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CELLULAR VULNERABILITIES OF GLIOBLASTOMA

Joni Merisaari

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To my Family

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ABSTRACT

Glioblastoma (GB) is the most fatal and frequent malignant brain tumor, and it is driven by multiple oncogenic pathways. Despite intensive screening of genomic, transcriptomic, metabolic, and post-translational landscape of GB, targeted therapies have provided no improvements for the survival of GB patients. This incurability of GB is due to its infiltrative growth, intratumoral heterogeneity and intrinsic resistance towards treatment modalities which are driven by its sub-populations, such as glioblastoma stem cells (GSCs). Therefore, it is crucial to try to understand the mechanisms of GBs cellular resistance and potential vulnerabilities of GSCs.

In this thesis we demonstrate alternative targets for GB therapy. Protein phosphatase 2A (PP2A) is inhibited in GB by non-genetic mechanisms, therefore, its therapeutic reactivation is possible. We described that small molecule reactivators of PP2A (SMAPs) efficiently cross the blood-brain barrier (BBB) and exhibit robust cytotoxicity towards heterogenous GB cell lines. Furthermore, we present specific kinases which inhibition induce synthetic lethality under PP2A reactivation. Collectively, these studies present SMAPs as a novel therapy for GB and propose an alternatives for multikinase inhibitors.

In GB, nanoparticles have been researched for their potential to circumvent insufficient drug properties. However, opposed to traditional utilization of nanoparticles, we discovered an alternative use of them in GB. We demonstrated that mesoporous silica nanoparticles (MSNs) functionalized with polyethylenimine (PEI) induce cell death specifically in GSCs. The PEI-MSNs accumulated in the lysosomes of GSCs and caused lysosomal membrane permeabilization potentially through proton sponge effect. Furthermore, we determined that PEI-MSNs efficiently cross the BBB in mice. In summary, this thesis presents a novel therapy concepts for GB.

KEYWORDS: glioblastoma, protein phosphatase 2A, blood-brain barrier, mesoporous silica nanoparticles, polyethylenimine, glioblastoma stem cells

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TIIVISTELMÄ

Glioblastooma (GB) on yleisin ja pahanlaatuisin aivosyöpä, jossa useat onkogeeniset signalointipolut ovat yliaktiivisia. Huolimatta genomiikan, transkriptomiikan, metabolomiikan ja translaation jälkeisten muutosten intensiivisestä seulonnasta GB:ssa, kohdennetut hoidot eivät ole tuottaneen lisäelinaikaa GB-potilaille. GB:n hoidon vaikeus johtuu sen infiltratiivisesta kasvusta, kasvaimen sisäisestä heterogeenisyydestä ja synnynnäisestä resistenssistä hoitoja vastaan. Syynä näihin on usein glioblastooman kantasolut. Tästä syystä, on erittäin tärkeää pyrkiä ymmärtämään GB:n solutason resistanssimekanismeja ja glioblastooman kantasolujen potentiaalisia heikkouksia.

Tässä väitöskirjassa esitämme uusia kohteita GB:n hoitoon. GB:ssa proteiinifosfataasi 2A (PP2A) on estetty muilla tavoin kuin geneettisillä mekanismeilla. Tästä syystä sen terapeuttinen uudelleenaktivointi on mahdollista. Osoitimme tutkimuksissamme, että pienimolekyyliset PP2A aktivaattorit (SMAP) läpäisevät veri-aivoesteen ja ovat sytotoksisia GB:n heterogeenisiä solulinjoja kohtaan. Tämän lisäksi selvitimme, minkä kinaasien hiljentäminen altistaa GB-soluja entisestään PP2A:n aktivaatiolle. Yhteenvetona tutkimus esittää SMAP lääkkeet uutena terapiamuotona GB:n hoitoon ja ehdottaa vaihtoehtoja multikinaasiestäjille.

Nanopartikkelitutkimus GB:aan liittyen on pääasiassa pyrkinyt parantamaan lääkkeiden ominaisuuksia. Me löysimme kuitenkin vaihtoehtoisen tavan käyttää nanopartikkeleita GB:ssa. Osoitimme, että mesohuokoiset piioksidi-nanopartikkelit, jotka on pinnoitettu polyetyyliemiinillä, aiheuttavat solukuoleman glioblastooman kantasoluissa. Kyseiset nanopartikkelit kerääntyivät glioblastooman kantasolujen lysosomeihin ja aiheuttivat sen membraanin tuhoutumisen ”proton sponge” efektin avulla. Kokonaisuudessaan väitöskirja esittää uusia heikkouksia glioblastooman kantasoluissa.

