10).

The survival time is affected by the origin of the tumor (1). Primary glioblastoma arises de novo, meaning that the tumor is characterized as grade IV from the point of origin. These tumors correspond most frequently with IDH-wildtype and have a rapid progression. Secondary glioblastoma, usually IDH-mutant, originates from low-grade gliomas, and gradually develops into GBM, causing a slower progression of the disease. IDH gene mutations is in such way a prognosic factor. Primary GBM mainly strikes the elderly (median age 60 years) and constitute about 90% of all cases of GBM. Secondary GBM mainly manifests in younger and middle-age people (median age 45 years) and make up the last 10% cases of GBM tumors.

Another element with impact on the prognosis for GBM patients is methylation of the MGMT promoter. It occurs in approximately 36% of the primary- and 75% of the secondary tumors (11). MGMT is an enzyme responsible for DNA-repair subsequent to exposure to alkalyating chemotherapeutic agents, such as temozolomide. The MGMT gene might be silenced during tumor progression by methylation of its promoter. Silencing of the gene results in lack of DNA repair after treatment with chemotherapy and concequently to an increased effect of temozolomide.

1.1.3 Treatment

There is a well established treatment regimen for GBM. Initally surgery is carried out, followed by treatment with cytostatica, usually temozolomide, and radiation therapy (12). The goal of the surgery is maximal safe resection, along with preservation of the neurological function. High-grade gliomas does, in general, infiltrate the surrounding tissue. This makes complete resection difficult. Additional goals are to confirm a pathological diagnosis and test the tumor for MGMT promotor methylation and IDH mutation, performed through a biopsy.

Temozolomide is the most frequently used chemotherapeutic agent and is given in cycles concominant with, and six months post, radiation (12). Radiation treatment usually lasts for six weeks (13). When it comes to nutritional therapy, The European Society for Clinical Nutrition and Metabolism (ESPEN) have no spesific recommendations for GBM in their guidelines (14). It is mentioned in the report that a ketogenic diet (KD) have been tested as a treatment for GBM in pilot trials, but no therapeutic effect has been demonstrated and compliance is low. A question for research stated in the ESPEN guidelines is «Effects of fasting or fasting mimicing diets on wanted and unwanted effects of anticancer agents» (14, p. 14).

Considering that GBM tumors have a high mortality rate, in spite of the existing treatment regimen, there are grounds to do research on new treatment options.

1.2 Inducing immunogenic cell death

A cancer cell has multiple hallmarks that potentially can be utilized (15). One of them is the ability to evade immune destruction. The theory is that the immune system constantly has the cells and tissues of the body under surveillance to avoid formation of cancerous cells. Seeing as tumors are able to form in immunocompetent beings, the cancer cells must somehow avoid detection and eradication by the immune system. A potential way of treating cancer could be to awake the immune system and trigger an immune response (16). This could be managed by the use of an immunogenic cell death (ICD) inducer.

ICD is a cell death where the immune system is activated against the tumor (16, 17). Daily, billions of human cells die and are replaced without the immune system noticing. These cells undergo a regulated cell death, which long have been considered a non-immunogenic process. However, under certain circumstances, stress can make the regulated cell death cause an inflammatory response. When a cancer cell is exposed to an ICD inducer, the endoplasmic reticulum stress response is activated and reactive oxygen species (ROS) are produced, causing the cell to die (16, 17). This results in presentation of damage-associated molecular patterns (DAMPs) and antigens on the cell surface. DAMPs attract immature dendritic cells (DC) and stimulate their maturation into active antigen presenting immune cells. The DCs engulf dead cancer cells and migrate to the lymph nodes, where they activate the adaptive immune system through an antigen presentation, inducing cytotoxic T-lymphocyte reactions

responses. This leads to the death of additional cancer cells. The process of ICD is illustrated in figure 2.

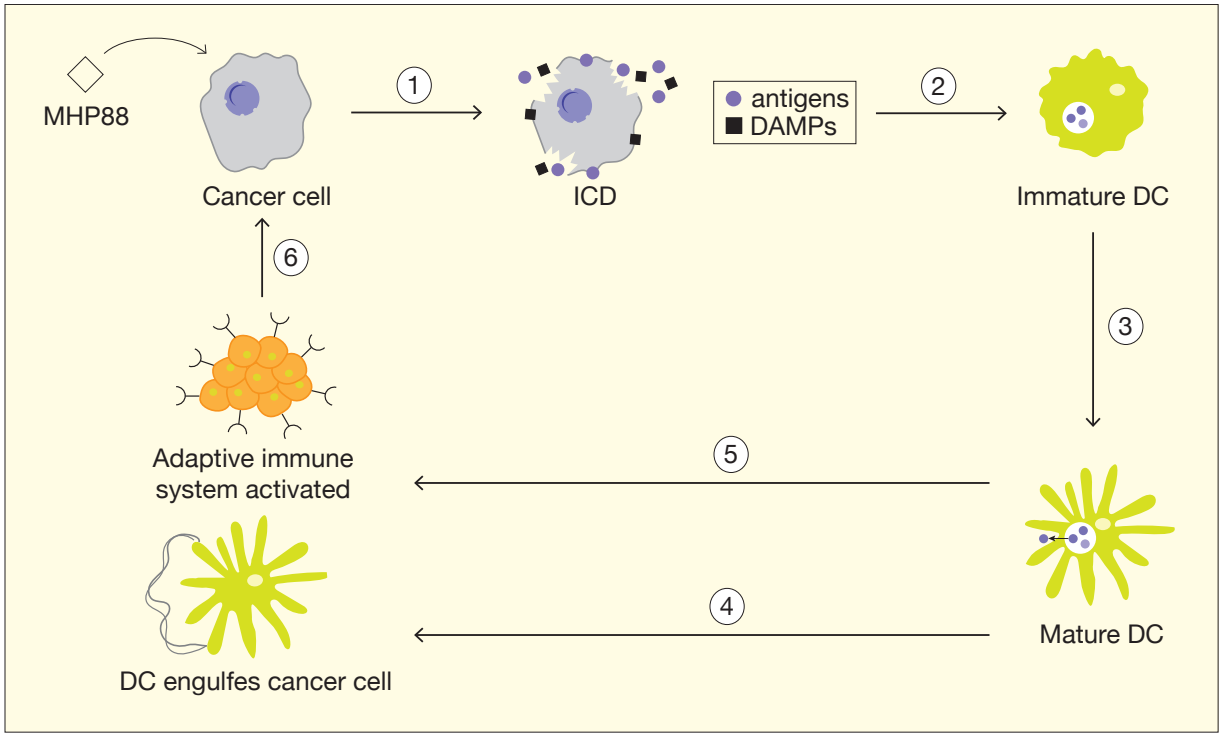

Figure 2. Immunogenic cell death
The figure illustrates the main steps of ICD (16, 17). 1) An ICD inducer (here: MHP88) is introduced to the cancer cell. The endoplasmic reticulum stress response is activated, and ROS are produced, making the cell rupture and leak DAMPs and antigens. 2) DAMPs attract immature DCs which arrives at the site of the cancer cell and takes up the antigen. 3) The immature DC is consequently activated into a mature DC. 4) The mature DC engulfs dead cancer cells. 5) The mature DC also migrates to the lymph nodes where it presents the cancer cells' antigen on its surface to T-lymphocytes, activating them. 6) The activation of T-lymphocytes awakens the adaptive immune system and results in a gradual immunity and the death of additional cancer cells. DAMPs, Damage-associated molecular patterns; DC, Dendritic cell; ICD, Immunogenic cell death; ROS, Reactive oxygen species.

1.2.1 MHP88

MHP88 is a natural marine compound patented and owned by UiT The Arctic University of Norway (UiT) (18). It has structural similarities to LTX-401, an oncolytic compound shown to work as an ICD inducer in mouse studies (19, 20). Unpublished studies, carried out by others in the Tumor Biology Research Group (TBRG), have shown that MHP88 induces all major hallmarks of ICD, including the release and exposure of DAMPs, in vitro. This indicates that the compound has potential as a future therapeutic agent.

It is desired by others in the TBRG to carry out mouse studies on MHP88 as a possible treatment for GBM. To prepare for these experiments it is favorable to investigate the qualities of the compound further in vitro. Hence, MHP88 was utilized in the present thesis.

1.3 Cancer cell metabolism - The «Warburg effect»

Another hallmark of cancer is a reprogrammed energy metabolism (15). The brain is under normoglycemic conditions a large consumer of glucose (21, p. 380). However, during prolonged starvation or when on a ketogenic diet (KD), ketone bodies become the brain cells' primary source of energy. This is not the incidence for cancer cells however. They are heavily dependent on glucose regardless of variations in nutritional supply, due to a feature of their metabolism known as the Warburg effect (15).

During aerobic conditions, cells produce adenosine triphosphate (ATP) from glucose through glycolysis in the cytosol and the TCA cycle and oxidative phosphorylation in the mitochondria. Glucose is first converted to pyruvate through the glycolysis. Next, pyruvate is converted to acetyl-CoA and oxidized to CO_2 and water in the mitochondria (21, p. 213, 248). A total of 36 ATP molecules are produced from one molecule of glucose during this process. Cells without mitochondria, such as erythrocytes, rely solemnly on the glycolysis for energy production. When oxygen is scarce, all cells are highly dependent on energy production through the glycolysis, with lactate as the end product. This anaerobic process is known as fermentation and generates only two molecules of ATP from one molecule of glucose, which is 18 times less than the maximum energy potential of a glucose molecule (21, p. 217).

Cancer cells do not alter their energy metabolism based on the presence of oxygen (22). They confer glucose to lactate either way. This feature was initially recognized by Otto Warburg in the 1920s and is referred to as the «Warburg effect» or «aerobic glycolysis». Otto Warburg hypothesized that cancer cells favor aerobic glycolysis due to an impaired respiration (22). He alleged that the respiratory system is injured permanently in cells proliferating into cancer cells, leading to an energy deprivation and increased rates of fermentation. The detection of aerobic glycolysis is the origin to the hallmark «reprogrammed energy metabolism» (15). An illustration of the Warburg effect, with comparison to oxidative phosphorylation and anaerobic glycolysis, is presented in figure 3.

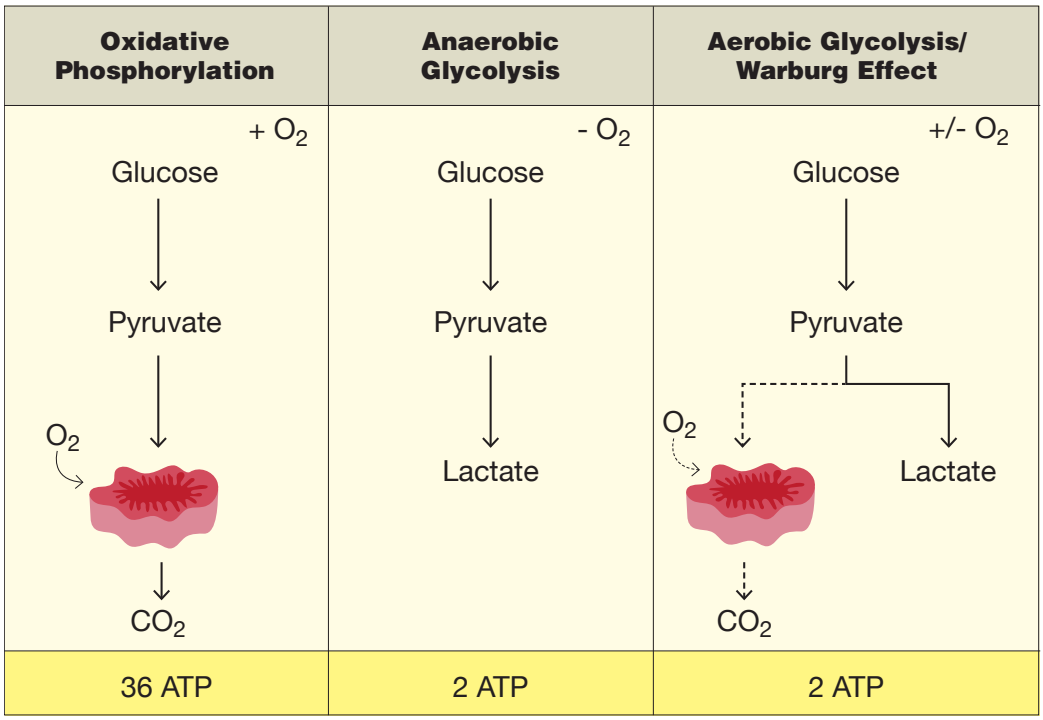

Figure 3. The Warburg effect
Normal cells will in the presence of oxygen produce 36 molecules of ATP from one molecule of glucose through glycolysis in the cytosol and oxidative phosphorylation in the mitochondria (21, p. 217, 213, 248). During hypoxia the cells solemnly relies on the glycolysis for energy production with lactate and 2 ATP molecules as the end products. This mean of energy production is known as anaerobic glycolysis due to the hypoxia. Cancer cells do not alter their energy metabolism based on the presence of oxygen (22). Glucose is converted to lactate and 2 ATP molecules through the glycolysis either way. This feature is known as the Warburg effect or aerobic glycolysis, seeing as the glycolysis occurs in the presence of oxygen. ATP, Adenosine triphosphate.

It has been demonstrated that tumors have oxidative capacities, disproving this part of Warburg's hypothesis (23-26). In other words, cancer cells can potentially exploit the full energy potential of a glucose molecule. Consequently, research have been conducted to explore how aerobic glycolysis could be favorable to cancer cells (15, 27). It is not apparent what the advantages of the Warburg effect are, but several theories have been proposed and examined. Some of them are presented in the following paragraphs.

Proliferating mammalian cells possess the same metabolic nature as cancer cells (28). Aerobic glycolysis is therefore not a unique feature of cancer cells, but rather a feature of all proliferating cells. The glycolysis has intermediates that can be extracted for use in different biosynthetic pathways to produce amino acids, lipids and nucleotides (27). Consequently, the Warburg effect might be related to a requirement of energy and biomass for proliferation. Intermediates are extracted from both the glycolysis and TCA cycle for biomass production. However, a large portion of the carbon is used for lactate production. This makes the reaction pathway partly inconsistent with the need of carbon for biomass production (29).

Lactate is hypothesized to favor the development of cancer by decreasing the pH in the tumor microenvironment (30). H^+ diffuses into the adjacent healthy tissue and causes alterations to the tissue structure that allows for cancer cell invasion. In addition, increased rates of glycolysis deprive the tumor-infiltrating lymphocytes, which are immune cells programmed to kill tumor cells, of glucose (31). This stimulates to further growth and invasiveness of the tumor. The advantages of lactate formation could at this explain the Warburg effect. However, an issue is that the Warburg effect is thought to be an early event in oncogenesis that occurs prior to the tumor cells becoming invasive (27). In addition, it is demonstrated that glycolysis is preferred in unicellular organisms with no potential of invasiveness (32). Consequently, the lactate theory is not sufficient to account for the total Warburg effect either.

Another product from the glycolysis, ATP, could partially explain of why aerobic glycolysis is preferable to cancer cells (27). ATP production from one glucose molecule during aerobic glycolysis is low compared with the mitochondrial respiration, but the synthesis happens 10-100 times faster. It has been demonstrated that aerobic glycolysis increases with high ATP demands, while the mitochondrial respiration remains constant (33). This might give the cancer cells an advantage when nutrients are scarce. However, proliferating cells have a higher demand of the reducing agent NADPH for biosynthesis than of ATP (27). The demand of ATP is therefore not a rate-limiting factor.

Presumably, there are several explanations to why aerobic glycolysis is favorable in cancer cells. What is evident is that glucose is of significant importance. The reprogrammed energy metabolism could therefore be considered a possible target for cancer treatment. By reducing the cells' access to glucose, one could theoretically weaken them. This could increase their susceptibility towards an ICD inducer, such as MHP88, and thus two hallmarks of cancer could be exploited simultaneously. A KD is purposed as a potential approach to target the reprogrammed energy metabolism.

1.4 The ketogenic diet

In addition to glucose, normal cells can utilize fatty acids for energy production through ß-oxidation in the mitochondria (34). The breakdown of fatty acids yields acetyl-CoA units, which can enter the TCA cycle and oxidative phosphorylation. When there are excessive fatty acids in the body, the liver converts the surplus of acetyl-CoA to ketone bodies (21, p. 380). These can be utilized by extrahepatic tissues for energy production. The brain can readily

change its primary energy substrate from glucose to ketone bodies. However, it has been questioned whether GBM cells are capable of utilizing these metabolites. This chapter elaborates the ketogenesis and the KD as a potential adjuvant treatment for GBM.

1.4.1 A fasting mimicking diet

A KD has high contents of fats, moderate contents of proteins and low contents of carbohydrates relative to a standard diet (35). There are different types of the diet. On a classic KD, fat usually contributes with 70-90 energy percent (E%), meaning that 70-90% of the calories are provided from fat. Equivalent, carbohydrates contribute with 3-16 E% and proteins with 6-15 E%. Admission of the diet creates a metabolism similar to hunger or long-term fasting (36, p. 239-248). When the body starves, several metabolic adaptations are made to manage the situation for as long as possible. The blood level of insulin falls, and the level of glucagon rises. This causes the blood glucose to drop, the glycogen storages to be depleted and the adipose tissue to become the main provider of energy. Fatty acids are oxidized to acetyl-CoA and the surplus is used to produce ketone bodies in the liver. In addition, amino acids, lactate and glycerol fuels the formation of glucose through the gluconeogenesis in the liver and partly in the kidneys. Similar adaptations occur when admitting a KD, which mimics the fasting metabolism (35). The diet is thought to take advantage of the Warburg effect when used as an adjuvant treatment for cancer patients by inflicting an energy deprivation that is supposed to sensitize the cancer cells to medical treatment (37, 38). The diet is also alleged to inhibit tumor angiogenesis, promote tumor cell apoptosis through increased oxidative stress and decrease inflammation. Some of these mechanisms are proposed to be caused by ketone body signaling, indicating that the ketone bodies can provide a direct effect upon the tumor cells.

1.4.1.1 Increased lipolysis

During normal metabolic conditions, glucose works as the cells' primary energy source (21, p. 220, 380). When the circulatory levels of glucose drop however, the gluconeogenesis will provide the cells with energy for a while, until ß-oxidation of fatty acids and ketogenesis become the main providers of energy. The small amounts of glucose available is spared for neurons, erythrocytes and proliferating cells (39).

When fasting or admitting a KD, the blood levels of glucagon rise (21, p. 370-371). This leads to increased lipolysis, the metabolic pathway where triacylglycerol is hydrolyzed into glycerol and fatty acids. During fasting, triacylglycerol is derived from the body's adipose

tissue stores. When dieting, it is provided from dietary fat and to some extent from the adipose tissue stores, depending on meals (40). The triacylglycerol is transported in chylomicrons from the small intestine and in very low-density lipoproteins from the liver and broken down to glycerol and fatty acids by lipoprotein lipase (21, p. 370-371). Free fatty acids from the adipose tissue stores are transported in the blood, bound to albumin. The fatty acids are primarily imported into the mitochondrial matrix of cells in the liver, skeletal muscles, heart and kidneys. Glycerol is used as a precursor for glucose synthesis in the gluconeogenesis.

Most tissues can utilize fatty acids as an energy source, but the rate of fatty acid oxidation is highest in skeletal muscles and the liver (21, p. 371-372). Erythrocytes cannot utilize fatty acids due to lack of mitochondria. The brain has limited access to fatty acids because of the blood-brain barrier. In the mitochondria, fatty acids undergo ß-oxidation with acetyl-CoA as the end product (21, p. 373-374, 379-380, 39). This leads to great amounts of acetyl-CoA, which enters the TCA cycle by condensing with oxaloacetate to form citrate. When fasting or admitting a KD, the production of acetyl-CoA is greatly enhanced due to the increased lipolysis. The pool of oxaloacetate becomes correlative reduced and at some point, it is no longer sufficient to produce citrate. As a response, the surplus of acetyl-CoA is used to produce the ketone bodies, ß-hydroxybutyrate (BHB) and acetoacetate, in the mitochondria of hepatocytes and, to some extent, in astrocytes, enterocytes and kidney epithelia.

1.4.1.2 Synthesis and oxidation of ketone bodies

The first step in the formation of the ketone bodies is condensation of two molecules of acetyl-CoA into one molecule of acetoacetyl-CoA. In the second step, acetoacetyl-CoA is condensed with a third molecule of acetyl-CoA, forming ß-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The third and last step entails HMG-CoA being split into one molecule of acetyl-CoA and one molecule of acetoacetate. BHB is produced from acetoacetate through the oxidation of NADH to NAD^+. Acetoacetate is, based on this, the only real keto compound. However, since the transformation of acetoacetate to BHB is rapid and BHB is the most abundant of the two in the blood, both are referred to as ketone bodies. When the blood concentration of ketone bodies is high, acetoacetate is spontaneously decarboxylated to acetone, which the body disposes of through the breath. Ketone bodies are also, to some extent, produced from the catabolism of ketogenic amino acids in the liver (21, p. 297).

Even though the ketone bodies primarily are synthesized in the liver, the hepatocytes cannot utilize them for energy production due to lack of the rate-limiting ketolytic enzyme, succinyl-CoA:3-keto acid (3-oxoacid) CoA transferase (SCOT) (21, p. 380). This leads to a net flow of ketone bodies to the extrahepatic tissues. After an overnight fast the blood concentration of ketone bodies can reach 0.1 mmol/l. Levels above 0.2 mmol/l are defined as ketosis (41). When on a KD, normal blood levels of ketone bodies are 5-6 mmol/l. Plasma glucose concentrations falls accordingly, from about 4.5-6 mmol/l to 3.5-4.5 mmol/l.

In extrahepatic tissues, ketone bodies are taken up by monocarboxylate transporters (MCT) and imported into the mitochondria (42). Their metabolism is directly related to their concentration in the blood (21, p. 380). BHB is converted to acetoacetate, which condenses with succinyl-CoA and forms succinate and acetoacetyl-CoA. The latter is split into two molecules of acetyl-CoA, which can derive ATP through the TCA. This process, known as ketolysis, is illustrated in figure 4.