AVAINSANAT: glioblastooma, proteiinifosfataasi 2A, veri-aivoeste, mesohuokoinen piioksidi-nanopartikkeli, glioblastooman kantasolut

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Abbreviations

ABC-transporter	ATP-binding cassette transporters
Acetyl-CoA	Acetyl-coenzyme A
Akt	Protein kinase B
AML	Acute myeloid leukemia
ANP32a	Acidic leucine-rich nuclear phosphoprotein-32A
ATRX	α thalassemia/mental retardation syndrome X-linked
BAD	BCL-2-associated death promoter
BAK	BCL2 antagonist/killer 1
BAX	BCL-2-associated X protein
BBB	Blood-brain barrier
BBI	Bromodomain inhibitor
BCL-2	B-cell lymphoma 2
bEND3	Mouse brain micro vessel endothelial cells
BH	BCL2 homology
BTB	Blood-tumor barrier
CAD	Cationic amphiphilic drugs
CDK	Cyclin-dependent kinases
CIP2A	Cancerous inhibitor of PP2A
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
Cyt c	Cytochrome c
DCA	Dichloroacetate
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
EC	Endothelial cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAS	Fas cell surface death receptor
FBS	Fetal bovine serum

FUS	Focused ultrasound
GARP	Gene Activity Ranking Profile
GB	Glioblastoma
G-CIMP	Glioma CpG Island Methylator Phenotype
GSC	Glioblastoma stem cell
HCC	Hepatocellular carcinoma
HEAT	Huntington/elongation/A-subunit/TOR
HIF	Hypoxia-inducible transcription factor
HIFko	Immortalized mouse astrocytes
I3C	Indole-3-carbinol
IC_{50}	Half maximal inhibitory concentration
IDH	Isocitrate dehydrogenase
IGBP1	Immunoglobulin-binding protein 1
iHAP	improved heterocyclic PP2A activator
LCMT1	Leucine carboxyl methyltransferase 1
LMP	Lysosomal membrane permeabilization
LOF	Loss-of-function
LogP	Log octanol-water partition coefficient
MGMT	O6-methylguanine-DNA methyltransferase
MOMP	Mitochondrial outer membrane permeabilization
MRI	Magnetic resonance imaging
MSN	Mesoporous silica nanoparticle
mTOR	Mammalian target of rapamycin
Na-FI	Sodium-fluorescein
NF1	Neurofibromin 1
NSCLC	Non-small cell lung carcinoma
PDC	Pyruvate dehydrogenase complex
PDGFRA	Platelet-derived growth factor receptor A
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PDPK1	Protein 3-phosphoinositide-dependent protein kinase1
PEG	Polyethylene glycol
PEI	Polyethylenimine
PFA	Paraformaldehyde
P-gp	P-glycoprotein
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidy-linositol-3, 4-bisphosphate
PIP3	Phosphatidylinositol-3, 4, 5-bisphosphate
PME-1	Protein phosphatase methylesterase 1
PP2A	Protein phosphatase 2A

PPase	Protein phosphatases
PPZ	Phenothiazines
PS80	Polysorbate 80
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modifications
RB	Retinoblastoma protein
ROS	Reactive oxygen species
RT	Room temperature
SET	Inhibitor-2 of PP2A
SETBP1	SET-binding protein
SMAP	Small molecule reactivator of PP2A
TEM	Transmission electron microscopy
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TMZ	Temozolomide
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor
WHO	World health organization

List of Original Publications

This thesis by Joni Merisaari is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Joni Merisaari**, Oxana V. Denisova, Milena Doroszko, Vadim Le Joncour, Patrik Johansson, William P.J. Leenders, David B. Kastrinsky, Nilesh Zaware, Goutham Narla, Pirjo Laakkonen, Sven Nelander, Michael Ohlmeyer, Jukka Westermarck. Preclinical monotherapy efficacy of blood-brain barrier permeable small molecule activators of tumor suppressor PP2A in glioblastoma. *Brain communications,* 2020; Jan 11;2(1):fcaa002.