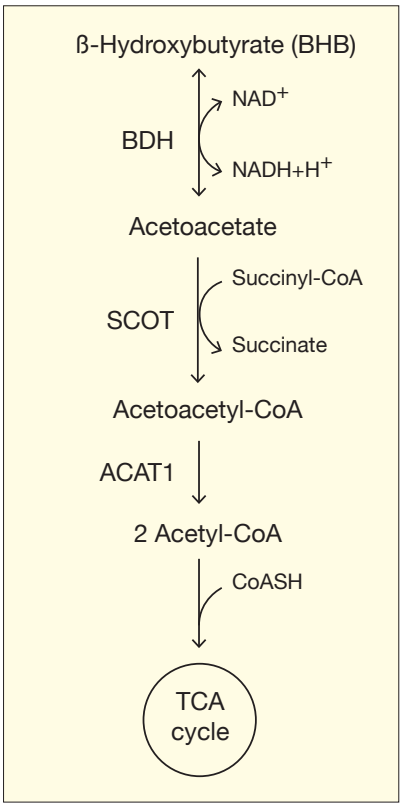

Figure 4. Ketolysis
The figure depicts the breakdown of ketone bodies, known as ketolysis (21, p. 380). The process takes place in extrahepatic tissues with oxidative capacity. The first step entails the reversible breakdown of BHB to acetoacetate, catalyzed by BDH1. Further, acetoacetyl-CoA is formed from acetoacetate. This is the rate-limiting step of the breakdown of ketone bodies, and is catalyzed by SCOT. Finally, two molecules of acetyl-CoA are formed from acetoacetyl-CoA and these can enter the TCA cycle and generate ATP by being oxidized to CO_2 and water. ACAT1, acetoacetyl-CoA Thiolase; ATP, Adenosine triphosphate; BDH, ß-hydroxybutyrate dehydrogenase; BHB, ß-hydroxybutyrate; SCOT, Succinyl-CoA:3-ketoacid-CoA transferase; TCA, Tricarboxylic acid.

One hypothesis is that GBM cells cannot utilize ketone bodies as a source of energy due to their altered metabolism (37). Consequently, a state of ketosis will lead to an energy deprivation, which in turn makes the cells vulnerable and more susceptible to medical anti-neoplastic treatment. Brain cancer cells are of specific interest since they, under normoglycemic conditions, have great access to glucose (21, p. 380). It is possible that a glucose deprivation will have a more prominent effect on brain cancer cells than other cancerous cells.

1.4.2 A treatment for refractory epilepsy

The KD is already an established treatment option for patients with refractory epilepsy. These patients fail to obtain seizure control with anticonvulsant drugs or suffer from severe side effects from the medication (43). As a consequence, the KD has become an alternative therapy. The diet has documented effect on reduction in seizure frequency and remission in children (44, 45). It is unknown whether it is the direct effect from ketone bodies, the reduction of carbohydrates and/or calories or other factors that provides the antiepileptic effect (43).

The mentioning of epilepsy is relevant due to a common side effect of brain tumors being epileptic seizures (46). Among GBM patients, the incidence of epilepsy ranges from 30-50%. It can be challenging to treat these seizures due to multiple factors. The antiepileptic medication can interact with anticancer-drugs and consequently be contraindicated. Multidrug-resistance proteins can transfer the antiepileptic medication out of the brain and lead to a refractory epilepsy. Side-effects can occur in connection with surgery and/or radiation therapy. Of the clinical trials conducted on GBM and KD, only one case report is found to mention epilepsy (47). The report states that the patient's epileptic seizures ceased after he started on a KD. Two other studies reported that the patients became neurologically stable after starting the KD, without epileptic seizures being specifically mentioned (48, 49).

The fact that the KD is already being used as a treatment option for epilepsy, and that epilepsy is a side effect of GBM, can be viewed as a benefit when conducting research on KD as an adjuvant treatment for GBM.

1.4.3 A potential adjuvant treatment for cancer

Weber et al. (50) did, in 2019, a review of preclinical and clinical studies on KD as an adjuvant treatment for cancer. Their conclusion was that «the KD seems to create an unfavorable environment for cancer cell proliferation» (50). The authors pointed out that the diet appears to have a therapeutic effect in combination with anti-cancer treatment. However, due to heterogeneous and weak study designs, no conclusions could be drawn with certainty. The same applies to the subgroup of clinical and preclinical studies conducted on KD and GBM.

The clinical studies are few and have low evidence (47-49, 51-55). This is caused by a high risk of bias in relation to lack of blinding, randomization and comparison, small sample sizes and incomplete outcome data. The studies have little basis for comparison, because the diet is applied at various times in the clinical pathway and have unlike composition and duration. In addition, the patients use different or no anticancer-treatment concomitant with the KD. The majority of the studies report of patients who have had an improved progression free survival after starting the KD as an adjuvant treatment (47-49, 52, 53). Despite of challenges in elucidating what might have caused the effect, these results encourage further research. One of the two studies that did not demonstrate an improvement put two patients on a KD without concomitant treatment at recurrence (55). This is not the most noteworthy finding, as the diet is hypothesized to have effect as an adjuvant treatment.

In vivo research has demonstrated that a restricted or unlimited KD in combination with different treatments have the potential to reduce tumor growth and promote survival (54, 56-60). Two of the studies also found a minor effect when the mice were fed only an unlimited KD (56, 57). Others have found reduced tumor growth when using only a caloric restricted KD (61, 62). It has also been demonstrated that the KD leads to reduced expression of proteins involved in tumor progression (63), enhances immunity against the tumor (64) and reduces the production of ROS (65). However, not all results are consistent. One study found no improved survival when using the KD as the only treatment for GBM in mice (66), while another demonstrated oxidation of ketone bodies in the tumor (67).

In the clinical trial where the two patients had no improvement on the KD, the GBM cells were found to express ketolytic enzymes (55). Mauer et al. (66) had the same finding in their study. However, they also found that the ketone bodies did not protect the GBM cells against hypoglycemia (66). Chang et al. (68) found that GBM tumors have heterogeneous expression

of both ketolytic and glycolytic enzymes. They suggested that the expression of enzymes could contribute to decide who would be best considered for treatment with the KD. Other in vitro findings are that ketolytic enzymes are downregulated in GBM (69), the KD inhibits growth of GBM cells (70, 71), GBM cells utilize ketone bodies and fatty acid for energy production and that the KD does not reduce tumor growth (72).

The KD appears to be safe and feasible in both preclinical and clinical trials and could be regarded a possible adjuvant treatment for GBM. However, based on the fluctuating results there are grounds to do more research on the diet as an adjuvant treatment option for GBM patients. It is desired by others in the TBRG to perform mouse studies on KD and MHP88. To prepare for this, an in vitro study is necessary. Cell lines do not require ethical considerations and are simple and inexpensive to conduct research on, compared to animals and humans (73, 74, p. 6-7). Consequently, it is relatively easy to launch and carry out cellular experiments. In addition, the researcher is provided with an unlimited amount of pure cell material, making it possible to repeat experiments and reproduce results (73). Weber et al. (50) conclude in their review with a need of more molecular studies to further investigate the potential mechanisms of the KD.

2 Aims of the study

The primary aim of the present thesis was to investigate if the metabolic alterations caused by a KD have impact on GBM cells and the function of MHP88. Two of the hallmarks of cancer was utilized to answer the research questions: reprogrammed energy metabolism and the ability to evade immune destruction. This was carried out through changing the cells culturing conditions and by using the ICD inducer, MHP88, owned and patented by UiT. An additional goal was to prepare for future mouse studies with MHP88 and KD. The first research question was:

1. Does the ketone body ß-hydroxybutyrate (BHB) have an adjuvant effect to MHP88 on glioblastoma multiforme (GBM) cells?

An additional goal was to prepare for future mouse studies with the murine glioblastoma cell line GL261-luc2. Hence, a bioluminescence experiment was included in the thesis. The second research question was made in relation to this experiment:

2. Does the murine glioblastoma cell line GL261-luc2 express the luciferase gene *luc2* at the same level throughout cell culturing?

3 Materials

3.1 Equipment

Name	Company	Catalogue number
Cell scraper	Thermo Fischer Scientific	179693
Cover glasses	Brand	BR723015
F96 Microwell™, White	Thermo Fischer Scientific	236105
Glass Bottom 24-Well Plate	MatTek Corporation	P24G-1.0-13-F
Hemocytometer	Marienfeld-Superior	0640211
Immobilion-P Transfer Membranes	Millipore	IPVH00010
NuPAGE™ 10% Bis-Tris Gel, 1.5 mm x 10 well	Thermo Fischer Scientific	NP0315BOX
Tissue Culture Plate, 6 well, clear	Falcon	353046
Tissue Culture Plate, 96 well, clear	Falcon	351172
Whatman Chromatografy paper, 3 MM Chr	Sigma-Aldrich	WHA3030917

3.2 Instruments

Name	Company
Bioruptor® Plus	Diagenode
Bioruptor® Water Cooler	Diagenode
Celldiscoverer 7	ZEISS
CLARIOstar Plus	BMG Labtech
Combimag Ret	IKA
LAS3000	FujiFilm Life Science
Light microscope	Leica
Microplate Reader	VWR
PowerEase 500 Power Supply	Thermo Fischer Scientific
Sension+ PH31 Advanced GLP laboratory pH & ORP meter	Hach
Test Tube Rotator	Labinco
Titramax 1000	Heidolph
XCell SureLock™ Mini-Cell and XCell II TM Blot Module	Thermo Fischer Scientific

3.3 Media, serum and trypsin

Name	Company	Catalogue number
Dulbecco's Modified Eagle Medium (DMEM) high glucose	Sigma-Aldrich	D6429
Fetal Bovine Serum (FBS)	Sigma-Aldrich	F2442
Minimum Essential Medium Eagle (MEM)	Sigma-Aldrich	M4655
RPMI-1640 Medium	Sigma-Aldrich	R8758
RPMI 1640 Medium, no glucose	Gibco	11879020
Trypsin – EDTA Solution	Sigma-Aldrich	T4049

3.4 Reagents and antibodies

Name	Company	Catalogue number
Albumin from bovine serum (BSA)	Sigma-Aldrich	A9647
Anti-ß-actin antibody, mouse monoclonal	Sigma-Aldrich	A1978
Anti-biotin HRP-linked antibody	Cell Signaling Technology	7075
Anti-OXCT1/SCOT antibody, rabbit	Abcam	Ab105320
Biotinylated Protein Ladder	Cell Signaling Technology	7727
Bright Glow™ Luciferase Assay	Promega	E2610
Bromphenol Blue	Sigma-Aldrich	B5525
CellTiter 96® AQ_{eous} One Solution Reagent	Promega	G3582
Chemiluminescent Peroxidase Substrate-3	Sigma-Aldrich	CPS350-1KT
DC Protein Assay	Bio-Rad	5000111
Dithioireitol (DTT)	Sigma-Aldrich	10197777001
Fibronectin, solution	Sigma-Aldrich	F1141
Glycerol 99.5%	Sigma-Aldrich	G7893
Glycine	VWR	M103
Goat Anti-Rabbit IgG (H+L), Mouse/Human, ads-HRP	SouthernBiotech	4050-05
G-418 Solution	Roche	04727878001
HRP-conjugated goat anti-mouse	BD Biosciences	554002
Methanol	VWR	20847

MHP88	UiT The Arctic University of Norway	
NuPAGE™ MES SDS Running Buffer (20X)	Thermo Fischer Scientific	NP0002
Phosphate Buffered Saline (PBS) Tablets	VWR	E404
Re-Blot Plus Mild Antibody Stripping Solution	Millipore	2502
(R)- 3-Hydroxybutyric acid (BHB)	Sigma-Aldrich	54920
Seeblue™ Pre-stained Protein Standard	Thermo Fischer Scientific	LC5625
SIGMA*FAST*™ Protease Inhibitor Cocktail Tablets	Sigma-Aldrich	S8830
Sodium Chloride (NaCl)	VWR	27810.295
Sodium Deoxycholate	VWR	27836.135
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich	05030
Sodium Hydroxide (NaOH)	Sigma-Aldrich	30620
Tris Base	Millipore	648310
Tris-HCl	Merck	1.08219.0100
Triton™ X-100	Sigma-Aldrich	X100
Tween 20	Sigma-Aldrich	P1379

3.5 Solutions

Name	Contents
Blocking Buffer	5% w/v fat free milk powder in TBS-T
Blotting Buffer	0.7% w/v Tris Base, 3.6% w/v Glycine, 25% v/v methanol in MilliQ water
Phosphate Buffered Saline (PBS)	1 Phosphate Buffered Saline tablet in 100 ml water
Radioimmunoprecipitation assay (RIPA) buffer	25 mM v/v Tris-HCl, 150 mM v/v NaCl, 0.1% v/v SDS, 0.5% w/v sodium deoxycholate, 1% v/v Triton™-X100 in MilliQ water
Running Buffer	5% v/v NuPAGE MES SDS Running Buffer (20X) in MilliQ water
Tris-Buffered Saline - Tween (TBS-T)	3% v/v 5 M NaCl, 2% v/v 1 M Tris pH 7.4, 0.1% v/v Tween in MilliQ water
1 M Tris pH 7.4	15.8% w/v TrisHCl, 10 M NaOH in MilliQ water
5 M Natrium Chloride (NaCl)	29.2% w/v NaCl in MilliQ water
5X SDS sample buffer	0,25 M v/v Tris-HCl pH 6.8, 10% v/v SDS, 20% v/v Glycerol, 0.01% v/v Bromphenol Blue in MilliQ water

4 Methods

4.1 Culturing of cell lines

Five different cell lines and primary hepatocytes from mouse were used in the present thesis. Two cell lines were derived from a murine GBM (GL261, GL261-luc2), one from murine cardiac muscle cells (HL-1) one from a human GBM (U251) and one from human fibroblasts (MRC-5). To maintain a cell culture the cells have to be subcultured (75). This implies a part of the culture to be transferred into fresh growth medium. This allows for further propagation of the cell line. Following is a description of how the cell lines were cultured. The given medium compositions will later in the present thesis be referred to as the cells' standard medium.

GL261

The murine glioblastoma cell line GL261 was developed by Seligman and Shear in 1939, when they implanted methylcholanthrene pellets into the brain of 20 mice (76). It is a syngeneic mouse model derived from GBM in C57BL/6 mice. The model shares several important characteristics with human GBM regarding histopathological markers, genetic mutations and growth pattern (invasive growth). In addition, it does not require a deficient immune system in the host, making it even more similar to a human GBM (77). The cell line was bought from Leibniz Institute DSMZ – Collection of Microorganisms and Cell Cultures (ACC 802).

GL261 was cultured in 15 ml Dulbecco's Modified Eagle's Medium (DMEM) high glucose, supplied with 10% fetal bovine serum (FBS) in a T75 flask. The cells grew adherently in culture and were subcultured 3 times a week with a ratio of 1:6 to 1:8. During subculturing the medium was removed and the cells were rinsed with 10 ml room tempered phosphate buffered saline (PBS) once. 2 ml of prewarmed Trypsin-EDTA solution was added for 4-5 minutes to detach the cells from the flask. The superfluous cells were removed and subsequently fresh, prewarmed medium was added. The cells were kept in a humidified incubator holding 37 ^{o}C and 5% CO_2.

GL261-luc2

The murine glioblastoma cell line GL261-luc2 is a modified version of GL261, received from Scheck laboratory. It is stably transfected with the pGL4.54 [*luc2*/CMV/Neo] vector, which contains the firefly luciferase reporter gene *luc2* (78). *Luc2* encodes a firefly luciferase

enzyme that catalyzes the oxidation of luciferin to oxyluciferin, causing emission of green or yellow light. This feature makes the cell line useful for bioluminescence imaging both in vivo and in vitro (79).

GL261-luc2 was cultured in 15 ml DMEM high glucose, supplied with 10% FBS in a T75 flask. The cells grew adherently in culture and were subcultured 2-3 times a week with a ratio of 1:3 to 1:6. During subculturing the medium was removed and the cells were rinsed with 10 ml room tempered PBS once. 2 ml of prewarmed Trypsin-EDTA solution was added for 2-3 minutes to detach the cells from the flask. The superfluous cells were removed and subsequently fresh, prewarmed medium was added. Every 8th passage 100 µl/ml of the selection marker Geneticin (G418) was added to the medium. The cells were kept in a humidified incubator holding 37 ^{o}C and 5% CO_2.

U251

The human glioblastoma cell line U251 was established from a GBM tumor in a 75 years-old male in 1975 (80, 81). It has been carried out research on this cell line through injections in mice for several decades. Magnetic Resonance Imaging (MRI) conducted during these studies have shown similarities between the injected tumor in mice and the human GBM. U251 is known to possess the most prominent features of the human GBM, regarding histology, immunohistochemistry and genetics, including oncogenic pathways and tumor suppressors (77). The cell line was bought from Merck Life Science AS (09063001).

U251 was cultured in 15 ml DMEM high glucose, supplied with 10% FBS in a T75 flask. They grew adherently in culture and were subcultured 2-3 times a week with a ratio of 1:3 or 1:4. During subculturing the medium was removed and the cells were rinsed with 10 ml room tempered PBS once. 2 ml of prewarmed Trypsin-EDTA solution was added for 3-4 minutes to detach the cells from the flask. The superfluous cells were removed and subsequently fresh, prewarmed medium was added. The cells were kept in a humidified incubator holding 37 ^{o}C and 5% CO_2.

MRC-5

The human fibroblast cell line MRC-5 was generated from lung tissue, taken from a 14-week-old Caucasian male fetus in September 1966 (82). MRC-5 is frequently utilized as a control in cell assays (83). The cell line was bought from American Type Culture Collection, Rockville, MD, USA (CCL-171).

MRC-5 was cultured in 15 ml Minimum Essential Medium Eagle (MEM) supplied with 10% FBS in a T75 flask. They grew adherently in culture. The cells were subcultured 2-3 times a week with a ratio of 1:3 or 1:4. During subculturing the medium was removed and the cells were rinsed with 10 ml room tempered PBS twice. 2 ml of prewarmed Trypsin-EDTA solution was added for 3-4 minutes to detach the cells form the flask. The superfluous cells were removed and subsequently fresh, prewarmed medium was added. The cells were kept in a humidified incubator holding 37 ^{o}C and 5% CO_2.

HL-1

HL-1 is a cardiac muscle cell line established from a subcutaneous cardiomyocyte in C57BL/6J mouse (84). An ampulle of cells was received as a gift from Professor Ellen Aasum (IMB, UiT). The cells were lysed in RIPA buffer + 1% protease inhibitor, sonicated and stored at -20 ^{o}C until they were used in Western blotting.

Primary hepatocytes

Primary hepatocytes from mice, isolated from the liver, was received as a gift from Professor Karen Kristine Sørensen (IMB, UiT). The cells were lysed in RIPA buffer + 1% protease inhibitor, sonicated and stored at -20 ^{o}C until they were used in Western blotting.

4.2 Counting and seeding of cells

During subculturing, excessive cells were converted into a centrifuge tube. Approximately 10 µl was inserted on each side of a hemocytometer, in-between the hemocytometer and a cover glass. Further, the hemocytometer was placed under a light microscope and the counting proceeded. On each side of the hemocytometer there are nine quadrants containing 16 routes (4x4). Three out of nine quadrants were counted diagonally, starting from the upper left corner. All cells crossing the upper and the left line of the quadrants were counted. This procedure was repeated for the opposite side of the hemocytometer. When the counting was done, an average of the six routes was calculated (cells per route). Each of the nine quadrants measure 1 mm x 1 mm x 0.1 mm. This constitutes a volume of 0.1 mm^3 or 10^{-4} ml. Hence, to estimate the number of cells per ml, the number of cells per route was multiplicated by 10^4.

To find the optimal cell density, each cell line was seeded with 10,000, 20,000 and 30,000 cells/well in a 96 well plate. The cells were left in the incubator overnight and examined in a light microscope the next day. Photos were taken under the light microscope in

order to evaluate the most appropriate cell number. It was desired that the cells had reached 80% confluency (100% confluency means full coverage of the growth surface by the cells).

Protocol

Day 1

1. 10,000, 20,000 and 30,000 cells were seeded in 100 µl DMEM high glucose + 10% FBS (GL261, GL261-luc2, U251) or MEM + 10% FBS (MRC-5) in triplicates in a clear 96 well plate.
2. The well plate was left in the incubator overnight.

Day 2

3. The cells were photographed under a light microscope.

Having established a procedure for cell culturing, counting and seeding of cells, methods to answer the research questions were next carried out.

4.3 Live-cell Imaging of GL261-luc2

When on a KD the blood levels of glucose drop as described in chapter 1.4.1.2. To mimic ketosis in vitro, it is therefore essential to lower the cells' access to glucose. A live-cell imaging was performed with the murine glioblastoma cell line GL261-luc2 to investigate how long the cells could endure culturing in media holding physiological glucose concentrations (3.5 and 5 mmol/l).

Live-cell imaging is, as the name indicates, photographing of viable cells (85). The cells, usually in their culturing medium, are photographed a number of times over a period, creating a time-laps. It is important that the cells are kept in in optimal culturing conditions (here: 37^{o} C and 5% CO_2) to maintain the culture. In this experiment, brightfield microcopy was utilized. The cells were cultured in a glass bottom 24 well plate. Glass was preferred rather than plastic to ensure pictures of good quality. Consequently, the wells had to be treated with fibronectin prior to the seeding to allow adhesion of the cells. The machine Celldiscoverer 7 was used for the experiment.

Medium with 3.5 mmol/l glucose and 5 mmol/l glucose (RPMI 1640 Medium no glucose + RPMI-1640 Medium + 10% FBS) were used to mimic the physiologic blood glucose when

on, respectively, a KD and a standard diet (41). DMEM high glucose + 10% FBS, containing 23 mmol/l glucose was used as a negative control. RPMI 1640 Medium no glucose + 10% FBS was used as a positive control. Due to the content of glucose in FBS (5.7 mmol/l) the total glucose concentration of the medium became 0.57 mmol/l (86). However, the medium is referred to as glucose free to simplify matters. Figure 5 illustrates the layout of the well plate.

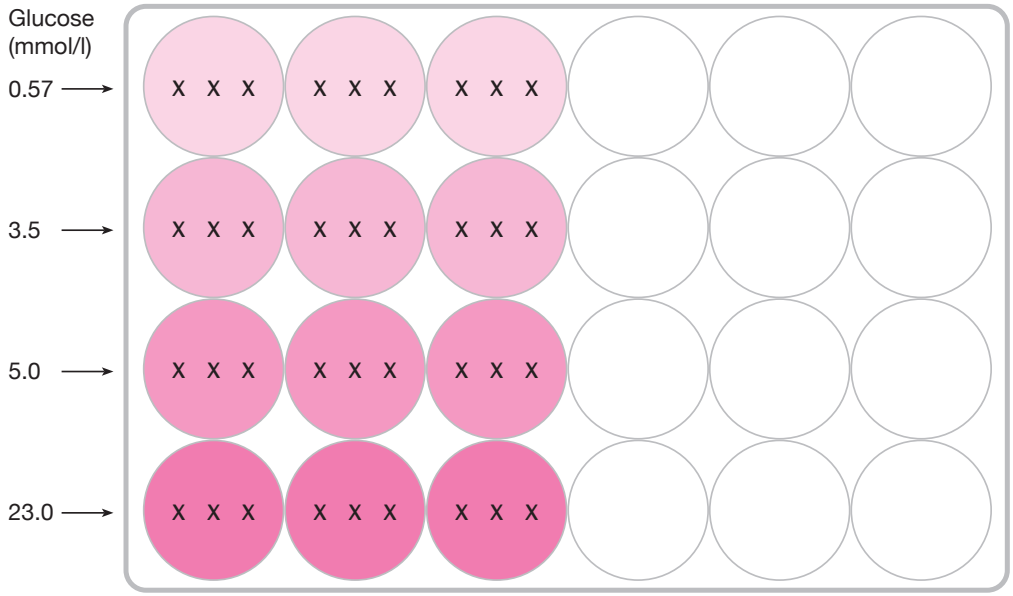

Figure 5. Layout of Live-cell Imaging
The figure illustrates the different glucose concentrations the cells were exposed to during the live-cell imaging. The crosses (x) depict where the wells were photographed.

Protocol

Day 1

Seeding of cells

1. 2 ml of 0.5 mg/ml fibronectin was diluted in 4.8 ml PBS
2. 300 µl of the fibronectin was pipetted into 12 wells in a glass bottom 24 well plate.
3. The plate was left in the incubator for 4 hours to let the fibronectin coat the wells.
4. The fibronectin was removed, and each well was washed with 300 µl PBS.
5. 150,000 GL261-luc2 cells were seeded in 1 ml DMEM high glucose + 10% FBS in the 12 wells.
6. The well plate was left in the incubator overnight.

Day 2

Stimulation with media

7. 4 ml of media containing the following was made:
    - 0.57 mmol/l glucose: 3600 µl RPMI 1640 Medium no glucose + 400 µl FBS

- 3.5 mmol/l glucose: 2560 µl RPMI 1640 Medium no glucose + 1040 µl RPMI-1640 medium + 400 µl FBS
- 5 mmol/l glucose: 2000 µl + 2560 µl RPMI 1640 Medium no glucose + 1600 µl RPMI-1640 medium + 400 µl FBS
- 23 mmol/l glucose: 3600 µl DMEM high glucose + 400 µl FBS

8. The medium was removed, and the cells were washed with 500 µl RPMI 1640 Medium, no glucose.
9. The four media made in step 6 was added to three wells each, in a volume of 1 ml.

Live-cell Imaging

10. The well plate was inserted into the Celldiscoverer 7. CO_2 was set to 5% and the temperature to 37 ºC. Photos were taken every 15 minutes for 24 hours at the spots where the crosses are marked in figure 5.

4.4 MTS assay, a cell cytotoxicity assay

In addition to the blood glucose being lowered on a KD, the blood level of ketone bodies rises (41). Ketosis was imitated in vitro through cultivation in a glucose reduced medium with BHB, as it is the most abundant ketone body in the blood (21, p. 379-380). The compound (R)-3-Hydroxybutyric acid was utilized as it is the isomer the brain utilizes for energy production (87). The cells received 1, 5 and 10 mmol/l BHB. 5 mmol/l was used because the predicted therapeutic effect of ketone bodies in human cancer is 2.5-7 mmol/l (88). 1 and 10 mmol/l were included to examine if the amount of BHB present would have an impact on the result.

To investigate if these culturing conditions would affect the function of MHP88, the *CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay* (henceforth referred to as MTS assay) was performed. It is a cell cytotoxicity assay. The cells were cultured in a 96 well plate in different media. Following, MHP88 was supplied to the culture with a concentration gradient (2-128 µg/ml) for four hours. Finally, the MTS reagent was added to the culture for one hour. The reagent contains a yellow compound which is reduced to formazan by viable cells (89). Formazan is purple. Consequently, a culture with viable cells will turn purple and a culture with dead cells will remain yellow. Absorbance can be measured at 490 nm to calculate the percentage of viable cells present in the culture. Wells containing primarily living and dead cells are used as references of respectively 100% and 0% absorbance (90). Based on this, the

half-maximal (50%) inhibitory concentration (IC_{50}) value of a drug can be calculated. This value reflects the concentration of the drug required to kill 50% of the cells in the culture.

The MTS assay reagent consists of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron coupling agent, phenazine ethosulfate (PES) (89). MTS is a salt, which in the presence of PES, is reduced to formazan by the cells' NAD(P)H dependent mitochondrial dehydrogenase enzyme. This is demonstrated in figure 6. Tetrazolium compounds are mainly reduced by the mitochondrial electron transport chain (91). Thus, the assay measures metabolic activity in the cells' mitochondria. As only the living cells produce formazan, the quantity produced is equivalent to the number of viable cells.

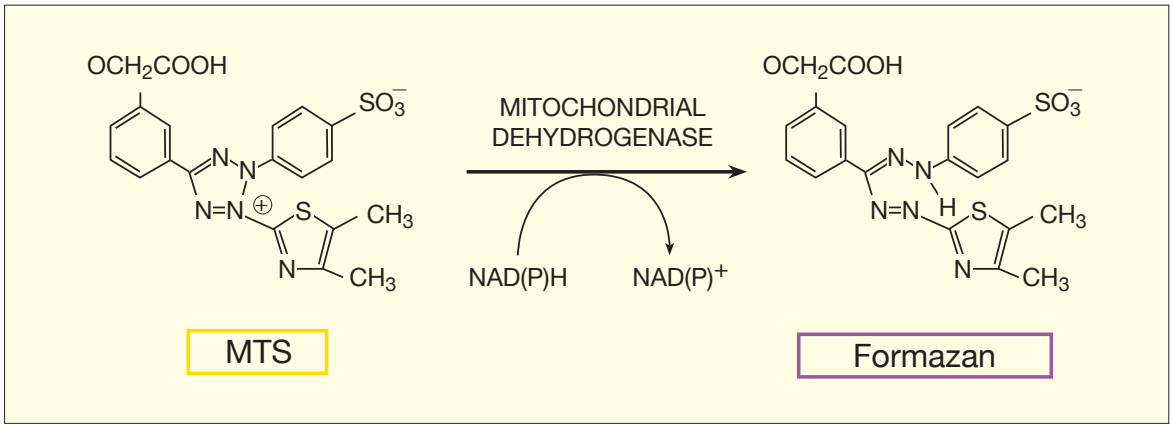

Figure 6. Reduction of MTS to formazan
The figure illustrates how MTS is reduced to formazan (89). When viable cells are exposed to MTS and the electron coupling agent PES, the latter transfers electrons from NADH or NADPH to MTS. The reaction is catalyzed by mitochondrial dehydrogenase. Consequently, MTS can only be reduced when added to a culture containing viable cells. MTS is a yellow compound which turns purple when reduced to formazan. Accordingly, there is a relation between the number of viable cells and the change in color. Following the reaction, this can be utilized by measuring the absorbance at 490 nm. PES, Phenazine ethosulfate. NA(P)D, Nicotinamide Adenine (Phosphate) Dinucleotide; NAD(P)H, Nicotineamide Adenine Dinucleotide (Phosphate), reduced.

The aim of performing MTS assays was to investigate if the cell cytotoxicity of MHP88 would become altered when the cells' standard medium was replaced with a glucose free- or reduced medium, containing BHB in different concentrations.

4.4.1 MTS assay with standard medium

In order to have a basis of comparison, the assay was first carried out with cells cultured in their standard medium. This generated results that could be compared to the assays later conducted with a glucose reduced medium and BHB. The assay was repeated three times with the murine glioblastoma cell lines GL261 and GL261-luc2, the human glioblastoma cell line U251 and the human fibroblast cell line MRC-5.

Protocol

Day 1

Seeding of cells

1. 20,000 cells (GL261, GL261-luc2, MRC-5) or 10,000 cells (U251) were seeded in 100 µl DMEM high glucose + 10% FBS (GL261, GL261-luc2, U251) or MEM + 10% FBS (MRC-5) in 24 wells in a clear 96 well plate.
2. The well plate was left in the incubator overnight.

Day 2

Preparation of reagents

3. A stock solution of 1 mg/ml MHP88 was prepared in serum free RPMI-1640 Medium.
4. From the stock solution of MHP88, the following dilutions were made:
    a. 64 µg/ml: 936 µl serum free RPMI-1640 + 64 µl stock solution
    b. 32 µg/ml: 500 µl serum free RPMI-1640 + 500 µl a
    c. 16 µg/ml: 500 µl serum free RPMI-1640 + 500 µl b
    d. 8 µg/ml: 500 µl serum free RPMI-1640 + 500 µl c
    e. 4 µg/ml: 500 µl serum free RPMI-1640 + 500 µl d
    f. 2 µg/ml: 500 µl serum free RPMI-1640 + 500 µl e
5. The positive control, 20 µl/ml Triton™ X-100, was diluted in serum free RPMI-1640.

Stimulation with MHP88

6. The medium was removed, and the cells were washed with 100 µl serum free RPMI-1640.
7. The cells, in triplicates, were stimulated with 100 µl of the six dilutions of MHP88, serum free RPMI-1640 (negative control) or diluted Triton™ X-100 (positive control). The admission time of MHP88 was logged.
8. The well plate was left in the incubator for 4 hours.

MTS assay

9. 20 µl of the MTS reagent was added to each well. The well plate was left in the incubator for 1 hour.
10. The absorbance was read at 490 nm by the use of a microplate reader. The cells were mixed for 5 seconds prior to the reading.

4.4.2 MTS assay with a glucose free medium and BHB

An MTS assay in a glucose free medium with BHB was attempted with the murine glioblastoma cell line GL261-luc2. The cells were cultured in a glucose free medium (RPMI 1640 Medium glucose free + 10% FBS) with 1, 5 or 10 mmol/l BHB. The assay was supposed to examine if the cell cytotoxicity of MHP88 would become altered when the cells were deprived of glucose and given access to ketone bodies. Here, the protocol for day 1 and 2 of this experiment is presented. On day 3 the cells were dead, and the experiment was consequently ended. The cells were supposed to be exposed to MHP88 (diluted in a glucose free medium with BHB) and the MTS reagent, similar to that of day 2 in the protocol in chapter 4.4.1. This part is left out of the following protocol.

Protocol

Day 1

Seeding of cells

1. 20,000 GL261-luc2 cells were seeded in 100 µl DMEM high glucose + 10% FBS in 51 wells in a clear 96 well plate.
2. The well plate was left in the incubator overnight.

Day 2

Preparation of media

3. A stock solution containing 2 ml RPMI 1640 Medium no glucose with 40 mmol/l BHB was prepared.
4. From the stock solution of BHB, 1.7 ml of the following was made:
    a. Medium with 1 mmol/l BHB and 10% FBS:
    170 µl FBS + 43 µl stock solution + 1487 µl RPMI 1640 Medium no glucose
    b. Medium with 5 mmol/l BHB and 10% FBS:
    170 µl FBS + 210 µl stock solution + 1320 µl RPMI 1640 Medium no glucose
    c. Medium with 10 mmol/l BHB and 10% FBS:
    170 µl FBS + 420 µl stock solution + 1110 µl RPMI 1640 Medium no glucose

Stimulation with glucose free medium and BHB

5. The medium was removed, and the cells were washed with 100 µl RPMI 1640 medium no glucose.
6. 100 µl of the three solutions made in step 2 was pipetted into respectively 15 wells each.

7. 100 µl of RPMI 1640 medium no glucose with 10% FBS was pipetted into the last 6 wells. These were the control wells.
8. The well plate was left in the incubator for 24 hours.

4.4.3 MTS assay with a glucose reduced medium and BHB

Since the cells did not survive in a glucose free medium, the MTS assays was instead carried out with a medium holding 3.5 mmol/l glucose and 0, 1, 5 or 10 mmol/l BHB. The intention was to mimic the blood levels of both glucose and ketone bodies achieved on a KD and see how this affected the cell cytotoxicity of MHP88. To better be able to differentiate the effect of BHB from a glucose reduction a group of cells were left with only the glucose reduced medium. The assay was repeated three times with the murine glioblastoma cell lines GL261 and GL261-luc2, the human glioblastoma cell line U251 and the human fibroblast cell line MRC-5. Figure 7 illustrates the layout of the 96 well plate.

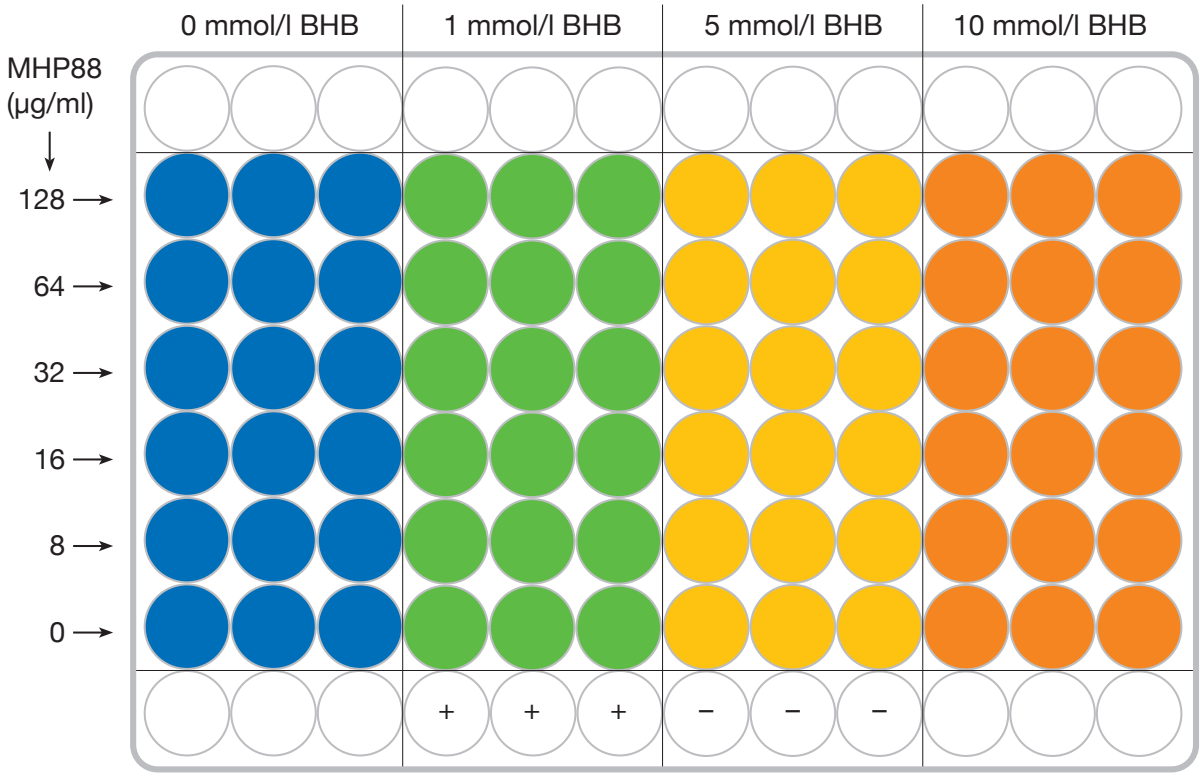

Figure 7. Layout of MTS assays with glucose reduced medium and BHB
The figure illustrates how the cells were seeded and stimulated during the MTS assays. All wells contained cells in a glucose reduced medium with either 0 mmol/l BHB (blue wells), 1 mmol/l BHB (green wells), 5 mmol/l BHB (yellow wells) or 10 mmol/l BHB (orange wells). After 16-18 hours of cultivation the cells were stimulated with MHP88 in a concentration gradient from 2 to 128 µg/ml for four hours. The positive control wells (+) were stimulated with *Triton™ X-100* and the negative (-) with low glucose medium. Finally, the MTS reagent was added for one hour and absorbance was read at 490 nm. BHB, ß-hydroxybutyrate.

Protocol

Day 1

Seeding of cells

1. 20,000 cells (GL261, GL261-luc2, MRC-5) or 10,000 cells (U251) were seeded in 100 µl DMEM high glucose + 10% FBS (GL261, GL261-luc2, U251) or MEM + 10% FBS (MRC-5) in 78 wells in a clear 96 well plate.
2. The well plate was left in the incubator for 5-6 hours.

Preparation of media

3. 40 ml of medium with 3.5 mmol/l glucose + 10% FBS was made by mixing 10.4 ml RPMI-1640 Medium, 4 ml FBS and 25.6 ml RPMI 1640 Medium no glucose. This medium is henceforth referred to as low glucose medium.
4. A stock solution containing 2 ml low glucose medium with 40 mmol/l BHB was prepared.
5. From the stock solution of BHB, 2 ml of the following were made:
    a. Medium with 3.5 mmol/l glucose, 1 mmol/l BHB and 10% FBS:
    1950 µl low glucose medium + 50 µl stock solution
    b. Medium with 3.5 mmol/l glucose, 5 mmol/l BHB and 10% FBS:
    1750 µl low glucose medium + 250 µl stock solution
    c. Medium with 3.5 mmol/l glucose, 10 mmol/l BHB and 10% FBS:
    1500 µl low glucose medium + 500 µl stock solution

Stimulation with low glucose medium and BHB

6. The medium was removed, and the cells were washed with 100 µl low glucose medium.
7. 100 µl of low glucose medium was pipetted into 18 wells (0 mmol/l BHB).
8. 100 µl of the three solutions made in step 5 was pipetted into respectively 18 wells each (1 mmol/l BHB, 5 mmol/l BHB, 10 mmol/l BHB).
9. 100 µl of low glucose medium was pipetted into the last 6 wells (controls).
10. The well plate was left in the incubator for 16-18 hours.

Day 2

Preparation of media and reagents

11. From the stock solution of BHB, 1.0 ml of the following were made:
    a. Medium with 2 mmol/l BHB
       950 µl low glucose medium + 50 µl stock solution
    b. Medium with 10 mmol/l BHB
       750 µl low glucose medium + 250 µl stock solution
    c. Medium with 20 mmol/l BHB
       500 µl low glucose medium + 500 µl stock solution
12. A stock solution of 1 mg/ml MHP88 was prepared in low glucose medium.
13. From the stock solution of MHP88, the following were made:
    a. 256 µg/ml: 1042 µl low glucose medium + 358 µl stock solution
    b. 128 µg/ml: 700 µl low glucose medium + 700 µl a
    c. 64 µg/ml: 700 µl low glucose medium + 700 µl b
    d. 32 µg/ml: 700 µl low glucose medium + 700 µl c
    e. 15 µg/ml: 700 µl low glucose medium + 700 µl d
14. The positive control, 20 µl/ml Triton™ X-100 was diluted in low glucose medium.

Stimulation with BHB and MHP88

15. The medium was removed from the cells.
16. 50 µl of low glucose medium was pipetted into the 18 wells constituting the 0 mmol/l BHB group.
17. 50 µl of medium with 2 mmol/l BHB was pipetted into the 18 wells constituting the 1 mmol/l BHB group.
18. 50 µl of medium with 10 mmol/l BHB was pipetted into the 18 wells constituting the 5 mmol/l BHB group.
19. 50 µl of medium with 20 mmol/l BHB was pipetted into the 18 wells constituting the 10 mmol/l BHB group.
20. 50 µl of each concentration of MHP88 was pipetted into 3 wells in each of the four groups. 50 µl of low glucose medium was pipetted into the 3 remaining wells in each group.
21. 100 µl of the diluted Triton™ X-100 was pipetted into the 3 positive control wells. 100 µl low glucose medium was pipetted into the 3 negative control wells.
22. The well plate was left in the incubator for 4 hours.

MTS assay

23. 20 µl of the MTS reagent was added to each well. The well plate was left in the incubator for 1 hour.
24. The absorbance was read at 490 nm by the use of a microplate reader. The wells were mixed for 5 seconds prior to the reading.

4.4.4 Statistical analysis

An Independent-Samples T Test was run in SPSS to estimate if there were a significant difference in the IC_{50} value of MHP88 when the cells were cultured in a glucose reduced medium with BHB compared to their standard medium. This was done separately for each cell line. Levine's test was used to find out if equal variances was assumed. Excel was used to estimate standard deviations.

4.5 Western blot

Western blot is a technique used in cell- and molecular biology to identify and quantify specific proteins extracted from a cell culture or tissue (92). The method involves several steps explained in the following chapters. In the present thesis, Western blotting was used to investigate if the glioblastoma cell lines (GL261, GL261-luc2, U251) expressed the rate-limiting ketolytic enzyme, SCOT. This enzyme is essential for the breakdown and utilization of BHB and acetoacetate (93).

To find the most suitable controls for the experiment, the Human Protein Atlas was utilized as a work of reference (94, 95). Primary hepatocytes from mice were used as negative control because they lack the enzyme. The murine cardiac muscle cell line, HL-1, was used as positive control, as their SCOT enzyme is known to have high catalytic activity. The human fibroblast cell line MRC-5 was included to work as a control for the human glioblastoma cell line U251. Fibroblasts are shown to have low expression of the enzyme.

Table 1. Status of SCOT in Western blot samples

Cell type	HL-1, (+ ctrl)	Hepatocytes, (- ctrl)	GL261-luc2	GL261	MRC-5 (+ ctrl)	U251
Status of SCOT	+	-	?	?	+	?

Table 1 presents the samples used in the Western blot, and the previous knowledge of the expression of SCOT. HL-1 is a murine cardiac muscle cell line, the hepatocytes were primary cells derived from mice, GL261-luc2 and GL261 are murine glioblastoma cell lines, MRC-5 is a human fibroblast cell line and U251 is a human glioblastoma cell line. (+) expression, (-) no expression, (?) unknown expression. Ctrl, control; (-), negative; (+), positive; SCOT, Succinyl-CoA:3-ketoacid (3-oxoacid) CoA transferase.