II Oxana V. Denisova, **Joni Merisaari**, Laxman Yetukuri, Amanpreet Kaur, Mikko Taipale, Mikael Jumppanen, Michael Ohlmeyer, Carina von Schantz-Fant, Krister Wennerberg, Jukka Westermarck. Triple kinase/phosphatase targeting of PI3K/AKT pathway and mitochondria is required for cytotoxicity across heterogenous glioblastoma cells. *Manuscript*

III Neeraj Prabhakar*, **Joni Merisaari***, Vadim Le Joncour, Markus Peurla, Didem Şen Karaman, Eudald Casals, Pirjo Laakkonen, Jukka Westermarck, Jessica M. Rosenholm. Circumventing Drug Treatment? Intrinsic Lethal Effects of Polyethyleneimine (PEI)-Functionalized Nanoparticles on Glioblastoma Cells Cultured in Stem Cell Conditions. *Cancers,* 2021, May 27;13(11):2631.
*Equal contributions

1 Introduction

Protein phosphorylation is the most common post-translational modification (PTM). The protein phosphorylation plays an important role in cellular signaling by regulating multiple pathways which are responsible for the cell's proliferation and survival. It is controlled by a balance between protein kinases and phosphatases (Khoury, Baliban, and Floudas 2011). However, the balance is disrupted in cancer by alterations in molecular signaling by various mechanisms. Inhibition of protein phosphatase (PPase) activity, which are often responsible for suppressing oncogenic activity, can lead to cancer (Hanahan and Weinberg 2011). One such is protein phosphatase 2A (PP2A), which is considered to be an important tumor suppressor gene. Inactivation of PP2A is a precondition for the malignant transformation of human cells. Various PP2A complexes are formed by a scaffolding (A), a regulatory (B) and a catalytic (C) subunit, and in total there are over 90 different combinations of PP2A with variable substrate specificity. This fact well resembles the importance of PP2A as a modulator of cellular signaling (Eichhorn, Creyghton, and Bernards 2009; Westermarck 2018). In cancer, and especially in glioblastoma (GB), PP2A is rarely mutated. Whereas, it has been mainly shown to be downregulated by its endogenous inhibitors such as protein phosphatase methylesterase 1 (PME-1), cancerous inhibitor of PP2A (CIP2A) and inhibitor-2 of PP2A (SET) (Kaur et al. 2016; Tomiyama et al. 2019). Small molecule reactivators of PP2A (SMAPs) have been developed. SMAPs have been shown to increase dephosphorylation capabilities of PP2A and to have antitumoral effect towards multiple *in vivo* models (Sangodkar et al. 2017; Kauko et al. 2018).

Every year there are approximately 100 000 people worldwide diagnosed with a diffuse glioma (Bray et al. 2018). GB has the highest incidence among diffuse gliomas and is considered to be the most aggressive primary brain tumor with an abysmal survival between 12 to 15 months in adults (Louis et al. 2016). GB is a disease driven by multiple oncogenic pathways such as highly mutated pro-survival RTK/PI3K/PTEN pathway (Brennan et al. 2013). In the past decade, there has been vital information obtained from the genomic, transcriptomic, post-translational and metabolic landscape of GB (Verhaak et al. 2010; Brennan et al. 2013; Q. Wang et al. 2017; TCGA 2008; H. Yan et al. 2009; Killela et al. 2013; Noushmehr et al.

2010). Understanding all this information has provided important knowledge regarding the mechanisms that drive GB progression, recurrence, and its resistance to therapies. In 2016 world health organization (WHO) updated the classification of GB based on tumor morphology and molecular alterations (Louis et al. 2016). Thus far, targeted monotherapies have provided little to no improvements for the survival of GB patients (H. Zhang et al. 2019). Therefore, it is crucial to try to understand this discontinuity between preclinical studies and clinical treatment.

In GB, nanoparticles have been heavily researched for their potential to circumvent insufficient drug properties. Nanoparticles have been shown to improve blood-brain barrier (BBB) penetration and pharmacokinetic properties of classical drug molecules. Furthermore, nanoparticles can be used to guide the drugs towards the site of interest to improve potency and reduce undesired peripheral effects (Ragelle et al. 2017). Mesoporous silica nanoparticles (MSNs) have demonstrated their potential to be used as drug carriers in cancer because of their good biocompatibility and non-toxic behavior up to high doses (Jafari et al. 2019).