4.5.1 Preparing of sample lysates

Prior to performing a Western blot, sample lysates must be prepared in order to extract the proteins from the cells or tissue in question (96). The RIPA buffer is commonly used for this purpose. It was supplied to the cell layer and the cell lysates were collected by scraping. The buffer contains the detergent sodium dodecyl sulfate (SDS), which causes the proteins to denature by disrupting their non-covalent interactions. SDS also coats the proteins with a negative charge by binding to them. Protease inhibitor was added to the buffer to avoid degradation of the target proteins by endogenous proteases, once the cell was lysed. The cell samples were sonicated to ensure complete cell lysis. Sonication is a process where ultrasound waves are used to transfer energy into the samples. It disrupts cellular membranes.

Protocol

Day 1

1. GL261, GL261-luc2, MRC-5 (750,000 cells/well) and U251 (375,000 cells/well) were seeded in triplicates in 6 well plates in 3 ml DMEM high glucose + 10% FBS (GL261, GL261-luc2, U251) or MEM + 10% FBS (MRC-5) and left in the incubator overnight.

Day 2

2. 100 x protease inhibitor was made by mixing one Protease Inhibitor Cocktail Tablet with 1 ml Milli Q water.
3. 200 µl RIPA buffer with 1% protease inhibitor was prepared per well.
4. The cells were washed twice with 1 ml PBS.
5. 200 µl of the RIPA buffer with 1% protease inhibitor was added to each well.
6. A cell scraper was used to remove the cells from their growth surface and the cell extracts were pipetted into 1.5 ml Eppendorf tubes.
7. The cells were sonicated for 10 minutes at 4 ^{o}C. Time on: 20 sec. Time off: 20 sec.
8. The samples were aliquoted and stored at -20 ^{o}C.

4.5.2 Protein assay

The concentrations of proteins in cell lysates are unknown (92). To be able to interpret the results of a Western blot it is important that the amount of protein is equal across all samples, or else they cannot be correctly compared. By using a protein standard as a reference, the protein concentrations of the various samples can be calculated. The DC protein assay from Bio-Rad was used for this purpose with albumin from bovine serum (BSA) as the standard of reference.

Protocol

1. A protein standard was prepared by diluting 2 mg/ml BSA in 1 ml RIPA buffer.
2. The following dilutions of the protein standard was made:
    a. 1.4 mg/ml: 10 µl RIPA buffer + 70 µl 2 mg/ml BSA
    b. 1.2 mg/ml: 10 µl RIPA buffer + 60 µl b
    c. 1.0 mg/ml: 10 µl RIPA buffer + 50 µl c
    d. 0.8 mg/ml: 10 µl RIPA buffer + 40 µl d
    e. 0.6 mg/ml: 12,5 µl RIPA buffer + 37.5 µl e
    f. 0.4 mg/ml: 15 µl RIPA buffer + 30 µl f
    g. 0.2 mg/ml: 20 µl RIPA buffer + 20 µl g
    h. RIPA buffer (blank)
3. All samples were diluted 1:10 in RIPA buffer.
4. 20 µl of reagent S was added to each ml of reagent A needed for the run (reagent A').
5. 5 µl of standards and samples (undiluted and diluted) were pipetted in triplicates in a 96 well plate.
6. 25 µl of the reagent A' was added to each well followed by 200 µl of reagent B.
7. The plate was mixed for 15 minutes using Titramax 1000 at 300 revolutions per minute (rpm)
8. Absorbance was read at 750 nm by the use of a microplate reader.
9. The protein quantity was calculated based on the standard curve for BSA.
10. The samples were diluted in RIPA buffer to contain the same quantity of proteins and stored at -20 ^{o}C until they were used in Western blotting.

4.5.3 Gel electrophoresis

As cells and tissue contain multiple proteins it is essential to separate them in order to perform identification and quantification (92). This was achieved through an electrophoresis, which separates the proteins based on their molecular weight (MW). The samples were

pipetted into wells on a polyacrylamide gel already placed in a mini cell, coupled to a power supply. An electric current causes the well-side of the gel to become negatively charged and the proteins to migrate towards the other, positively charged, end of the gel. Small proteins migrate easily and thereby more rapidly than large proteins. Hence, the proteins are separated on the gel according to their size.

Prior to the electrophoresis, the samples were diluted in a loading buffer containing glycerol (to let the samples sink in the wells) and bromphenol blue (to make it possible to observe the proteins during the separation) (92). DTT was supplied to help solubilize the proteins (96). Finally, the samples were heated to denature the proteins (92). A protein ladder was included to have a reference of molecular weight.

Protocol

1. 20% 5xSDS sample buffer and 500 mmol/l DTT were added to each sample.
2. The samples and a biotinylated protein ladder were heated at 100 ^{o}C for 5 minutes in a Combimag Ret.
3. The comb and tape were removed from the gel plate before it was placed in the buffer core in the lower buffer chamber of the XCell SureLock® Mini-Cell.
4. The inner and outer chamber were filled with running buffer.
5. 25 µl of the samples were loaded into one well each. 10 µl of the biotinylated protein standard and 7 µl of a seablue ladder were loaded into the two outermost wells.
6. The Mini-Cell lid was placed on top of the chamber, and the gel was run according to the NuPAGE gel program on the PowerEase 500 Power Supply for 40 minutes.

4.5.4 Blotting

After the proteins had been separated on the gel, they were transferred onto a nitrocellulose membrane. This was achieved by making a blotting module where the gel was placed in the middle of a positive electrode and the membrane. An electric current drags the negatively charged proteins against the positive cathode, from the gel and onto the membrane (92). The blotting module also included filter paper for the protection of the gel and membrane, and blotting pads to ensure contact between the membrane and the gel.

Protocol

1. Two filter papers and several blotting pads was prewetted in blotting buffer.
2. A transfer membrane was soaked in methanol for 3 seconds, in water for 10 seconds and in blotting buffer for minimum 5 minutes.
3. The gel was retrieved and placed on a filter paper.
4. The blotting module was put together in the cathode core of the XCell II™ Blot module accordingly: blotting pads, filter paper, gel, transfer membrane, filter paper, blotting pads. Air bubbles were removed underway.
5. The module was placed in the lower buffer chamber.
6. The inner chamber was filled with running buffer and the outer with cold water.
7. The Mini-Cell lid was placed on top of the chamber and blotting proceeded according to the NuPage Blot program on the PowerEase 500 Power Supply for 1 hour.

4.5.5 Blocking and detection

After the proteins had been transferred to the nitrocellulose membrane a blocking procedure was performed. The aim of this step is to evade a nonspecific binding of antibodies (92). Dried non-fat milk was used for this purpose. The dairy proteins bind to the membrane, blocking a potential binding of antibodies.

Subsequently, the membrane was incubated with the primary antibody anti-SCOT/OXCT1. The antibody binds to its target protein on the membrane (92). To be able to detect where the antibody was bound, the membrane was exposed to an enzyme horse radish peroxidase (HRP) conjugated secondary antibody, which binds to the primary antibody. When, in the next step, a luminol-based chemiluminescent substrate was poured over the membrane, HRP catalyzed the oxidation of luminol causing detectable light to be emitted. This is illustrated in figure 8. Here, LAS-3000 was used to detect the light.

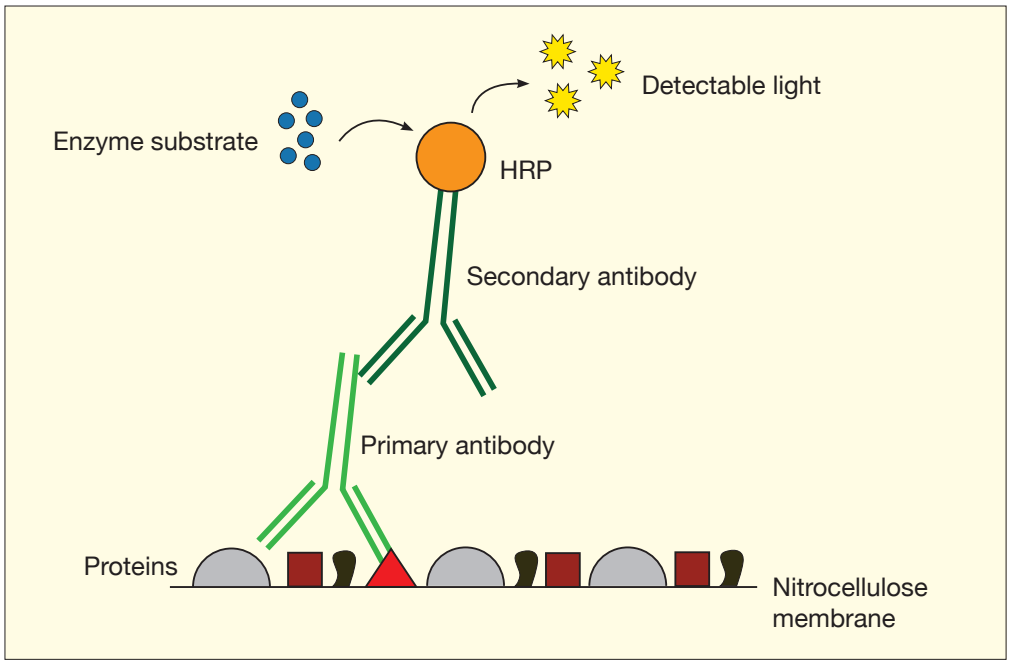

Figure 8. Detection of proteins during Western blotting
The membrane is soaked in blocking buffer containing primary antibody, leading to the detection and binding of the protein in question (92). Further, the membrane is exposed to a secondary antibody, conjugated to HRP. The secondary antibody binds to the primary antibody. When a luminol-based chemiluminescent substrate is poured over the membrane, HRP catalyzes the oxidation of luminol causing detectable light to be emitted. Finally, the light signal can be detected and captured. HRP, Horse Radish Peroxidase.

Protocol

Day 1

1. The membrane was soaked in TBS-T for 5 minutes and thereby transferred to a 50 ml tube containing 5 ml blocking buffer. It was left for 1 hour on a test tube rotator in room temperature.
2. Anti-OXCT/SCOT1 was diluted 1:500 in blocking buffer. The membrane was exposed to the diluted antibody overnight on a test tube rotator at 4 ^{o}C.

Day 2

3. The membrane was washed in TBS-T for 5 minutes on the test tube rotator x 3.
4. Anti-rabbit IgG and anti-biotin HRP-linked antibody was diluted, respectively, 1:2000 and 1:1000 in 5 ml blocking buffer. The membrane was exposed to the diluted antibodies for 1 hour on the test tube rotator in room temperature.
5. The membrane was washed in TBS-T for 5 minutes on the test tube rotator x 3.

6. 2 ml of the Chemiluminescent Peroxidase Substrate-3 was prepared by mixing 1 ml Chemiluminescent Reagent with 1 ml Chemiluminescent Reaction Buffer. The substrate was room tempered.
7. The membrane was incubated in the room tempered substrate for 5 minutes.
8. Excess liquid was drained from the membrane and it was wrapped in a plastic film.
9. LAS3000 from Fuji was used to detect and photograph the emitted light.

4.5.6 Stripping and reprobing of the membrane

If it is desirable to detect more than one protein on the membrane, it can be stripped of the antibody and reprobed with a new one (97). This can be utilized to detect proteins of similar size or to run a loading control. The latter is done to ensure that no loading or protein transfer error has occurred, and to verify that the samples contain similar amounts of protein. The loading control is the antibody of a protein that is present in all cell types. Here, the antibody of ß-actin was used.

Protocol

1. 5 ml stripping buffer was made by diluting Re-Blot Plus Mild Solution (10X) 1:10 in water.
2. The membrane was incubated with stripping buffer for 15 minutes on the test tube rotator in room temperature.
3. The membrane was rinsed in water x 3.
4. The membrane was blocked in 5 ml blocking buffer for 2x5 minutes on the test tube rotator.
5. Anti-ß-actin was diluted 1:4000 in blocking buffer. The membrane was incubated with the primary antibody overnight on the test tube rotator at 4 ^{o}C.
6. Step 4-9 in the protocol for blocking and detection was carried out. The secondary antibody, HRP-conjugated goat anti-mouse, was diluted 1:3000.

4.6 Bioluminescence measurements

The murine glioblastoma cell line GL261-luc2 is transfected with the pGL4.54 [*luc2*/CMV/Neo] vector, containing the firefly luciferase reporter gene *luc2* (78). *Luc2* encodes a firefly luciferase enzyme that catalyzes the oxidation of luciferin to oxyluciferin, causing emission of green or yellow light. This feature can be utilized to, among other things, perform bioluminescence imaging (79). Bioluminescence is a process where living organisms

produce and emit light. The reaction catalyzed by the firefly luciferase is an oxidation reaction of firefly luciferin, as shown in figure 9. When doing experiments in mice, one can insert GL261-luc2 into the brain and thereafter insert luciferin into the tail (79). The luciferase enzyme will catalyze the oxidation of luciferin into visible light. This enables the scientist to observe the development of the tumor with the use of bioluminescence imaging.

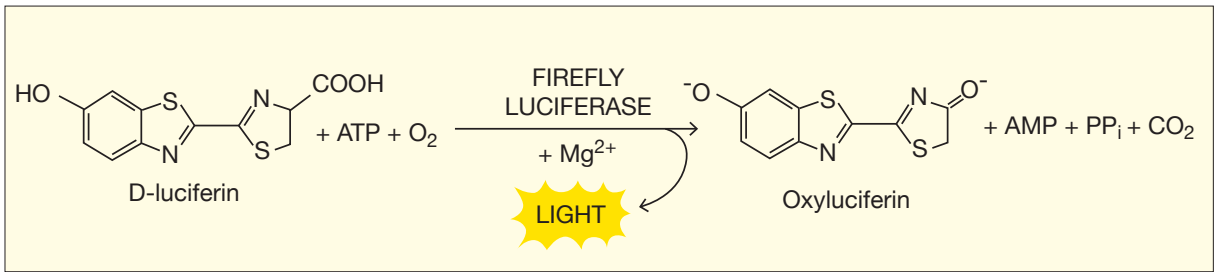

Figure 9. Oxidation of luciferin to oxyluciferin and light
The figure depicts a reaction catalyzed by the enzyme firefly luciferase, where luciferin is oxidized to oxyluciferin and light (79). The murine glioblastoma cell line GL261-luc2 is transfected with a vector containing the reporter gene *luc2*, which encodes a firefly luciferase enzyme. When luciferin is added to the cell culture, the oxidation reaction occurs, and light is produced.

According to the growth protocol received from Scheck laboratory reselection with geneticin (G418) is recommended every 8th to 10th passage to stimulate the selection of the transfected cells. G418 is an antibiotic that induces cell death by binding to the ribosome and causing inhibition of protein synthesis in both eukaryotic and prokaryotic cells (98). The pGL4.54 [*luc2*/CMV/Neo] vector contains a marker that gives resistance to G418 (99). Consequently, the antibiotic only kills untransfected cells when supplied to the culture.

The luminescence signal emitted from GL261-luc2 was measured to verify that the cell line did express *luc2*, and to examine whether the expression was stable in the weeks between selection. This was done to prepare for future mouse studies on MHP88 and KD with the cell line. G418 was expected to kill the untransfected cells in the culture, and thereby promote a culture with mainly transfected cells (98). The number of tumor cells in vitro correlates with the luminescence signal from the luciferase enzyme (100). Since the antibiotic was added only every 8th passage, it was desirable to test if the luminescence signal, and thereby the number of transfected cells, varied after selection with G418.

Table 2. Timing of selection and bioluminescence measurements

P-number	8, 4*	1	2	3	4	5	6	7	8	1
Selection (G418), Bioluminescence (BL)	G418	BL		BL		BL		BL	G418	BL

Table 2 illustrates how the timing of bioluminescence measurements (BL) was related to selection of the murine glioblastoma cell line GL261-luc2 with G418. The selection marker was added to the culture every 8th passage to stimulate the selection of cells transfected with the *luc2* gene, as described in the growth protocol received from Scheck laboratory. Bioluminescence was measured the first passage after G418 was added and then every other passage until it was added once again. A last measurement was performed after G418 was added the second time to see if it corresponded with the first measurement. *Measurements were carried out from two batches of GL261-luc2 cells. With batch 1 G418 was added the 8th passage after thawing, while with batch 2 it was added the 4th passage after thawing.

Bioluminescence measurements were performed with cells from the first passage after G418 was added, then every other passage until reselection with G418. An additional measurement was carried out the first passage after reselection. Two rounds of measurements were performed with two different cell batches. With batch 1 G418 was added the 8th passage after thawing, while with batch 2 it was added the 4th passage after thawing. GL261 was used as a negative control. GL261-luc2 was seeded with 5000, 10,000 and 20,000 cells/well. GL261 was seeded with 20,000 cells/well. A few modifications were made with batch 2 when seeding cells. Hence, two alternatives are presented in the protocol.

Protocol 1

Day 1

Seeding of cells 1

1. GL261-luc2 were seeded with 5000, 10,000 and 20,000 cells/well in 100 µl DMEM high glucose + 10% FBS in duplicates in a white 96 well plate.
2. GL261 cells were seeded with 20,000 cells/well in DMEM high glucose + 10% FBS in duplicates.
3. 100 µl of medium was pipetted into three empty wells to be used as a control.
4. The well plate was left in the incubator until next day.

Seeding of cells 2

1. Cells were seeded as explained above, but in triplicates. In addition, every other well was used both horizontal and vertical to leave empty wells between the cells. This was done to avoid that the luminescence signals from the wells would affect eachother.

Day 2

Preparation of reagent and cells

5. One bottle of Bright-Glo™ Buffer was mixed into one bottle of Bright-Glo™ Substrate, creating the reagent. Aliquotes of 1 ml was made the first time. These were stored at -70 °C.
6. Both cells and reagent were equilibrated to room temperature for maximum reproducibility.

Bioluminescence measurement

7. The settings on the CLARIOstar Plus were set to:
    - Emission: no filter
    - Optic: top optic
    - Setling time: 0.1 seconds
    - Measure interval time: 10.00 seconds
    - Pause before plate reading for 120 seconds (to allow complete cell lysis)
    - Shake metode: double orbital, 500 rpm, shake before plate reading
8. 100 µl of the reagent was added to each well. The plate was inserted into the CLARIOstar Plus and the reading was started immeadiatly.
9. To remove backround reading, the average luminescence from the three wells containg medium was substracted for the luminescence of the wells containing cells.

5 Results

The main intention with the present thesis was to investigate if the ketone body BHB could have an adjuvant effect to MHP88 on GBM cells. This was attempted answered by conducting variations of MTS assays. In the process of developing a protocol for the MTS assays, it was experimented with the content of glucose in the cell media. The first result presented here is from culturing of cells in a glucose free medium, as it formed the basis of the other experiments. A live-cell imaging was performed to examine if, and for how long, the cells could endure culturing in a glucose reduced environment. Prior to performing the MTS assays, cells were seeded in a 96 well plate to find the optimal cell number/well. To better be able to discuss the results from the MTS assays, a Western blot was carried out to investigate if the GBM cells did express the rate-limiting ketolytic enzyme, SCOT. In addition, a pH measurement of the different cell media used in the MTS assays was made.

Last, a bioluminescence measurement was conducted with the murine glioblastoma cell line GL261-luc2 to find out if the cells expressed *luc2* at a continuous level throughout cell culturing.

5.1 GL261-luc2 cultured in a glucose free medium with BHB

The MTS assays with BHB and MHP88 was originally to be performed with a glucose free medium for all cell lines. When the murine glioblastoma cell line GL261-luc2 was cultured in this medium however, the cells died. Figures 10a-d display pictures of the cells cultured in a glucose free medium and respectively 0, 1, 5 and 10 mmol/l BHB. The cells had detached from the well surface, changed shape from elongated to spherical and were floating in the wells. Based on the cells' appearance there seemed to be no differences between the wells regarding the quantity of BHB provided. Figure 10e shows a well containing GL261-luc2 cells cultured in standard medium for 48 hours, included to give a reference of living cells. These cells were adherent in the wells.

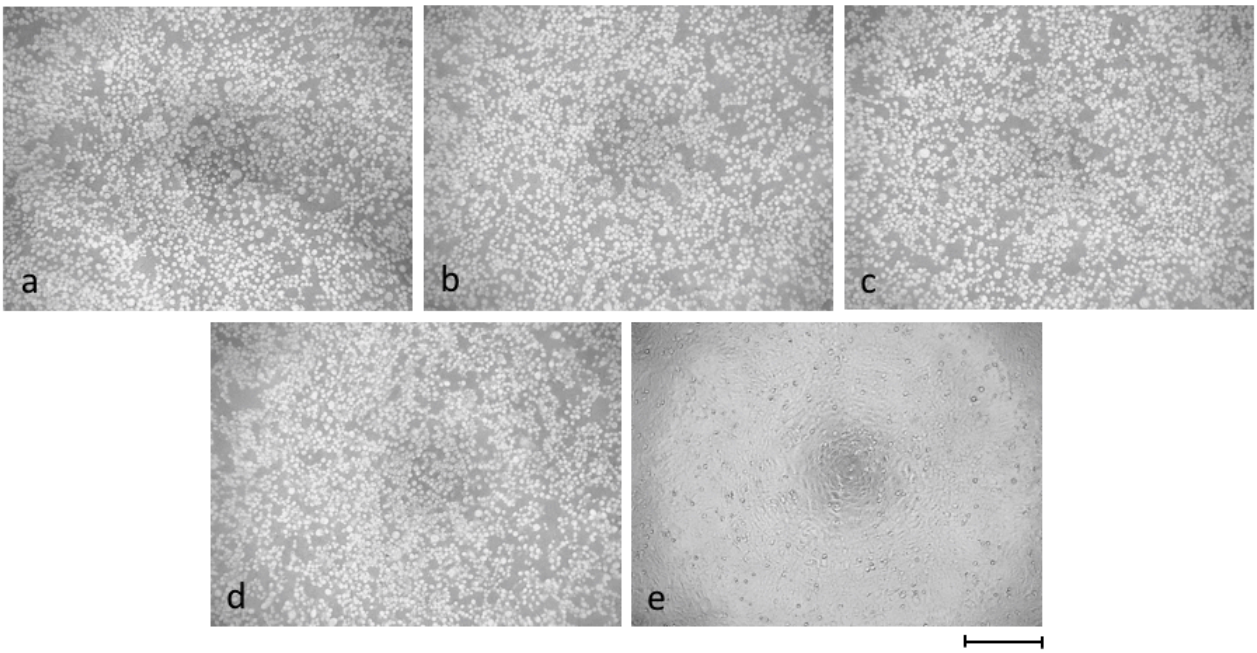

Figure 10. GL261-luc2 after cultivation in a glucose free medium with BHB
The pictures were taken after the murine glioblastoma cell line GL261-luc2 had been cultured in a plastic 96 well plate for 48 hours. The first 24 hours the cells were cultured in DMEM high glucose + 10% FBS. The next 24 hours the cells were cultured in the following: **a)** RPMI 1640 Medium no glucose + 10% FBS, **b)** RPMI 1640 Medium no glucose + 10% FBS + 1 mmol/l BHB, **c)** RPMI 1640 Medium no glucose + 10% FBS + 5 mmol/l BHB, **d)** RPMI 1640 Medium no glucose + 10% FBS + 10 mmol/l BHB, **e)** DMEM high glucose + 10% FBS. These cells were included in the figure to give a standard of comparison. Scale bar 200 µm. BHB, ß-hydroxybutyrate; FBS, Fetal Bovine Serum.