This thesis explores the potential of PP2A reactivation as a GB therapy. The study also identifies potential kinases which are responsible for the synthetic lethality in combination with PP2A reactivation. Synthetic lethality is that when a combination of deficiencies in two or more target genes lead to cell death, whereas a deficiency in only one does not. Here we show a triple therapy including inhibition protein kinase B (Akt) and pyruvate dehydrogenase kinase (PDK) combined with PP2A reactivation demonstrates efficacy across multiple heterogenous GB cell lines and glioblastoma stem cell (GSC) lines. Additionally, therapy potential of polyethylenimine (PEI) functionalized MSNs is examined and shown to induce cell death of GSCs through proton sponge effect.

2 Review of the Literature

2.1 Cancer

Term “cancer” accounts for a group of diseases which can be described by abnormal growth of cells and potential to invade into the neighboring tissue or even other organs. Tumors can be divided into benign (lacks ability to invade or metastasize) and malignant neoplasia’s. Development and malignant progression of a tumor is a long and slow process and premalignant tumor cells need to achieve multiple alterations in specific genes, referred as oncogenes or tumor suppresser genes, or their end products to continue towards malignancy. These alterations can develop on multiple levels by either genetic or epigenetic modifications, or by PTMs of proteins. Genetic alterations can be caused by somatic mutations, such as point mutations, or insertion or deletion of base-pair in DNA or by chromosomal modifications such as copy number alterations. Epigenetic abnormalities can disrupt the structure of chromatin by methylation of DNA promoter regions or histone modifications. PTMs, such as phosphorylation or ubiquitination, can further alter protein activity and functions. Due to accumulation of alterations in cancer cells, genomic instability arises which further supports premalignant cells progress towards the hallmarks of cancer development, where tumor cells ensure continuous proliferation, avoid cell death, gain immortality, ensure stable supply of nutrients and energy, avoid immune destruction, and invade to tissues. On top of genomic instability, inflammation enables tumor growth by paracrine signaling supplying proliferative and survival signals (Hanahan and Weinberg 2000, 2011).

2.1.1 Hallmarks of cancer

Hallmarks of cancer were first described in 2000 by Hanahan and Weinberg, where they suggested six essentials mechanisms for cancer development (Hanahan and Weinberg 2000). These six were later followed by two new hallmarks in 2011 (Hanahan and Weinberg 2011). These hallmarks are:

1) “Sustaining proliferative signaling”: cancer cells need to achieve chronic cell proliferation, which is driven by dysregulated signaling networks. This is caused by alterations (e.g. mutations) in genes responsible for proliferative signaling, also

known as oncogenes. One of the most common oncogene is an epidermal growth factor receptor (EGFR) which drives signaling towards proliferation through its downstream pathways KRAS-RAF-MEK-MAPK and PI3K-AKT-mTOR.

2) “Evading growth suppressors”: to counterbalance the previous hallmark, there are genes which hinder the cell proliferation, defined as tumor suppressor genes. They either block or divert the initiation of cell division caused by proliferative signaling. In cancer, inhibition of these genes, such as retinoblastoma protein (RB) or tumor protein 53 (TP53), which are master regulators of cell cycle, is required for tumor progression.

3) “Resisting of cell death”: an obstacle for cancer’s aberrant growth is an intrinsic cellular mechanism for cell death in abnormal circumstances. Most common mechanism in cells is programmed cell death, known as apoptosis. To achieve aberrant and large mass growth, cancer cells must evade apoptosis by either activating anti-apoptotic factors such as B-cell lymphoma 2 (BCL-2) or by inhibiting pro-apoptotic factors such as Bcl-2-associated death promoter (BAD) or Bcl-2-associated X protein (BAX).

4) “Enabling replicative immortality”: third barrier for cancer progression comes from a structural problem of human chromosomes, telomeres. Telomeres are located at the end of chromosomes and shorten by every cell-division cycle. After telomere decay normal cells eventually go to irreversible quiescence state (senescence) or progress into cell death. Cancer cells are able to circumvent this innate problem of cells to achieve immortality by upregulation of telomerase reverse transcriptase (TERT), which is otherwise repressed in postnatal somatic cells. This enzyme prevents telomere erosion by adding telomere repeat to the end of telomeres.

5) “Inducing angiogenesis”: to support cancer’s exponential growth it needs to achieve a steady supply of nutrients and oxygen. This is induced by upregulation of vascular endothelial growth factor (VEGF) or hypoxia-inducible transcription factor (HIF) both of which promote angiogenesis. Additionally, tumor cells utilize vascular co-option where the tumor cells seek and grow close to existing blood vessels.

6) “Activating invasion and metastasis”: for cancer to progress further from localized tumor it must invade nearby tissues and metastasize to distant organs. This is achieved by invading into blood or lymphatic vessels where malignant cells must survive in the harsh environment of the circulatory system. Cancer cells promote alterations in the expression of extracellular matrix (ECM) adhesion proteins.

7) “Deregulating cellular energetics and metabolism”: cancer is often riddled with hypoxic conditions. Cancer cells utilizes excessive glycolysis followed by lactic acid fermentation instead of oxidative phosphorylation. This happens even in the presence of abundant oxygen. Oxidative phosphorylation is more efficient than glycolysis, but increased glycolysis leads to the increased generation of metabolites

that benefit proliferation. This is known as Warburg effect, discovered already 90 years ago.

8) "Avoiding immune destruction": for premalignant cells to survive and progress they must avoid the surveillance done by the immune system cells, such as natural killer cells or cytotoxic T-lymphocytes, that would otherwise eliminate them because of their aberrant phenotype. This is achieved by either presenting cell-of-origins antigens which immune system will not attack or by producing novel antigens, which is possible due to high mutagenicity, which immune system fails to react against to. (Hanahan and Weinberg 2011, 2000).

2.1.2 Molecular mechanisms of cell death

Important for cellular balance and healthy growth is cell death. Cells regulate themselves by inducing cell death after aberrations in their functions. Inhibition of cell death is one of the main causes of cancer, and therefore, crucial for malignant progression.

2.1.2.1 Apoptosis

Apoptosis is a form of programmed cell death where the cell dies with minimal effect to its surrounding cells to avoid induction of inflammation (Cruickshanks et al. 2013). In apoptosis, the cell goes through morphological changes such as shrinkage of cellular components, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. This happens in a regulated manner resulting in vesicles which are discharged by neighboring immune cells such as phagocytes. Apoptosis can be stimulated through intra- or extracellular signals which initiate intrinsic or extrinsic apoptosis, respectively. Both pathways eventually result in an activation of caspase proteases responsible for the cleavage of cellular proteins and eventual cell death. The caspases can be divided to initiator caspases (caspase-2, -8, -9 and -10) which activate the effector caspases (caspase-3, -6 and -7) after their activation through either intrinsic or extrinsic apoptosis. Effector caspases are responsible for the cleavage of cellular proteins (Cruickshanks et al. 2013).

Intrinsic apoptosis can be initiated by numerous extracellular signals, such as DNA damage, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) overload, replication stress, microtubular alterations or mitotic defects (Czabotar et al. 2014; Roos, Thomas, and Kaina 2016; Galluzzi et al. 2018). Intrinsic apoptosis execution begins by widespread mitochondrial outer membrane permeabilization (MOMP) (Tait and Green 2010) which results in a release cytochrome c (Cyt c) into the cytoplasm. The Cyt c binds to the apoptotic protease-activating factor 1 and creates an oligomer complex called apoptosome which is responsible for caspase-9

activation, which results in further effector caspase activation. All this is controlled by pro- and anti-apoptotic BCL-2-family proteins which are a group of proteins sharing one to four BCL2 homology (BH) domains (BH1, BH2, BH3, and BH4) (Czabotar et al. 2014). The BCL-2-family proteins are divided into three different categories 1) the pro-apoptotic effector proteins (BAK and BAX), 2) the anti-apoptotic proteins (BCL-2, BCL-XL and MCL1) and the pro-apoptotic BH3-only proteins (BAD, BID, BIM, PUMA and NOXA).