Due to the cell death, the MTS assays could not be carried out in the glucose free medium. A live-cell imaging was next performed to investigate the cells' lifetime in a glucose reduced medium.

5.2 Live-cell Imaging of GL261-luc2 in different glucose concentrations

It was desirable to investigate the cells' endurance to culturing in a glucose reduced medium prior to designing a new MTS assay. A live-cell imaging was carried out over 24 hours with the murine glioblastoma cell line GL261-luc2. The cells were seeded in their standard medium on day 1. On day 2 it was replaced with medium holding 23 mmol/l, 5 mmol/l, 3.5 mmol/l and 0.57 mmol/l glucose and photographing was initiated. The content of serum in the cell media contributed with 0.57 mmol/l glucose (86). The results are presented in figure 11.

Cells cultured in 23 mmol/l glucose worked as a negative control, as the medium utilized was the cells' standard medium. They appeared to have proliferated into a homogenous, confluent and smooth layer after 24 hours. The cells receiving 0.57 mmol/l glucose were included as a

positive control. When 24 hours had passed, these cells looked heterogeneous, uneven and disjointed. This indicated that cell death was initiated.

Medium holding 3.5 and 5 mmol/l glucose were utilized to mimic the fasting blood glucose on a KD and a standard diet, respectively. There appeared to be little difference in the proliferation of the cells in these two groups. After 24 hours they had proliferated into a layer partly resembling the negative control but appeared less confluent and more uneven. The photos display that the cells were able to proliferate and survive in 3.5 and 5 mmol/l glucose and that cell death occurred only with 0.57 mmol/l glucose.

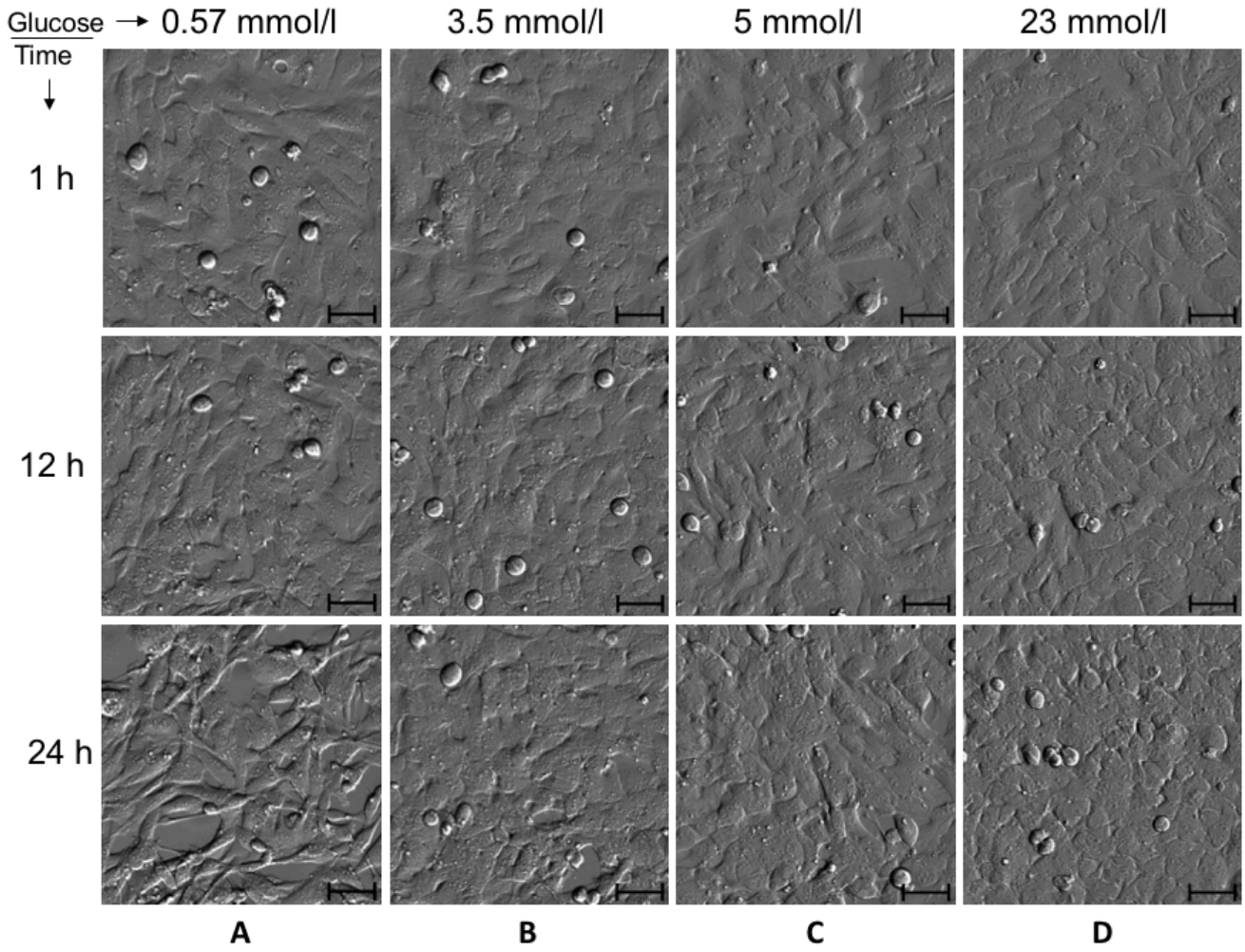

Figure 11. Live-cell Imaging results for GL261-luc2
The murine glioblastoma cell line GL261-luc2 was seeded with 150,000 cells/well in 12 wells in a glass bottom 24 well plate, coated with fibronectin. The cells were seeded in DMEM high glucose + 10% FBS and left in the incubator overnight. The following morning the medium was replaced with **A)** RPMI 1640 Medium no glucose + 10% FBS, **B-C)** RPMI 1640 Medium no glucose + RPMI-1640 Medium + 10% FBS or **D)** DMEM high glucose + 10% FBS. Following, a live-cell imaging was performed over 24 hours. Three pictures were taken of each well every 15 minutes. The figure depicts photos taken after 1, 12 and 24 hours. These pictures were carefully selected to represent the general observation made. Photos were taken with Celldiscoverer 7. Scale bar 50 µm. DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum.

With a basis in the live-cell imaging results, a new MTS assay protocol (see chapter 4.4.3) was developed with a glucose reduced medium holding 3.5 mmol/l glucose. The exposure time to the medium was set to 16-17 hours prior to adding MHP88, for all cell lines of interest. This timing was chosen because it was well within 24 hours and due to practical considerations.

5.3 Cell number per well during MTS assays

Prior to performing the MTS assays with the murine glioblastoma lines GL261 and GL261-luc2, the human glioblastoma cell line U251 and the human fibroblast cell line MRC-5, the optimal cell number per well in a 96 well plate was investigated. The four cell lines were seeded with 10,000, 20,000 and 30,000 cells/well, left in the incubator for 24 hours and then photographed. The results are displayed in figures 12-15. It was desired that the cells had reached 80% confluency after 24 hours. Based on the photographs, this appeared to be the incident with 20,000 cells/well for all cell lines. Hence, the cells were seeded with 20,000 cells/well in the initial MTS assays with standard medium.

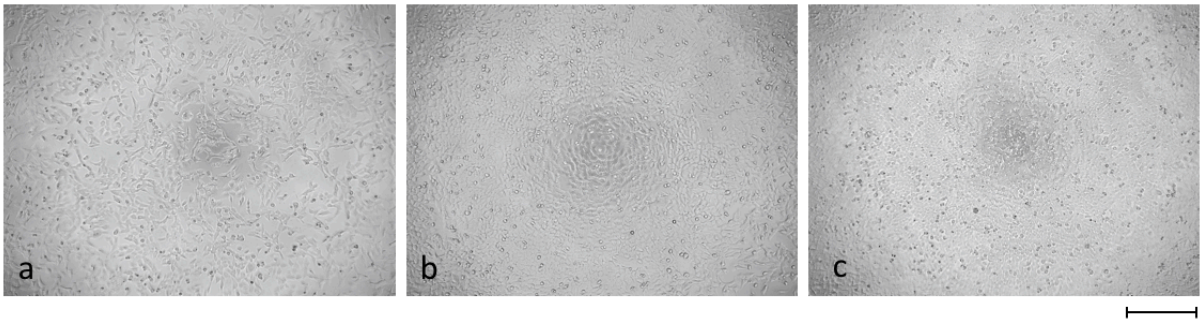

Figure 12. GL261 in a 96 well plate after 24 hours
The murine glioblastoma cell line was seeded in DMEM high glucose + 10% FBS in a 96 well plate and left in the incubator for 24 hours. **a)** 10,000 cells/well **b)** 20,000 cells/well **c)** 30,000 cells/well. Scale bar 200 µm.

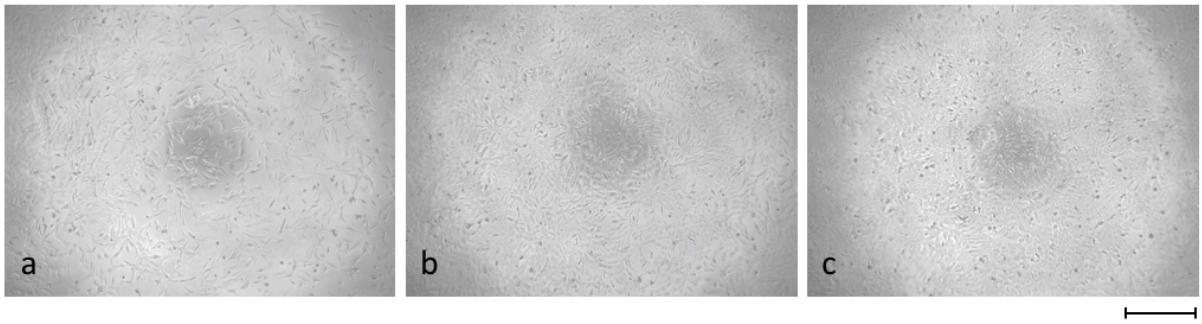

Figure 13. GL261-luc2 in a 96 well plate after 24 hours
The murine glioblastoma cell line GL261-luc2 was seeded in DMEM high glucose + 10% FBS in a 96 well plate and left in the incubator for 24 hours. **a)** 10,000 cells/well **b)** 20,000 cells/well **c)** 30,000 cells/well. Scale bar 200 µm.

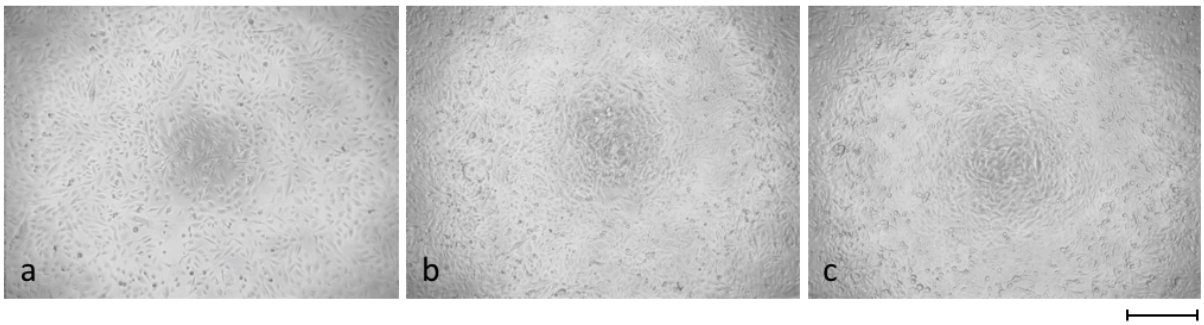

Figure 14. U251 in a 96 well plate after 24 hours
The human glioblastoma cell line U251 was seeded in DMEM high glucose + 10% FBS in a 96 well plate and left in the incubator for 24 hours. **a)** 10,000 cells/well **b)** 20,000 cells/well **c)** 30,000 cells/well. Scale bar 200 µm.

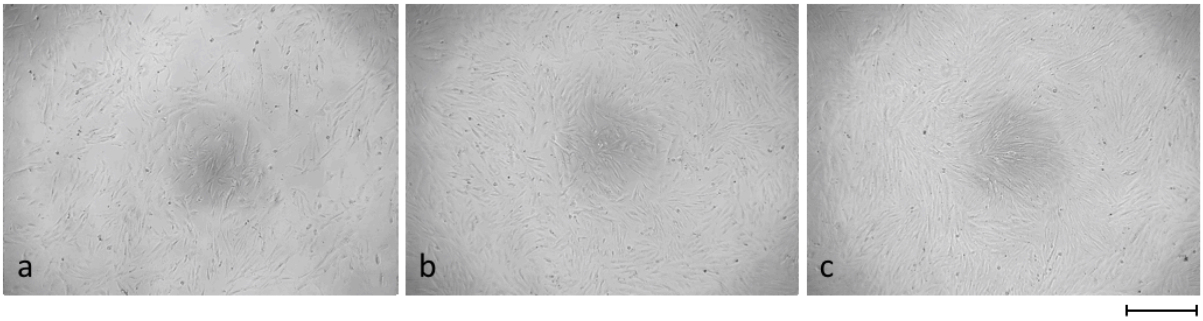

Figure 15. MRC-5 in a 96 well plate after 24 hours
The human fibroblast cell line MRC-5 was seeded in MEM + 10% FBS in a 96 well plate and left in the incubator for 24 hours. **a)** 10,000 cells/well **b)** 20,000 cells/well **c)** 30,000 cells/well. Scale bar 200 µm.

To ensure 20,000 cells/well was ideal, absorbance values from the MTS assays conducted with standard medium followed by serum free RPMI-1640 Medium and MHP88 were used as a verification. The absorbance values are based on the change in color after the MTS solution is added, as described in chapter 4.4. Figure 2 in the *CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay* protocol (see figure 22 in appendix) displays that there is a linear correlation between cell number and absorbance values for B9 hybridoma cells, until the absorbance reaches 0.8 (89). When the absorbance increases towards 1.2, this correlation is slightly diminished. Consequently, absorbance values up to 0.8 is ideal, but levels up to 1.2 is also acceptable. It was therefore desired that all absorbance values were to be kept below 1.2.

Table 3 shows absorbance values with 20,000 cells/well for GL261, GL261-luc2, MRC-5 and U251 and with 10,000 cells/well for U251. For U251, the absorbance values with 20,000 cells/well reached 1.9. As a consequence, the MTS assays was performed with 10,000 cells/well. The other cell lines were seeded with 20,000 cells/well in all MTS assays. The declining absorbance values with increasing concentrations of MHP88 is due to a reduction in the number of viable cells in the culture.

Table 3. Average absorbance values from MTS assays with MHP88

Concentration of MHP88	Average absorbance values for the cell lines (cell number/well)				
	GL261 (20,000)	GL261-luc2 (20,000)	MRC-5 (20,000)	U251 (20,000)	U251 (10,000)
64 µg/ml	0,12	0,12	0,13	0,20	0,17
32 µg/ml	0,13	0,13	0,15	0,24	0,19
16 µg/ml	0,15	0,24	0,44	1,15	0,37
8 µg/ml	0,40	0,36	0,95	1,82	0,95
4 µg/ml	0,49	0,38	1,17	1,83	1,0
2 µg/ml	0,51	0,40	1,18	1,9	1,1
0 µg/ml	0,48	0,40	1,14	1,7	1,1

Table 3 shows average absorbance values for the murine glioblastoma cell lines GL261 and GL261-luc2 and the human fibroblast cell line MRC-5 with 20,000 cells/well and the human glioblastoma cell line U251 with 20,000 and 10,000 cells/well in a 96 well plate. The absorbance was read at 490 nm after the cells had been cultured in their standard medium, followed by exposure to various concentrations of MHP88 (in serum free RPMI-1640 Medium) for four hours and the MTS reagent for one hour. The average absorbance values for GL261, GL261-luc2 and MRC-5 were calculated from three MTS assays per cell line with three wells per concentration of MHP88. Corresponding values for 10,000 and 20,000 U251 cells/well were, respectively, two and three MTS assays with three wells per concentration of MHP88.

5.4 Cell cytotoxicity of MHP88 and BHB

In the first round of MTS assays the cells were cultured in their standard medium and stimulated with MHP88 dissolved in serum free RPMI-1640. In the second round the cells were cultured and stimulated with MHP88 in a low glucose medium with 0, 1, 5 or 10 mmol/l. The second round contained in fact four different assays.

The hypothesis was that a KD starves cancer cells and that ketone bodies might have a direct effect in enforcing death upon the cells. Consequently, the IC_{50} value of MHP88 was expected to be lower in round two compared to round one. If the cells were unable to utilize ketone bodies but took damage of the low supply of glucose, it was expected that all four assays (0, 1, 5 and 10 mmol/l BHB) would generate the similar IC_{50} values of MHP88. If the ketone bodies had a direct effect upon the cancer cells, the IC_{50} value of MHP88 was expected to decline with increasing concentrations of BHB.

The mean IC_{50} value of MHP88 with standard deviations in all five assays (standard, 0, 1, 5 and 10 mmol/l BHB) for all four cell lines are presented in figure 16. Each assay was run three times for each cell line with triplicates of each dose of MHP88. An Independent-Samples T Test was run to estimate if there were a significant difference in the IC_{50} value of MHP88 alone, compared to MHP88 with BHB. This was done separately for each cell line and for each concentration of BHB. The null hypothesis was that the IC_{50} value of MHP88 was equal in all assays. $P \leq 0.05$ indicates a significant result.

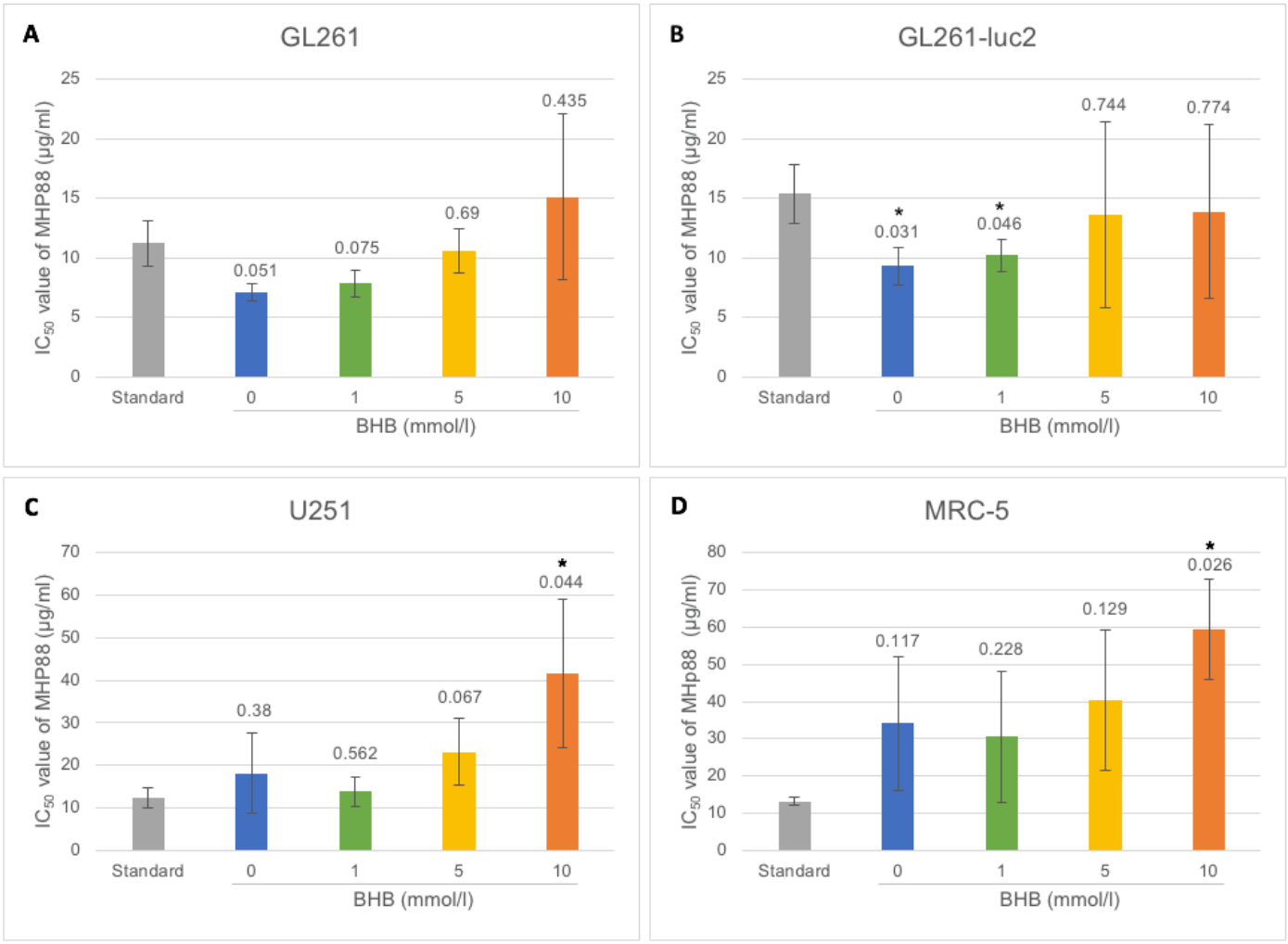

Figure 16. Comparison of mean IC_{50} values of MHP88 in different culturing media
Graphs A-D depict the mean IC_{50} values of MHP88 with standard deviations in five different MTS assays conducted with **A)** the murine glioblastoma cell line GL261, **B)** the murine glioblastoma cell line GL261-luc2, **C)** the human glioblastoma cell line U251 and **D)** the human fibroblast cell line MRC-5. In the first assay the cells were cultured in their standard medium and exposed to MHP88 in serum free RPMI-1640 Medium (grey columns). In the following assays the cells were cultured and exposed to MHP88 in 3.5 mmol/l glucose (blue columns), 3.5 mmol/l glucose + 1 mmol/l BHB (green columns), 3.5 mmol/l glucose + 5 mmol/l BHB (yellow columns) or 3.5 mmol/l glucose + 10 mmol/l BHB (orange columns). All means were calculated from three identical MTS assays with triplicates for each concentration of MHP88. An Independent-Samples T Test was run separately for each cell line. The IC_{50} values of MHP88 in the glucose reduced assays was individually compared with the IC_{50} value of MHP88 in the assay with standard medium. The p-value is presented above each column. *$p \leq 0.05$ implies significance. BHB, ß-hydroxybutyrate; IC_{50}, half-maximal inhibitory concentration.