In normal conditions BCL2 associated X, apoptosis regulator (BAX) shifts between the outer membrane of the mitochondria and the cytosol (Edlich et al. 2011). Whereas BCL2 antagonist/killer 1 (BAK) resides only at the outer membrane of the mitochondria. Furthermore, the anti-apoptotic proteins (BCL-2, BCL-XL and MCL1) are bound to the pro-apoptotic effector proteins (BAK and BAX), hence inhibiting their apoptotic effect. Upon induction of apoptosis, the pro-apoptotic BH3-only proteins (BAD, BID, BIM, PUMA and NOXA) shift to mitochondria and interact with the anti-apoptotic proteins (BCL-2, BCL-XL and MCL1). This allows the pro-apoptotic effector proteins (BAK and BAX) to remove themselves from the anti-apoptotic proteins and start to form large oligomers, as these oligomers get through the outer mitochondrial membrane they create pores and enhance the beforementioned MOMP (Edlich et al. 2011; Kuwana et al. 2005).

The extrinsic apoptosis is mainly initiated through by so called death receptors, which are activated by extracellular ligands (Galluzzi et al. 2018). The most common death receptors are Fas cell surface death receptor (FAS), and TNF receptor superfamily member 1A, and TRAIL receptors 1 & 2 (Wajant 2002; Walczak 2013; Von Karstedt, Montinaro, and Walczak 2017). In short, death receptor activation initiates caspase 8 or 10 activation through multiprotein complex with fas-associated protein with death domain called death-inducing signaling complex. Which then leads to further effector caspase activation and apoptosis (Galluzzi et al. 2018).

In cancer, apoptosis is often dysregulated through overexpression of anti-apoptotic factors, such as BCL-2 which has been recognized as an oncogene (Carneiro and El-Deiry 2020) as its overexpression in human tumors leads to growth and drug resistance (Fulda 2009). Transcription and phosphorylation of BCL-2 family proteins is regulated by cyclin-dependent kinases (CDKs) and p53 which are often dysregulated in tumors (Chipuk et al. 2004). Furthermore, other anti-apoptotic proteins such as BCL-XL and MCL1 are overexpressed in human tumors (Chipuk et al. 2004). For example, these can be treated with the pan-CDK inhibitor flavopiridol which inhibits MCL1 transcription and upregulates the pro-apoptotic BH3-only proteins (BIM, NOXA and BIK) in multiple myeloma (S. Chen et al. 2012). Extrinsic apoptosis death receptors are controlled in cancer through decoy receptors which compete for death ligands and cell surface area, therefore reducing apoptotic signals.

In lung, colon, and breast cancer, a decoy receptor called DcR3 is highly expressed and amplified, which acts as receptor for Fas ligands (Pitti et al. 1998; Carneiro and El-Deiry 2020).

2.1.2.2 Lysosomal membrane permeabilization

Lysosomal membrane permeabilization (LMP) and the cell death caused by it are crucial part of the thesis. Hence, I will go through it shortly. Lysosomes are membrane-enclosed organelles that contain hydrolytic enzymes and can be considered recycling centers of cells. They are responsible for degradation and recycling of unwanted intracellular proteins, DNA, RNA, carbohydrates, and lipids via autophagy. Furthermore, lysosomes are responsible for the degradation of extracellular objects taken in by endocytosis. Therefore, ensuring proper lysosomal activity is crucial for cellular homeostasis (F. Wang, Gómez-Sintes, and Boya 2018). On the contrary, the high levels of hydrolytic enzymes in lysosomes makes them harmful to the cell in case of their rupture. The role of lysosomes in cell death was proposed already soon after their discovery by Christian de Duve. He considered lysosomes to be "suicide bags" of the cell. This theory hypothesized that cell death could be caused by the release of the lysosomal hydrolases into the cytoplasm (de Duve 1959). The release of lysosomal contents, including cathepsins, can be caused by phenomenon called LMP. The leakage of lysosomal contents breaks down the cellular components, increases cytosolic acidity and can therefore induce cell death. Depending on how robust the leakage is, the cells can either die by necrosis or through controlled cell death mechanism such as apoptosis (F. Wang, Gómez-Sintes, and Boya 2018). LMP can be caused by either internal or external stimulates, such as lysosomotropic agents with detergent activity or by ROS (F. Wang, Gómez-Sintes, and Boya 2018). Lysosomotropic agents cause swelling of the lysosomes. This effect is also known as "proton sponge" where lysosomotropic agents absorb protons from the lysosome into their unprotonated amines. As a results lysosome absorbs more protons and hence increases influx of Cl^- ions and water, eventually leading to lysosomal membrane rupture (Vermeulen et al. 2018).