For the murine glioblastoma cell line GL261 there were no significant results ($p > 0.05$), as displayed in figure 16. This implies that the IC_{50} value, and at this the cell cytotoxicity, of MHP88 was statistically unaffected by the change in nutrient supply. Still, a trend could be observed with increasing cell cytotoxicity in the glucose reduced medium holding 0 mmol/l (p=0.051) and 1 mmol/l BHB (p=0.075) and a stable cell cytotoxicity with 5 mmol/l (p=0.69) and 10 mmol/l BHB (p=0.435).

A similar tendency was apparent for the murine glioblastoma cell line GL261-luc2. Here, the IC_{50} value of MHP88 was significantly lower when combined with low glucose medium with 0 mmol/l BHB (p=0.031) and 1 mmol/l BHB (p=0.046). In other words, MHP88 had a stronger cytotoxic effect upon these cells when combined with low glucose and a low BHB concentration. When the concentration of BHB was increased further however, the effect was abolished. The presence of 5 and 10 mmol/l BHB had no significant impact on the effect of MHP88. Both GL261 and GL261-luc2 had the largest standard deviations in IC_{50} values in the groups of cells receiving 5 and 10 mmol/l BHB.

For the human glioblastoma cell line U251 and the human fibroblast cell line MRC-5 the results appeared somehow different. To begin with, there were considerable large standard deviations for all cells cultured in the low glucose medium (except 1 mmol/l BHB for U251). Secondly, the IC_{50} values of MHP88 in the low glucose assays rose to much higher levels than in the corresponding assays with the other two cell lines. The IC_{50} values of the assays with MHP88 in standard medium was approximately equal.

For U251 the cell cytotoxicity of MHP88 was not statistically affected by the cells being cultured in a low glucose medium with 0 mmol/l (p=0.38), 1 mmol/l (p=0.562) or 5 mmol/l BHB (p=0.067). Still, there was a trend towards 5 mmol/l BHB lowering the IC_{50} value of MHP88. When the BHB concentration was increased to 10 mmol/l the cell cytotoxicity of MHP88 was statistically lowered (p=0.044). Based on these results, increasing concentrations of BHB appeared to cause increased tolerance towards MHP88.

MRC-5 was included as a control cell line. All low glucose assays generated IC_{50} values higher than the control, but the cell cytotoxicity of MHP88 was only significantly lowered when 10 mmol/l BHB was added to the medium (p=0.026). This corresponds with the finding made for U251, where increasing concentrations of BHB reduced the cell cytotoxicity of MHP88.

5.5 Expression of the rate-limiting ketolytic enzyme, SCOT

It was desirable to investigate the expression of the rate-limiting ketolytic enzyme, SCOT in the GBM cells after having found that BHB lowered the cell cytotoxicity of MHP88. This enzyme is essential for the breakdown of BHB and acetoacetate to acetyl-CoA (93). A Western blot was carried out for this purpose. The murine cardiac muscle cell line HL-1 was

used as positive control. These cells have large quantities of the enzyme as they utilize ketone bodies for energy production to a great extent (94, 95). Primary hepatocytes from mice were used as negative control as they do not metabolize ketone bodies due of lack of the enzyme. The primary antibody anti-OXCT1/SCOT was used.

5.5.1 The optimal dilution of anti-OCXT1/SCOT

A titration with anti-OXCT1/SCOT diluted 1:250, 1:500 and 1:1000 was first performed to find the optimal dilution. In the pictures in figure 17, a biotinylated protein ladder is displayed to the left and the positive control (HL-1) to the right. The pronounced band from HL-1 corresponds with the molecular weight of SCOT, which is 56 kilodalton (kDa). As can be seen, the band was weak when the antibody was diluted 1:1000. When diluted 1:500 the band became more pronounced and even more so when diluted 1:250. However, it was thought sufficient to use a dilution of 1:500 when investigating the expression of SCOT in the cell lines.

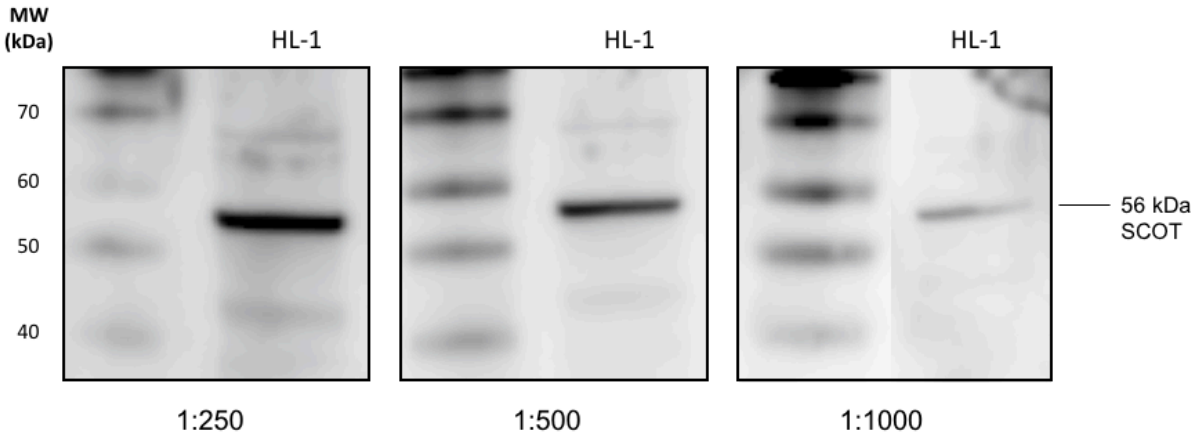

Figure 17. Titration with anti-OXCT1/SCOT
Cell lysate of the murine cardiac muscle cell line HL-1 was subjected to electrophoresis. The proteins were blotted onto a nitrocellulose membrane and SCOT (56 kDa) was detected by use of anti-OXCT1/SCOT diluted 1:250, 1:500 or 1:1000 in blocking buffer. The pronounced band seen in the lane with proteins from HL-1 corresponds with the molecular weight of SCOT (56 kDa). kDa, kilodalton; MW, molecular weight; OXCT1/SCOT, succinyl-CoA:3-keto acid (3-oxoacid) CoA transferase.

5.5.2 Protein content in the samples

Samples included in the Western blot were the murine glioblastoma cell lines GL261 and GL261-luc2, the human glioblastoma cell line U251, the murine cardiac muscle cell line HL-1, primary hepatocytes from mice and the human fibroblast cell line MRC-5. A protein assay was performed to find the protein content of the various samples. Next, the samples were diluted to hold similar protein concentrations. As displayed in table 4, HL-1 contained the

least protein (1.15 mg/ml). Despite this, the samples were diluted to reach the protein concentration of GL261, which contained the second least (1.35 mg/ml)). This was due to the positive control being included mainly to verify that the antibody did work. Hence, it was thought inexpedient to dilute the other samples to this protein level when the aim of the experiment was to detect proteins.

Table 4. Protein content in Western blot samples

Sample	HL-1	Hepatocytes	GL261	GL261-luc2	U251	MRC-5
Protein content (mg/ml)	1.15	6.58	1.35	1.98	1.47	1.73
Protein content at loading (mg/ml)	1.15	1.35	1.35	1.35	1.35	1.35

Table 4 displays the original protein content (mg/ml) and protein content at loading (mg/ml) of the samples used in the Western blot. These were the cardiac muscle cell line HL-1, primary hepatocytes from mice, the murine glioblastoma cell lines GL261 and GL261-luc2, the human glioblastoma cell line U251 and the human fibroblast cell line MRC-5. All samples, except HL-1, were diluted to reach 1.35 mg/ml protein.

5.5.3 Expression of SCOT and ß-actin

Having established the appropriate dilution for anti-OXCT1/SCOT and diluted the samples, a Western blot was next performed. As the hypothesis was that GBM cells cannot utilize ketone bodies for energy production, it was expected that the cells had none or little expression of the enzyme. However, the reduced cell cytotoxicity of MHP88 in the presence of BHB indicated that the cells might had metabolized the ketone body. The results are presented in figure 18. ß-actin was used as a loading control.

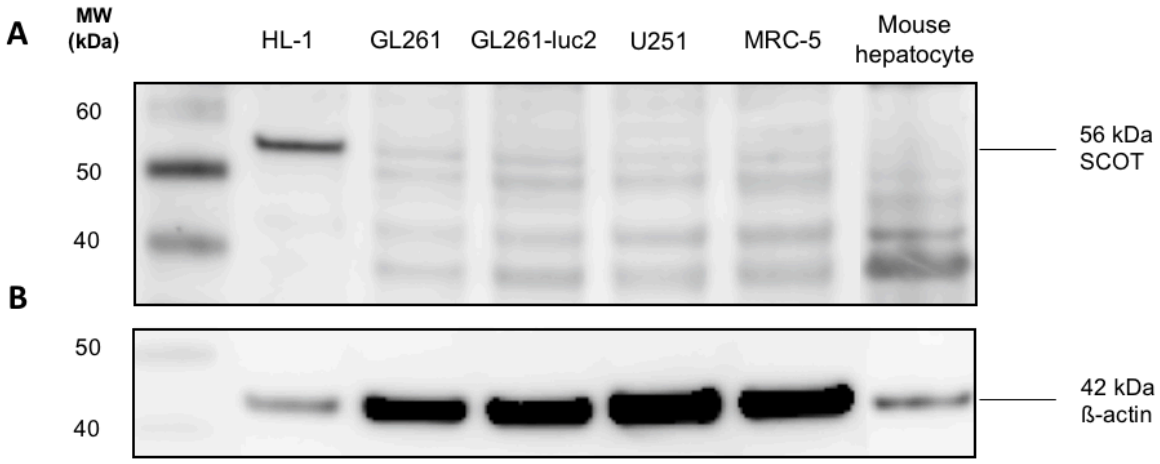

Figure 18. Expression of SCOT and ß-actin
A) Cell lysates of the murine glioblastoma cell lines GL261 and GL261-luc2, the human glioblastoma cell line U251, the human fibroblast cell line MRC-5 fibroblasts, the murine cardiac muscle cell line HL-1 and primary hepatocytes from mice were subjected to electrophoresis. The proteins were blotted onto a nitrocellulose membrane and SCOT (56 kDa) was detected by the use of anti-OXCT1/SCOT diluted 1:500 in blocking buffer. **B)** The membrane was stripped and reprobed with anti-ß-actin diluted 1:4000 in blocking buffer. ß-actin has a molecular weight of 42 kDa. MW, molecular weight; SCOT, succinyl-CoA:3-keto acid (3-oxoacid) CoA transferase.

SCOT was widely expressed in HL-1. In the GBM cells in comparison, there appeared to be low expression of the enzyme. The same was the incident for MRC-5. Just by looking at the membrane, the band from U251 appeared weaker than the others. The band that is present for all cell lines, right beneath 56 kDa, is a possible post-translational modification of the enzyme with a molecular weight of 52 kDa. The lowest band could be a splice variant with a molecular weight of 36.7 kDa. The band right above 40 kDa is of unknown origin.

ß-actin was present in all samples. The protein appeared to be equally expressed in GL261, GL261-luc2 and MRC-5. Compared to these samples U251 seemed to contain a bit more ß-actin, while HL-1 and the primary hepatocytes contained considerably less.

5.6 pH of the cell media used in the MTS assays

When performing the MTS assays, the molecular structure of BHB had not been a matter of consideration. Later on, attention was drawn to the fact that BHB is an acid. Increasing concentrations in the cell medium would presumably cause a correspondingly decreasing pH level. The pH of the different cell media used in the MTS assays was therefore measured. All values are presented in table 5.

Table 5. pH values of cell media used in the MTS assays

Cell medium	RPMI-1640	Low glucose +0BHB	Low glucose + 1BHB	Low glucose + 5BHB	Low glucose + 10BHB
pH	8.33	8.14	7.75	7.07	6.63

Table 5 displays the pH value of the cell media used in the different MTS assays. RPMI-1640 was used in the assay with only MHP88. Medium with 3.5 mmol/l glucose and 0 mmol/l BHB (0BHB), 1 mmol/l BHB (1BHB), 5 mmol/l BHB (5BHB) and 10 mmol/l BHB (10BHB) were used in the low glucose assays with BHB. BHB, ß-hydroxybutyrate.

It was next questioned if the decrease in pH could have a connection with the rise in the IC_{50} value of MHP88 with increasing concentrations of BHB. According to the Henderson Hasselbalch equation a compound exists in 50% unprotonated form and 50% protonated form when the pH of its surroundings equals its pKa value (101). The pKa of MHP88 is 10.6 (this is based on the compound's molecular composition). It is presumed that MHP88 has to cross the cell membrane to work its effect. In order for that to happen the molecule must be neutrally charged. So, it can be assumed that when the pH is 10.6, 50% of the present MHP88 will be able to cross the cell membrane and work its effect. When the pH deceases from the pKa value there will be a surplus of the protonated isoform of MHP88. If the pH decreases

with two units from the pKa there will only be 1% of unprotonated molecules present. The pH of RPMI-1640 Medium, as was used for the control assays, was 8.33. In the glucose reduced medium it was 8.14 with 0 mmol/l BHB, 7.75 with 1 mmol/l BHB, 7.07 mmol/l BHB and 6.63 with 10 mmol/l BHB. By use of the Henderson Hasselbalch equation it was found that the percentage of active MHP88 molecules were, in the same order as the given pH values $5x10^{-3}$, $3.5x10^{-3}$, $1.4x10^{-3}$,$3x10^{-4}$ and $1,1x10^{-4}$.

5.7 Luminescence signals from two batches of GL261-luc2 cells

The final experiment, bioluminescence measurement, was carried out to prepare for future mouse studies on the KD and MHP88. The expression of the luciferase gene *luc2* in the murine glioblastoma cell line GL261-luc2 is kept by regular selection with the selection marker G418. It was desirable to investigate if the number of transfected cells varied based on the time of selection. The untransfected cell line GL261 was used as a control and was anticipated to emit no light.

Bioluminescence was measured from two batches of GL261-luc2 cells. The growth protocol for GL261-luc2, received from Scheck laboratory, suggests that the cells are challenged with 100 µl/ml G418 the second passage after thawing and subsequently every 8^{th}-10^{th} passage. The first time GL261-luc2 was thawed a part of this information was overlooked and G418 was not added until the 8^{th} passage was reached. The first measurement was undertaken the first passage after selection. This passage is referred to as p1 in relation to the G418 selection cycle. Further, bioluminescence was measured every other passage until a new 8 passages had passed, and the selection marker was added once again. The cells were left in the incubator over the weekend after the second selection. On Monday morning, the cells were supposed to be seeded for a last bioluminescence measurement, but the flask contained no viable cells. Consequently, only four measurements were carried out. The results are presented in figure 19.

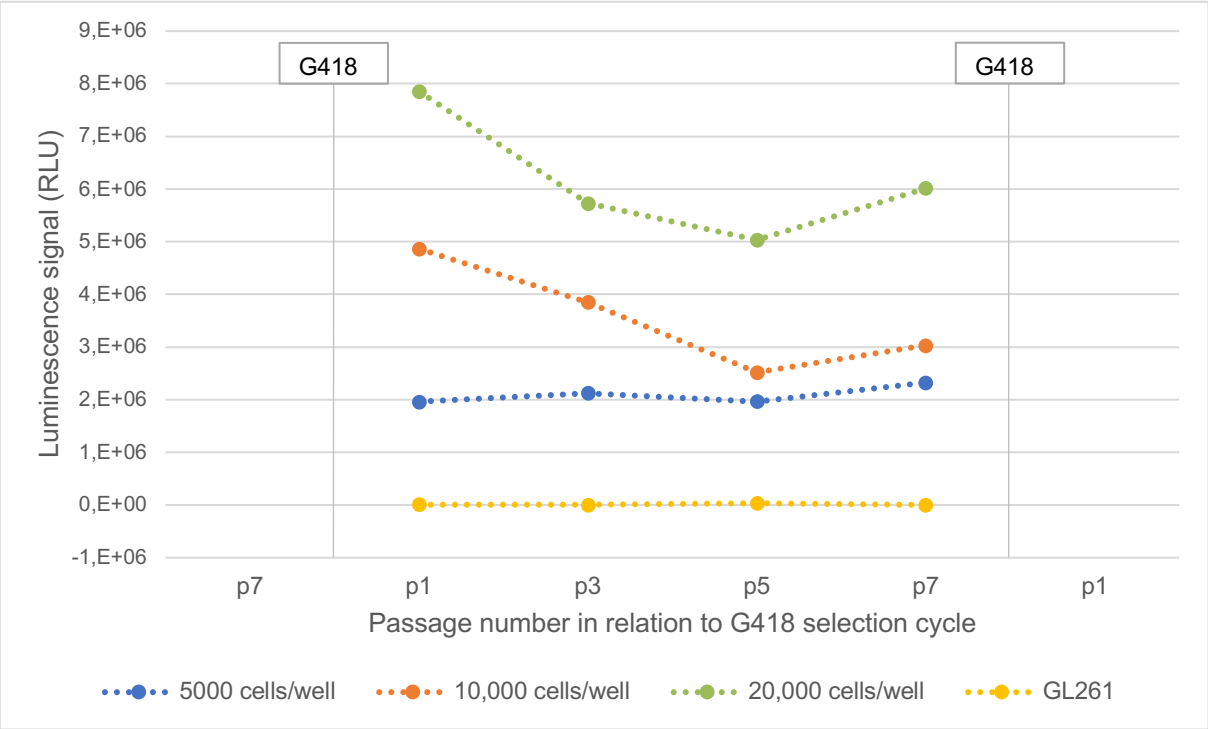

Figure 19. Luminescence signals from GL261-luc2, batch 1
The graph depicts luminescence signals from the murine glioblastoma cell line GL261-luc2 with 5000, 10,000 and 20,000 cells/well and the control, the untransfected cell line GL261, with 20,000 cells/well. Each pronounced dot represents the mean luminescence signal from two wells without background luminescence. The signal was measured 4 minutes after the Bright glow reagent was added. Four measurements were made over a period of three weeks. G418 was supplied to the culture the 8th passage after thawing and the first measurement was made the following passage (this passage is referred to as p1 in the G418 selection cycle). Subsequently, measurements were made every other passage until the 8th passage was reached and G418 was added once again. Measure interval time was 10 seconds/well. RLU, relative light units.

When a new batch of GL261-luc2 cells was thawed awareness had been drawn to the fact that the cells were to be challenged with 100 µl/ml G418 already the second passage. However, due to trouble with starting the culture, the cells were first challenged the 4th passage after thawing. Following, three bioluminescence measurements were carried out with the same timing as in the first round of measurements. Finally, a fifth measurement was carried out from the passage made after G418 was added the second time around. The results are presented in figure 20.

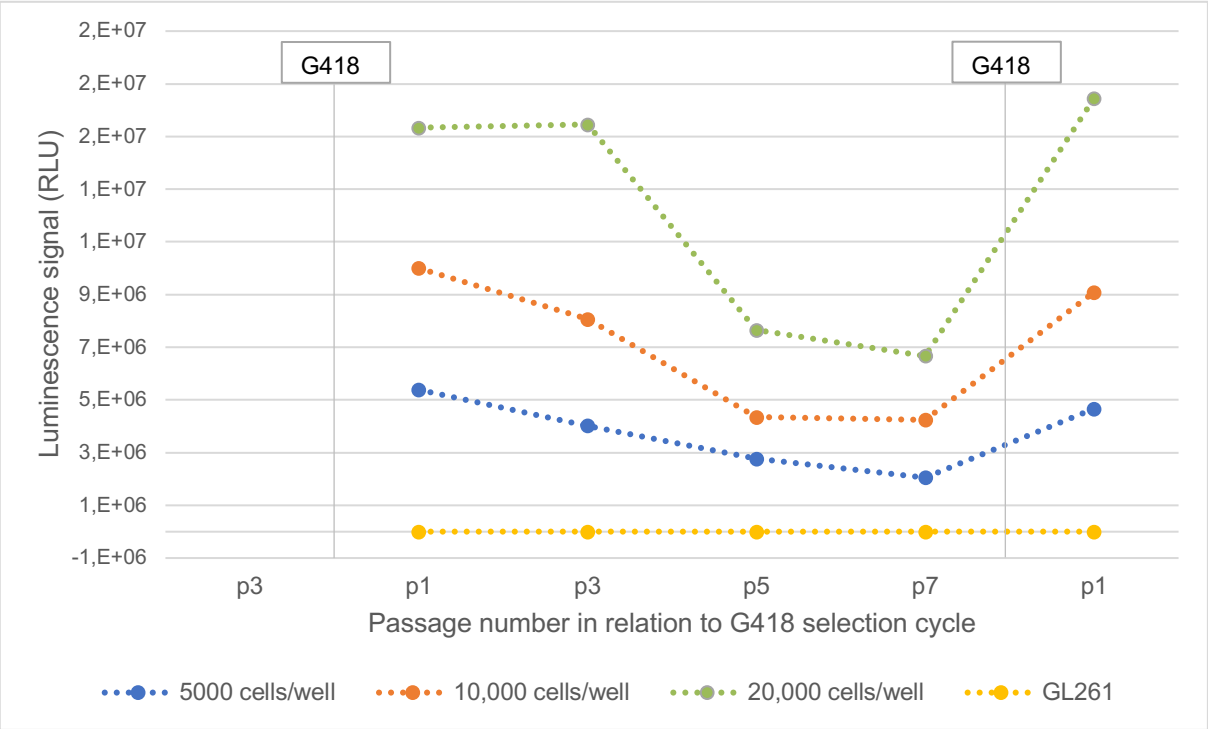

Figure 20. Luminescence signals from GL261-luc2, batch 2
The graph depicts luminescence signals from the murine glioblastoma cell line GL261-luc2 with 5000, 10,000 and 20,000 cells/well and the control, the untransfected cell line GL261, with 20,000 cells/well. Each pronounced dot represents the mean luminescence signal from three wells without background luminescence. The signal was measured 4 minutes after the Bright glow reagent was added. Five measurements were carried out over a period of three weeks. G418 was supplied to the culture during the 4th passage after thawing and the first measurement was made the following passage (this passage is referred to as p1 in the G418 selection cycle). Subsequently, measurements were made every other passage until the 8th passage was reached and G418 was added once again. The fifth measurement was carried out from the first passage after G418 was added the second time (p1). Measure interval time was 10 seconds/well. RLU: relative light units.

The luminescence signal from GL261-luc2 cells batch 2 in p1 and p3 was about twice as high as the corresponding signal from batch 1. The signal then became considerably lowered when p5 and p7 was reached. When G418 was added once more (p8), the signal reached a new high (p1). The luminescence signals from batch 1 was more continuous. However, there were a decrease in the signal until the last measurement (p7) where it increased a little. The luminescence signals from the wells containing 20,000 GL261-luc2 cells/well are compared in figure 21 to give a better impression of the differences.

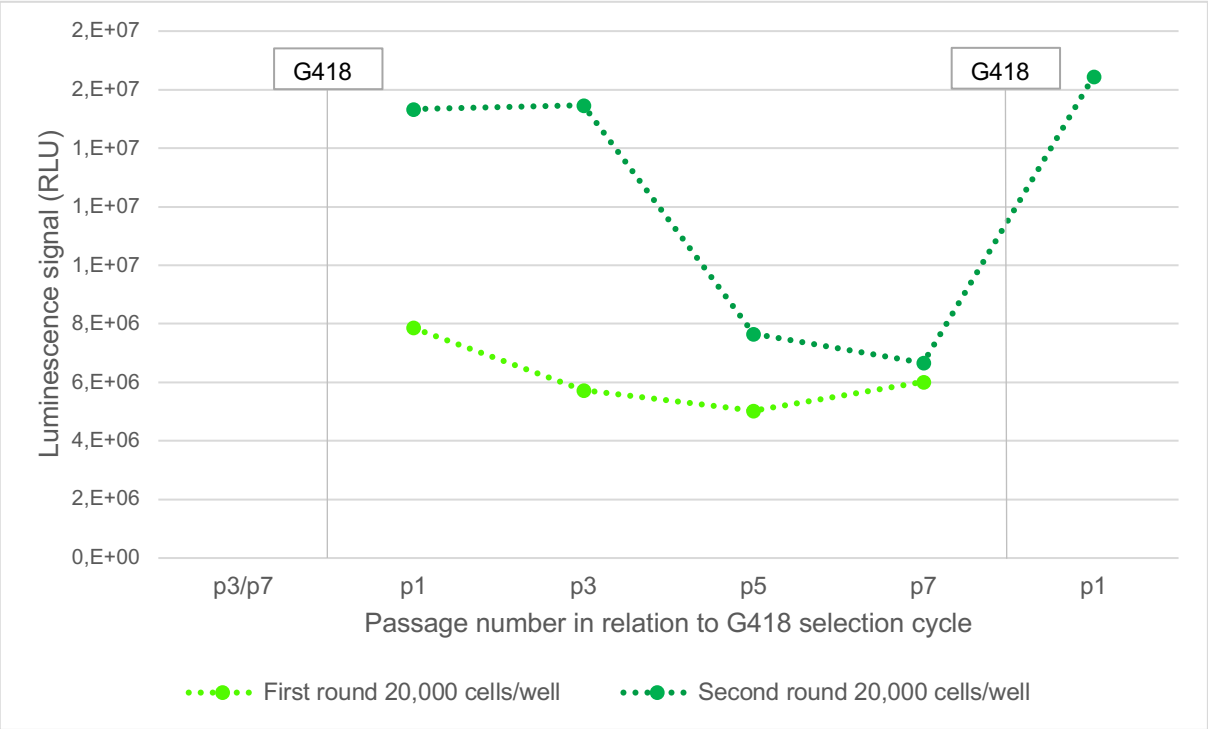

Figure 21. Luminescence signals from 20,000 GL261-luc2 cells, batch 1 and 2
The light green dots depict the luminescence signal emitted from the murine glioblastoma cell line GL261-luc2 batch 1 over four passages after G418 was added the 8th passage after thawing. Each pronounced dot represents the mean luminescence signal from two wells without background luminescence. The dark green dots depict the luminescence signal emitted from GL261-luc2 batch 2 over five passages. G418 was added the 4th passage after thawing and after the cells had been passaged another 8 times. Each pronounced dot represents the mean luminescence signal from three wells without background luminescence. Measure interval time was 10 seconds/well. RLU, relative light units.

6 Discussion

6.1 General discussion

6.1.1 Glucose deprivation vs. glucose reduction

The primary aim of the present thesis was to investigate the effects of ß-hydroxybutyrate (BHB) in combination with MHP88 on GBM cells, and at this conduct research on the ketogenic diet (KD) as an adjuvant treatment to MHP88. It was thought beneficial to deprive the cells of glucose to examine the effect of BHB. Consequently, the MTS assays with MHP88 and BHB were to be performed with cells cultured in a glucose free medium. However, the assays could not be carried out. An evaluation concerning the necessity of a glucose deprivation in order to answer the research questions was therefore made.

Glucose is said to fuel the growth of tumors (88), while ketone bodies are proposed to have a toxic effect (102, 103). It was therefore thought natural to deprive the cells of glucose to investigate the effect of ketone bodies. The purpose with the current MTS assay was to examine if the cell cytotoxicity of MHP88 would be affected when the GBM cells were cultured in a glucose free medium with BHB compared to a high glucose medium. The hypothesis was that if the cells were not able to utilize ketone bodies, the IC_{50} value of MHP88 would decrease, as a result of starvation. However, the planned assay was never conducted as the murine glioblastoma cell line GL261-luc2 died of cultivation in a glucose free medium for 24 hours.

Presumably, the cells did not tolerate the glucose deprivation. It could be caused by lacking abilities to oxidize ketone bodies, seeing as all cells suffered the same fate independent of the presence of BHB. Another explanation could be that the cell death was caused by the glucose deficiency alone. It might be that the culturing conditions became too harsh for the cells to survive in, independent of their abilities to utilize BHB. Research conducted by Nanette et al. (104) suggests that human GBM cells are incapable of survival when glucose is withdrawn. They demonstrated that a glucose withdrawal caused increased oxidation of fatty acids. This led to oxidative stress, resulting in apoptosis and cell death. Astrocytes, used as a control, survived an equivalent treatment. A similar finding was made for neuroblastoma cells in a different study (103). The cells had increased rates of apoptosis and reduced viability when cultured in a glucose free medium.

Another explanation to the cell death could be the seeding density. It has been demonstrated that it affects the GBM cells' ability to survive a 24 hour glucose withdrawal (105). Perhaps the outcome would have been different had the cells been seeded with a higher density.

There have also been made contradictory findings regarding a glucose deprivation in GBM cells. Wang et al. (106) found that 0.5 mmol/l glucose in the cell medium caused an immediate upregulation of the glucose transporter GLUT1, which increased the uptake of glucose in the cell. This mechanism was mediated through the enzyme glutamate dehydrogenase 1 (GDH1). The enzyme also enhanced the metabolism of glutamine in the absence of glucose and promoted tumor cell survival. Glutamine is a non-essential amino acid that make up one of the most abundant nutrients in the blood (107). It is the most versatile amino acid in the human body, with a vital role in providing carbon for the cells' biosynthetic and bioenergetic needs. Glutamine can be converted into the amino acid glutamate or its corresponding keto-acid α-ketoglutarate and simultaneously release free nitrogen. Nitrogen is vital for the synthesis of pyrimidines, purines and NADPH, and glutamine is in such way of particular importance in proliferating cells. Glutamine fuels the TCA cycle through α-ketoglutarate. In cases where the glycolysis cannot provide the TCA cycle with sufficient amounts of carbon, GDH1 becomes important for cell survival. By providing intermediates to the TCA cycle, glutamine is also a contributor to the production of ATP.

In the experiment conducted in the present thesis, glutamine was present in the cell medium. There were also 0.57 mmol/l glucose present, in spite of the cell medium being referred to as glucose free (the glucose came from the FBS). Still, the cells did not survive the cultivation. Goji et al. (108) demonstrated that glutamine, together with the amino acid cystine, induced NADPH depletion and accumulation of ROS in the absence of glucose. This occurred through the cystine/glutamate antiporter xCT and caused GBM cells to die. Based on this finding, there is a possibility that the cell death observed in the present thesis could be due to a glucose-deprivation induced cell death caused by glutamine and cystine.

Eckert et al. (109) suggested, based on their findings, that cell death in relation to glucose withdrawal was connected to PEA-15 (phosphoprotein enriched in astrocytes 15 kDa). The protein can be phosphorylated at a serine residue, located at the C-terminal end of the protein. This enables it to induce resistance to a glucose deprivation-induced apoptosis. In their study they found that PEA-15 was significantly upregulated and appeared to protect the GBM cells when 0.025 mmol/l glucose was present in the cell medium. However, when cells were

completely deprived of glucose cell death was initiated. It might be that different cell lines express different levels of the protein. Low PEA-15 expression could therefore have caused the cells to become apoptotic in the present thesis.

In short, different findings have been made in regards of the GBM cells' ability to survive in a glucose free environment. As it was desired to start the experiment with ketone bodies rather than experimenting further with the glucose free medium, the glucose concentration in the cell medium was increased. This appeared to be more appropriate for the aim of the thesis. When following a KD, the blood glucose is indeed being lowered but it is never brought to 0 mmol/l (21, p. 380). In spite of the diets' low quantity of carbohydrates, the fasting blood glucose is only lowered from 4-6 mmol/l to 3.5 mmol/l. Even though ketone bodies become the body's primary energy source, glucose is still required by glycolytic cells lacking oxidative capacity and also partly by the brain (36, p. 242-245). When on a KD the blood level of insulin falls, and the level of glucagon correspondingly rises. Glucagon stimulates the gluconeogenesis, a process where glucose is constituted from non-glucose precursors. This process takes place in the liver and kidneys. Glucose is regenerated from glycerol released from the breakdown of fat, lactate generated from glycolysis in glycolytic cells and from glycolytic amino acids. As a result, the blood glucose is maintained, however at a lower level.

Considering that the GL261-luc2 cells died when deprived of glucose and that the physiological blood glucose is partly maintained on a KD, a new protocol for the MTS assay was developed. The cells were cultured in medium holding 3.5 mmol/l glucose and various concentrations of BHB. These culturing conditions coincided better with the physiological environment during ketosis. Others who have implemented similar research in vitro have cultured the cells in 2.5 mmol/l glucose (70, 72, 110). The blood-brain barrier limits the exchange of glucose between the peripheral and cerebral compartments (110). 2.5 mmol/l is the concentration of glucose commonly found extracellularly in the brain. As the present thesis was written to achieve a master's in clinical nutrition it was thought natural to mimic the physiological blood glucose obtained on a KD, as it is the clinical focus when using the diet. However, as the glucose concentration that was used was considerably reduced from the cells' standard medium and since the aim primarily was to assess the effect of ketone bodies, it is not likely that this have had a significant impact on the results.

Prior to implementing the new MTS assay, it was investigated if the GL261-luc2 cells could sustain culturing in 3.5 mmol/l glucose through live-cell imaging. As can be seen in the

resulting photographs in figure 11, cells receiving a high glucose medium had proliferated into a smooth and homogenous layer. The cells deprived of glucose on the contrary appeared disjointed, heterogeneous and uneven. What was profound was the different appearance of the cells deprived of glucose in the failed MTS assay and in the live-cell imaging. This is most likely caused by a methodological difference. Prior to the planned MTS assay the cells were cultured in a plastic 96 well plate, while the cells used in the live-cell imaging were kept in a fibronectin-coated glass 24 well plate. The fibronectin presumably caused the latter to stick to its surface, causing the cells to appear adherent and elongated compared to the spherical, afloat cells in the 96 well plate.

Cells cultured in 3.5 mmol/l glucose had proliferated into a seemingly confluent and homogenous layer after 24 hours, indicating that the cells did tolerate the glucose reduction. This also applied to the cells cultured in 5 mmol/l glucose. The latter concentration was included to investigate if there were any difference in the cells' proliferation when they received the fasting blood glucose on a standard diet compared to a KD. Based on the results it did not appear so. However, as the method only generated pictures the interpretation was left for the researcher. There is no telling if the number of viable cells varied across the wells receiving 23, 5 and 3.5 mmol/l glucose. As the intention with the experiment primarily was to investigate if the cells could be cultured in 3.5 mmol/l glucose prior to the MTS assay, the method was thought adequate for the present thesis.

A study that researched a similar matter more thoroughly, by Maurer et al. (66), found that GBM cells were more capable of survival when cultured in 5 mmol/l glucose compared to 2.5 mmol/l glucose. This suggests that GBM cells can be weakened by a glucose restriction. It was investigated further in the MTS assays with 3.5 mmol/l glucose and no BHB conducted in the present thesis. For the human glioblastoma cell line U251 and the human fibroblast cell line MRC-5 the low glucose medium had no significant effect on the cell cytotoxicity of MHP88 ($p > 0.05$). It could mean that the cells were adequately nourished something that contradicts the finding in the last mentioned study (66). For the murine glioblastoma cell line GL261-luc2 however, the results suggest that the glucose reduction did affect the cells. The cell cytotoxicity of MHP88 increased in the presence of a glucose reduced medium compared to a high glucose medium ($p=0.031$). For the murine glioblastoma cell line GL261, there was not a statistically significant increase by definition ($p=0.051$), but the p-value was so low it must be taken into consideration that the cells might have been affected by the glucose reduction.

6.1.2 The cell cytotoxic effect of BHB and MHP88 combined

Having investigated the effect of a glucose reduction on GBM cells with various outcomes, BHB was added to the cell medium to examine if the ketone body had an adjuvant effect to MHP88. For GL261-luc2, the increased cytotoxic effect of MHP88 observed with a low glucose medium persisted when 1 mmol/l BHB was added to the culture. This was in accordance with a finding in glioma-stem like cells, made by Ji et al. (70), where 1 mmol/l BHB in a low glucose medium (2.5 mmol/l) caused declining numbers of cells in the culture. When they, in the mentioned study, increased the concentration of BHB to 5 and 10 mmol/l they found that the number of cells further declined. This was caused by inhibited proliferation and glycolysis, increased apoptosis and damaged mitochondria. Ketone bodies (BHB and acetoacetate) in a glucose free medium were also shown to increase apoptosis in neuroblastoma cells in a different study (103).

In the present thesis, supplementation of 5 mmol/l BHB to the glucose reduced cell medium did not significantly affect the cell cytotoxicity of MHP88 for any cell line. When the amount of BHB was increased to 10 mmol/l the cell cytotoxicity of MHP88 was significantly reduced in cultures with both U251- and MRC-5 cells. These findings were not in accordance with the hypothesis that GBM cells are weakened by cultivation in a glucose reduced medium with BHB, nor with the mentioned findings. Ji et al. (70) cultured their glioma-stem like cells in low glucose and BHB for seven days. Here, the cells were only cultured with BHB for 16-18 hours prior to being exposed to MHP88. Perhaps a longer cultivation time with BHB would have resulted in increased cell cytotoxicity of MHP88. In the already mentioned study by Maurer et al. (66) they cultured their GBM cells in low glucose medium (2.5 mmol/l) and BHB over seven days. However, they found that supplementation of a low glucose medium and 5 mmol/l BHB caused a declining cell density already after one day. They also discovered that supplementation of BHB did not protect the cells against the glucose reduction.

In comparison to the other studies, Maurer et al. (66) found no proapoptotic effect of BHB on GBM cells when using a glucose free medium. It might therefore be a cell type specific effect. If it is assumed that no apoptosis occurred and that the cells were unharmed by BHB, it is reasonable that the IC_{50} value of MHP88 value did not decline with increasing amounts of BHB. Still, it cannot account for how BHB seemingly decreased the cell cytotoxicity of MHP88 in high concentrations. It was therefore questioned if the results could be related to metabolism of fatty acids or ketone bodies.

A difference between the assay with standard medium and the assays with a glucose reduced medium was the presence of fetal bovine serum (FBS) in the latter during exposure to MHP88. All assays were supposed to be serum free and this mistake was detected late in the study course. FBS contains a manifold of nutrients and growth factors, amongst others triglycerides, which can be broken down to glycerol and fatty acids and used as bioenergetic fuel (21, p. 359, 86). In a study by Sperry et al. (72) they demonstrated that the fatty acid palmitic acid was oxidized in a glioblastoma cell culture with low glucose (2.5 mmol/l) and hence increased cell growth. If the cell lines used in the present thesis were able to metabolize fatty acids FBS would have supplied an extra source of nutrients. An additional MTS assays was performed with the four cell lines without serum (data not shown) to investigate if FBS did have an impact on the IC_{50} value of MHP88. Looking at the results it did not appear so but as only one assay was performed it cannot be said with certainty. However, the dead GL261-luc2 cells from culturing in a glucose free medium with 10% FBS indicate that the serum did not protect the cells. However, the little amount present might not have been sufficient to maintain cell growth in the absence of glucose anyways.

In the study by Sperry et al. (72) they also found that 2.5-5 mmol/l BHB promoted cell growth. To investigate if the cells might have oxidized BHB in present thesis the expression of the rate-limiting ketolytic enzyme SCOT was investigated. The glioblastoma cell lines appeared to have low expression of SCOT. The Human Protein Atlas shows that the expression of SCOT in glial cell is in fact low, compared to cardiomyocytes (94, 95). To perform an accurate comparison, primary astrocytes from mice should have been included as a control. Based on the present experiment it cannot be told if the expression of SCOT in the GBM cells deviated from astrocytes. Immunohistochemistry staining for ketolytic enzymes in two human GBM tumors revealed that one had low expression of SCOT while the other had seemingly high expression (55). The same authors analyzed several GBM specimens from humans by similar means and found that the expression of ketolytic enzymes most frequently was low, compared to adjacent non-neoplastic brain tissue (68). Still, there were differences across the various samples, suggesting that enzymes are heterogeneously expressed across tumors.

Here, SCOT was not quantified, but U251 appeared to have lower expression compared to the other glioblastoma cell lines. The loading control ß-actin displayed that the U251 sample contained slightly more proteins than GL261 and GL261-luc2. Hence, it is unlikely that the finding was caused by a loading error. The presence of SCOT was originally thought in

consistency with the cells being able to metabolize BHB. Maurer et al. (66) found that several glioma cell lines expressed not only SCOT, but also BDH1 and 2, both at the mRNA and protein level. In spite of this, the cells did not seem able to metabolize ketone bodies. Consequently, it cannot be determined if the GBM cells, nor the control cell line MRC-5, did utilize BHB for energy production on the basis of having detected SCOT.

Whether metabolization of ketone bodies and fatty acids have brought about the protective effect of BHB is in any case unlikely, at least for the U251 cell line. The mean IC_{50} value of MHP88 rose from 12.4 µg/ml in assay with standard medium to 41.6 µg/ml in the assay with the glucose reduced medium and 10 mmol/l BHB. That is near a quadruplication in the requirement for MHP88 to kill half the cells in the culture. It is unlikely that this effect can be attributed to BHB only as an energy metabolite. The cells in the assay with standard medium were cultured in a high glucose medium (23 mmol/l) and received 11 mmol/l glucose when MHP88 was added, indicating that their energy demand should be covered. Also, when Sperry et al. (72) increased the amount of BHB in the culture to 10 mmol/l the proliferation of the GBM cells was inhibited. It was assumed to be caused by the toxicity of BHB in such high concentrations. This finding is not in accordance with the result obtained here, where the presence of 10 mmol/l BHB in the cell media decreased the cell cytotoxicity of MHP88 in U251- and MRC-5 cultures.

Seeing as the presence of ketolytic enzymes does not imply oxidation of ketone bodies, that ketone bodies and fatty acids as energy metabolites are unlikely to have protected the cells from MHP88 and that 10 mmol/l BHB is expected to be toxic, neither of these theories seem to explain the reduced cell cytotoxicity of MHP88. By changing focus from cell metabolism to the chemistry of BHB a new explanation was deduced.

A feature of BHB that had not been taken into consideration when the MTS assays were conducted was its qualities as an acid. As decreasing pH causes increased protonation of MHP88, it is likely that BHB have reduced the number of functional MHP88 molecules in the culture. This can explain why the cell cytotoxicity of MHP88 was reduced with increasing concentrations of BHB. The matter was looked further into by PhD candidate Susannah von Hofsten (IMB, UiT). She discovered that the pH of the cell medium does seem to affect the function of MHP88, independent of the presence of BHB.

It is therefore a possibility that the pH alterations can be held accountable for parts of the results obtained in the experiments with BHB. Still, there are some elements which qualifies for further investigation. Primarily, the cytotoxic effect of MHP88 was not statistically influenced by the presence of 5 and 10 mmol/l BHB in the cultures containing GL261 and GL261-luc2 cells. If it is correct that the pH hampers the effect of MHP88 it could be reasoned that these culturing conditions have had an effect on the cells, as the IC_{50} value of MHP88 remained statistically unaffected. Secondly, the IC_{50} values of MHP88 with BHB rose to much higher levels in the human cell lines (U251, MRC-5) compared to the murine cell lines (GL261, GL261-luc2). This was curious, especially since assays with standard medium generated rather similar IC_{50} values across the cell lines.

Seeing as the alterations in pH caused by BHB seemed to hamper the effect of MHP88, the combination did not appear ideal. However, it is important to remember that this was the incident in a cell culture. In the body, the level of blood ketone bodies can reach 5-6 mmol/l when a KD is consumed (41). Ketoacidosis, which is blood ketone bodies $\geq$ 10 mmol/l, rarely occurs when dieting but is rather a complication of diabetes mellitus and chronic alcoholism (111). It was chosen to include such high levels in the experiment to investigate the potential effects of BHB. The increasing levels of blood ketone bodies on a KD does not affect the physiological pH on the same level as the cell medium because the blood has a buffer system (112). It is made up of chemical compounds, which counteracts a fall in pH when there are increasing numbers of acidic molecules in the blood. The variation in pH that occurred in the MTS experiment is consequently not transferable to the human body. However, as the pH in the blood normally lies between 7.35 and 7.45 the function of MHP88 could be affected. Another consideration is that lactate generated through glycolysis acidifies the tumor environment (30). When doing research on MHP88 in solid tumors this will be important to keep in mind. Thus, the finding of how pH affects the function of MHP88 is relevant for future mouse studies.

6.1.3 Selection with G418 affects the expression of *luc2* in GL261-luc2

The bioluminescence measurements were performed to prepare for future mouse studies. It was desirable to find out if the expression of *luc2* in the murine glioblastoma cell line GL261-luc2 was stable prior to injecting the cell line into mice. The growth protocol from Scheck laboratory states that the bioluminescence in vitro is approximately 6190 photons/sec/cell. It

is not said at what time in the G418 selection cycle this is valid. Consequently, it appears as the luminescence signal is expected to be stable.

In the bioluminescence measurements with GL261-luc2 batch 1, G418 was added in p8, while with batch 2 it was added in p4. This seem to have affected the results, as the signals from p1 and p3 were doubled from batch 1 to batch 2. Presumably, the timing of the first selection is decisive for the intensity of the luminescence signal. In definition by extension, the number of transfected cells decline after thawing if G418 is not added in an early passage. Ideally, the selection marker is to be supplied early after thawing.

Interestingly, there were a conspicuous decrease in the signal when the third and fourth measurement were carried out from batch 2. This suggests that the number of transfected cells is stable only for a short period of time after selection. In the measurements from batch 1 the trend was similar, even though the decrease was much less profound. In breast cancer cells it was demonstrated that the expression of *luc2* was stable for three months after a continuous selection with 800 µg/ml G418 over a time period of eight weeks (113). Stable expression of a luciferase gene in murine colon adenocarcinoma cells was achieved with the use of 400 µg/ml G418 (time period not given) (114). It might be that a longer selection process is required to achieve a stable culture of GL261-luc2 cells. Here, only 100 µg/ml of G418 was used. When a titration was performed with GL261 and G418, it was revealed that 80% of the untransfected cells survived (see appendix figure 23). Perhaps the number of transfected cells would be more stable if a higher dose of G418 was supplied over a longer period of time.

From batch 2, a fifth measurement was made after the second selection with G418. It was expected that the signal would increase. As displayed in the results, the hypothesis was confirmed. The signal reached a new high after the cells had been selected the second time around. This indicates that the luminescence signal and at this, the number of transfected cells, was stimulated by selection with G418. It would have been interesting to measure luminescence from batch 1 after the second selection with G418 as well. However, this could not be carried out as the cells died after the selection. There was not made a backup without the G418, seeing as the selection went unproblematic the first time around. It could be that the low luminescence signal from this batch was due to the cells being weakened, seeing as they died right after the measurements were completed. However, the cells had seemingly normal growth up to the point of the cell death.

6.2 Methodological considerations

6.2.1 Sources of bias in laboratory work

When working with cells, independent of the method in use, several factors can influence the outcome of the experiments. Imprecise counting and/or pipetting and an irregular growth pattern can affect the cell density in spite of the incubation time being constant. It is therefore challenging to obtain stable conditions throughout all experiments that involve viable cells, in spite of following the same protocol. This could affect the results. To deal with this risk of bias the cells were always seeded in triplicates, except for the bioluminescence measurement with GL261-luc2 batch 1 where the cells were seeded in duplicates. The MTS assays were repeated three times for each cell line and in the bioluminescence measurements the cells were seeded in three different densities.

Imprecise weighing and/or pipetting could also have led to deviations from the stated concentration of different reagents compared to the actual value. It was attempted kept stable by using a laboratory weight in a closed room and by using the same pipettes in all experiments.

6.2.2 Cell lines

Cell lines possess mainly the same functional features as primary cells but are easier to work with. (73). They supply an unlimited amount of a pure cell population, which allows for reproduction of experiments and results. Cell lines can also be altered to study the function of, for instance, a specific gene or protein. However, it is important to keep in mind that they are genetically manipulated and therefore somehow distinct from primary cells (73, 74, p. 7). This might alter their functionalities and phenotype. In addition, serial passages of a cell line can make it even more deviant from the primary cell. Results obtained from a cell line could originate from an abnormal function obtained during its formation and development.

Cells are kept in an artificial environment outside the human body that excludes interaction with other cell types (73). In addition, they are cultured in nutrient conditions that differs from the human body. An example is the glucose content of 25 mmol/l in DMEM high glucose (110). This represents severe hyperglycemia in the human body, which is a pathological condition. Mimicking ketosis in vitro might affect cell lines differently than primary cells as they are adapted to grow in inhumane nutrient conditions. Another challenge with cell culturing is the risk of contamination, primarily by the bacteria mycoplasma and through

cross-contamination with other cells (73). Results obtained with the use of a cell line are therefore not necessarily transferable to the primary cell or original tumor. Following thawing, all cell cultures utilized in the present thesis tested negative for mycoplasma infection.

It is also difficult to transfer in vitro results into clinical research (115). Results obtained in a cell lab are not always valid for a complex system such as the human body, where the cells interact with a number of biochemical processes. Taking this into consideration, it is not obvious that an in vitro study is the best approach to answer the research question. However, to implement a KD in daily life can be demanding, time-consuming and might affect the patient's quality of life (116). It is therefore crucial to consider the ethical aspect of doing clinical research on a KD as an adjuvant treatment in humans. Patients who are diagnosed with GBM have a poor prognosis, with the median survival at diagnosis being less than two years (2, 10). To carry out an experimental diet on this group of patients is consequently challenging. In addition, the potential mechanism of the diet is still unclear (50). In the review by Weber et al. (50), they conclude with a need of more molecular studies to further investigate the mechanisms of the KD. Additional in vitro research can thus be of great value to detect whether further research in animals and humans is appropriate. As MHP88 is a new potential anti-cancer agent, further in vitro research is required to investigate its qualities prior to prospective mouse studies and clinical trials.

6.2.2.1 The choice of cell lines for the present thesis

An aim with the present thesis was to prepare for future mouse studies with the murine glioblastoma cell line GL261-luc2. Hence, this cell line was used in the experiments. The murine glioblastoma cell line GL261 is frequently used in GBM research (117). It was included as a control in the bioluminescence measurements and to have an untransfected murine glioblastoma cell line to use in the MTS assays. As research in humans are the ultimate purpose with in vitro research, it was desirable to include a glioblastoma cell line of human origin (U251). This cell line is commonly used in xenogeneic mouse models of cancer (77). The human fibroblast cell line MRC-5 was included to have a nonmalignant cell line for comparison. MRC-5 is frequently utilized as a control in cell assays (83). It grows adherently, similar to the glioblastoma cell lines and is relatively easy and cheap to work with.

Primary hepatocytes from mice were included as the negative control in the Western blot. They were suitable for this objective as they do not express the rate-limiting ketolytic enzyme

SCOT (94, 95). The ß-actin bands from the Western blot show that the sample contained less proteins than the others, in spite of being diluted to reach a similar protein concentration. There was some trouble with the first blot performed with these cells (data not shown). When the electrophoresis was run the samples appeared to remain stuck at the top of the gel. Consequently, the proteins did not wander. It was questioned if the cells had been properly lysed. To deal with this problem the sample was lysed in RIPA buffer with 1% protease inhibitor and sonicated over again. A new protein assay was carried out, revealing that the sample contained six times the protein concentration last measured. The sample was therefore further diluted in RIPA buffer. As shown in figure 18 this was evidently not sufficient to obtain a proper sample with correct protein concentrations. Due to time limitations it was not possible to trouble shoot this further.

The last cell line included in the experiment was the murine cardiac muscle cell line HL-1. These cells were used as a positive control in the Western blot because of their high expression of SCOT (94, 95). Based on the result they appeared to function well for this purpose. Ideally, primary astrocytes from mice should have been included as well, seeing as GBM cells have their origin in these cells. It would have made it easier to settle if the expression seen in the GBM cells were low relative to that in astrocytes. The same apply to the MTS assay. Astrocytes would have been a more correct control for the GBM cells. HL-1 cells should also have been included in the MTS assay. Since it is known that these cells largely metabolize ketone bodies it would have been interesting to assess the effect of MHP88 in both the presence and absence of BHB and compare this with the GBM cells. Since HL-1 cells are difficult and expensive to culture this was not an alternative.

6.2.3 Preparing of cell media

The glucose reduced media were prepared by mixing RPMI 1640 Medium no glucose, RMPI-1640 Medium and FBS. The glucose content in the different cell media is given by the manufacturers. FBS has an unknown concentration of glucose. In the present thesis a value estimated from 39 samples of FBS was used (86). The glucose concentration of the serum was tried measured with an electronic blood glucose meter, without success. Hence, there were some insecurities related to this concentration. This, in combination with the medium being mixed in the lab could have led to small deviations in the glucose content from the stated values.

In the experiments with BHB, (R)-ß-hydroxybutyrate was added to the glucose reduced cell medium. This isomer was considered the most suitable as it is the one utilized by the brain for energy production (87). In the human body, both BHB and acetoacetate will be present when ketosis is induced (21, p. 380). As BHB is the most abundant ketone body in the blood it was used in the experiments. Besides, BHB is converted to acetoacetate prior to being oxidized. Consequently, if the cells can metabolize BHB they will also be able to metabolize acetoacetate. What could possibly have been lost is any potential direct effects of acetoacetate. Ideally, both ketone bodies should have been utilized in the experiments. However, as there was a limitation to the work load this was deprioritized. BHB was weighted and diluted in the laboratory. This could have cause small deviations in the actual concentration from the stated values. However, it ought not to have affected the results.

6.2.4 Live-cell imaging

Due to limited time and resources, live-cell imaging was carried out only once. This was seen fit as the experiment was performed mainly to obtain progression with the MTS assays. It was expected that the results generated from the live-cell imaging could be transferred to the other cell lines. The murine glioblastoma cell line GL261-luc2 was used as it was the cell line utilized when the initial glucose deprivation was tried out. When conducting the MTS assays with BHB it was indeed confirmed through a light microscope that the other cell lines were able to proliferate in 3.5 mmol/l glucose for several hours prior to the assay.

A limitation with live-cell imaging is that the results are left for the researcher to interpret as they occur only in pictures. This can lead to misinterpretation caused by expectations created by a hypothesis or research question. To reduce the risk of such bias the results were discussed with other members of the TBRG. The experiment was carried out to find out if, and for how long, the cells could survive culturing in a glucose reduced medium. This could easily be seen in the pictures and required little interpretation, hence the method was thought to be sufficient. Still, the cells were rather confluent in the wells when the imaging was initiated. Maybe it would have been easier to observe possible inequalities between the different wells if the cells had been seeded with a lower density.

6.2.5 MTS assay

MTS assay was utilized as it is easy to use, cost efficient, sensitive and demands little time (90). The method was therefore considered suitable as the researcher had no previous laboratory experience and many repetitions of the assay was required. By changing the cell

medium, it was possible to investigate the effect of MHP88 in both a standard medium and a glucose reduced medium with BHB.

The IC_{50} values of MHP88 had large standard deviations in several of the assays. This could be caused by the absorbance being affected by incubation time, cell density and volume of MTS solution (90). Even though the cut off for statistical significance was set at $p \leq 0.05$, p-values close to this cut off have been described as they were trending towards significance. The results were interpreted this way because when viewed as a whole, increasing concentrations of BHB appeared to lower the cytotoxic effect of MHP88. Hence, if one were to dismiss all of the results that generated a p-value above 0.05, the bigger picture would be lost. Further, it could be discussed if there was a point in adding a cut off when it is not completely complied with. Here, the p-value was used an indication for interpretation of the results, but not as an absolute standard.

In regards of the method, the assay measures the activity of NADH/NADPH dependent dehydrogenase enzymes in the electron transport chain in the mitochondria (91). Research has revealed that the mitochondria in GBM cells can be defect and the cells are alleged to have reduced respiratory capacity (69, 118, 119). The murine glioblastoma cell lines GL261 and GL261-luc2 had a considerably less pronounced change in color compared to the human glioblastoma cell line U251 and fibroblast cell line MRC-5. This is demonstrated by the low absorbance values shown in table 3. It indicates that less MTS (yellow) has been converted to formazan (purple) in these cells. This reflects low metabolic activity and/or a reduced number of mitochondria in the cells. Based on this information and the fluctuating results generated from the assays it can be question if the method was suitable to answer the research question.

6.2.6 Western blotting

Western blotting is a widely used technique for the detection of proteins in cell lysates by the use of antibodies (92). The method was used in the present thesis to investigate the expression of the rate-limiting ketolytic enzyme SCOT in various samples. It is a relatively simple procedure, but there are limitations. To begin with, the method is semi-quantitative as it only allows for a relative comparison of protein contents. This is due to variations in loading and transfer rates between samples on a blot and also across different blots. In addition, the emitted signal after exposure to the chemiluminescent reagent have no linear correlation with variations in sample concentrations. Consequently, the method does not provide a precise measurement of the target protein.

Unusual bands at unexpected positions can form due to protease degradation, which causes unspecific binding of the antibody (92). All samples were exposed to a protease inhibitor to avoid this. Still, there were bands of unknown origin present on the nitrocellulose membrane. A consideration that must be made prior to detection of the proteins is optimization of the antibody concentration. Too high concentrations cause white bonds, while too low concentrations cause weak bonds. The latter happened when anti-OXCT1/SCOT was diluted 1:1000. This was solved by performing a titration to find the optimum dilution of the antibody.

When stripping the membrane of antibodies, as was done prior to detection with anti-ß-actin, proteins can be lost (120). The bands with ß-actin, displayed in figure 18, could be affected by this. These bands were remarkably smaller for the controls. HL-1 was loaded with less proteins than the other samples and it was therefore expected that the sample contained less ß-actin. The sample containing primary hepatocytes, however, was loaded with the same protein concentrations as the other cell lines. The low expression of ß-actin could be due to the stripping procedure. It can also be caused by unequal loading or trouble with lysing of the sample as described in chapter 6.2.2.1. There is also a possibility that the samples did contain different levels of ß-actin (121).

The Western blot technique was learned at the end of the study period. For that reason, there was not enough time to repeat the experiment several times as originally planned. The presented results are based on only one round of blotting with all six samples. Ideally three rounds should have been performed to make sure the results were not incidental.

6.2.7 Bioluminescence measurements

The Bright-Glo Luciferase assay system was used to perform bioluminescence measurements. It is easy to use, requires little time and have high assay sensitivity (122). The assay conditions and timing of the reading can affect the results. These were kept stable throughout all measurements. Three different cell densities were included to easier detect researcher errors. In batch 1 the luminescence signal from 10,000 cells/well (figure 19) deviated from that from 5000 and 20,000 cells/well in p5 and p7. In batch 2 the signal from 5000 and 10,000 cells/well decreased in p3 while the signal from 20,000 cells/well was stable (figure 20). This indicate that the cell number/well might not have been stable throughout all measurements. However, by using three cell densities the likelihood of such an error affecting the interpretation of the results is reduced, compared to using only one.

In the protocol in chapter 4.6, two different ways of seeding cells in the 96 well plate are explained. With batch 1, wells next to each other on the plate were used. It was observed that the luminescence signal from the wells affected each other. This was obvious when looking at the signal from wells containing GL261 cells and was avoided with batch 2 by leaving empty wells between the wells holding cells. The luminescence signal was only affected to a small degree by using wells next to each other, so this ought not to have affected the basis of comparison for the two batches. Cells from batch 1 were seeded in duplicates and cells from batch 2 in triplicates. This was only done with consideration to the amount of reagent available.

7 Conclusion

The cell cytotoxicity of MHP88 was reduced or unaffected by culturing in a glucose reduced medium with BHB. It is possible that this can be attributed to a decrease in pH caused by BHB, as variations in pH appears to affect the function of MHP88. Further research is necessary to investigate the effect of BHB and MHP88 on GBM cells. Other findings in relation to a ketogenic diet as an adjuvant treatment to GBM was that GBM cells can endure cultivation in a medium holding 3.5 mmol/l glucose for 24 hours and that both human and mouse derived GBM cells appeared to express the rate-limiting ketolytic enzyme SCOT. However, the expression seemed to be low and the experiment need to be repeated in order to confirm the data. The luminescence signal from the GL261-luc2 culture appeared to be dependent on selection with G418, indicating that the *luc2* gene is not expressed at a constant level throughout culturing.

8 Future perspectives

Further research on new treatment options for GBM patients is of great value, as the tumor is one of the deadliest types of human cancer. What has been established in the present thesis is the importance of glucose for cell survival and imitation of ketosis in vitro. This ought to be considered when implementing future in vitro experiments on KD as an adjuvant treatment for GBM. A suitable method to further investigate the effects of a glucose reduction can be xCELLigence, as the method quantifies cell growth. A future perspective could also be to investigate the effects of BHB over different time intervals. The ketone body acetoacetate could be included to investigate the effect of the two ketone bodies both alone and combined. Brain cells from healthy mice should be included as control cells.

To take it a step further from cell research, tissue blocks from GBM tumors can be used to study gene expression with immunohistochemistry. It would make it possible to localize proteins involved in the ketone body metabolism, such as enzymes and transporters, in the actual tumor. It could also be utilized to locate PEA-15, a protein alleged to protect against a glucose withdrawal in GBM cells. However, this method in similarity with cell research cannot answer to the total effect of a KD. The purposed effect of the diet in clinical trials might not be caused by a direct effect of ketone bodies, but rather an altered microbiota or other systemic effects. A mouse study would be more suitable to investigate this. A future PhD project could be established for further research into possible mechanisms of the diet in mice. MHP88 also ought to be studied in mice to investigate if it causes immunogenic cell death in vivo as well as in vitro.

The ultimate goal would be to implement the KD in the clinic. As of now, ESPEN guidelines have no spesific recommendations regarding nutritional therapy for GBM patients. To implement a KD would require close follow-up by a clinical dietitian to ensure that all food requirements are covered. Monitoring of weight, body mass composition and blood cholesterol would also be essential, as the diet can cause weight loss and alter the blood lipid profile. To ensure that the patients adhere to the diet and that ketosis is induced, blood glucose and ketone bodies ought to be measured regularly. This is something the patients could do at home to minimize the burden of the diet.

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Appendix

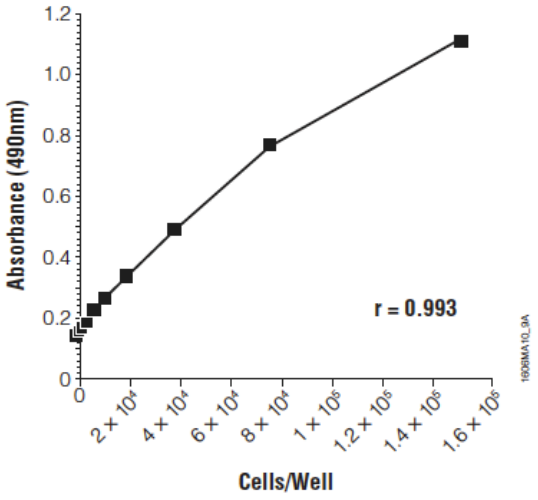

Figure 2. Effect of cell number on absorbance at 490nm measured using the CellTiter 96® AQ_{ueous} One Solution Assay. Various numbers of B9 hybridoma cells were added to the wells of a 96-well plate in RPMI containing 50µM 2-mercaptoethanol and supplemented with 5% FBS and 2ng/ml IL-6. The medium was allowed to equilibrate for 1 hour; then 20µl/well of CellTiter 96® AQ_{ueous} One Solution Reagent was added. After 1 hour at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance at 490nm was recorded using an ELISA plate reader. Each point represents the mean ± SD of 4 replicates. The correlation coefficient of the line was 0.993, indicating a linear response between cell number and absorbance at 490nm. The background absorbance shown at zero cells/well was not subtracted from these data.

Figure 22. Figure from Promega protocol (Appendix 1)
The graph is copied from the *CellTiter 96® AQueous One Solution Cell Proliferation Assay* protocol from Promega (89).

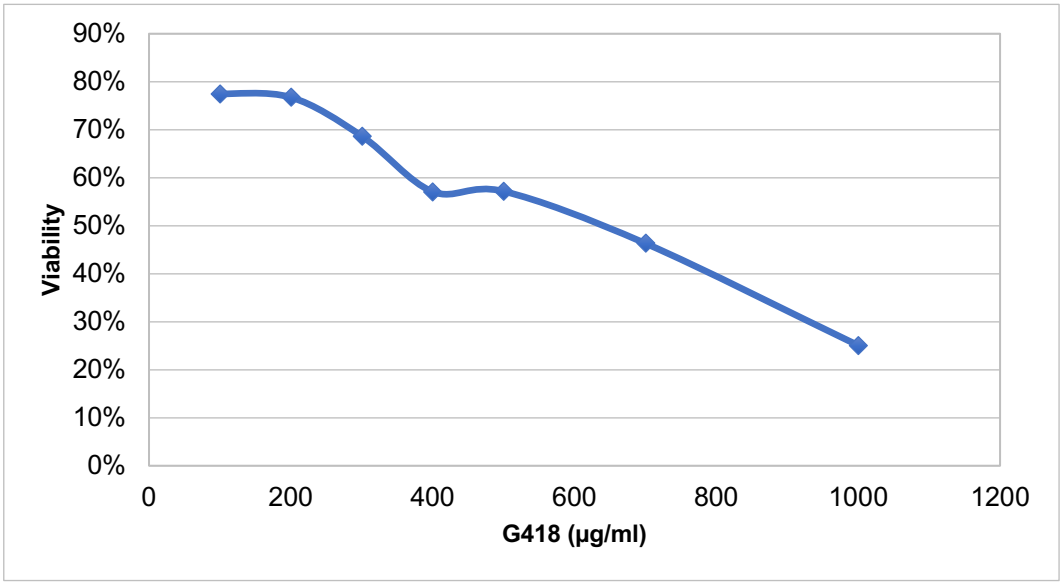

Figure 23. Titration with GL261 and G418 (Appendix 2)
The graph depicts the viability of the murine glioblastoma cell line GL261 after exposure to G418 in various concentrations for 48 hours. The cells were seeded with 20,000 cells/well in DMEM high glucose + 10% FBS in a 96 well plate.