In cancer, lysosomal functions are altered through changes in volume, composition, and cellular distribution to promote invasive growth, angiogenesis, and drug resistance (Serrano-Puebla and Boya 2018). However, these changes also can promote their sensitivity to LMP inducing compounds. For example, altered morphology of lysosomes can make them more vulnerable to the "proton sponge effect" (Kallunki, Olsen, and Jäättelä 2013). Cathepsins have increased expression in multiple cancers, such as melanoma, glioma, and lung cancer. This often makes the LMP more potent target because of excessive outburst of cathepsins (Vetvicka,

Vetvickova, and Benes 2004; Fukuda et al. 2005). Multiple ways have been suggested to therapeutically target LMP. Cationic amphiphilic drugs (CADs), such as antidepressants or antihistamines, induce LMP by increasing the swelling of the lysosomes after their get trapped in them through protonation (Petersen et al. 2013). Efficacy of antihistamines have been already shown in patients with non-small cell lung cancer (Ellegaard et al. 2016). In glioma, antihistamines were shown to reduce invasiveness and increase survival of mouse with intracranial GB xenografts (Le Joncour et al. 2019). As nanoparticles accumulate into lysosomes, they can be potential carriers of LMP inducing compounds. Furthermore, nanoparticles acting as CADs are susceptible for protonation hence can they can induce "proton sponge effect" themselves (M. Wang and Thanou 2010; J. Wang et al. 2017; Ding et al. 2017).

2.2 Glioblastoma

Diffuse gliomas are the most common brain tumors accounting for approximately 33 % of all brain tumors. Every year approximately 100 000 people worldwide are diagnosed with a diffuse glioma. Although this accounts for only less than 1 % of all new cancer cases each year, diffuse gliomas are considered one of the most atrocious cancer, as they have one of the highest and swiftest mortality (Bray et al. 2018). Among diffuse gliomas, GB is the most aggressive primary brain tumor among adults and has the highest incidence (Louis et al. 2016). Most common symptoms for GB include headaches, seizures, memory loss, confusion, and behavioral changes. These mainly emerge from increased intracranial pressure and neuronal damage caused by the tumor (Alifieris and Trafalis 2015).

Still, in the age of personalized medicine, the standard of care therapy for GB relies on classical cancer therapy by maximal safe surgical resection followed by radiotherapy, with adjuvant temozolomide (TMZ), if the tumors histopathology indicates vulnerability towards this chemotherapy (Stupp et al. 2009). This treatment scheme has been the golden standard over the last decades. After diagnosis of GB, with aforementioned treatment, typical overall survival of GB patient varies between 12 to 15 months with an abysmal 5-year survival rate between 3 to 7% (Ostrom et al. 2019). The challenges in GB treatment are well reflected in the scientific literature. Keyword "glioblastoma" resulted in 28338 publications over the last 10 years to August 2021. This is an enormous amount of knowledge regarding molecular mechanisms, driver genes and variable treatment modalities for GB. Nevertheless, translation of this knowledge has been unsuccessful so far towards better clinical outcomes. However, with new knowledge we have also learned why therapies do not succeed. GB is riddled with multiple different ways to evade successful cure: 1) complete resection of the intracranial tumor is often impossible

because of infiltrative tumor growth, 2) therapies need to cross the BBB 3) GB has high intra-/intertumoral heterogeneity and 4) intrinsic resistance towards traditional therapies (Zanders, Svensson, and Bailey 2019). These problems will further be introduced in the coming chapters.

2.2.1 Classification of Gliomas

Historically gliomas were categorized based on tissue morphology (cellular proliferation, mitotic characteristics, and necrotic areas) observed in the tumor. Gliomas were considered to originate from different glial cell of origin, therefore referred as gliomas (Sanai, Alvarez-Buylla, and Berger 2005). Gliomas were categorized to four different brain tumors based on their cell type: ependymomas (ependymal cells), oligodendrogliomas (oligodendrocytes), astrocytomas (astrocytes) and mixed gliomas. GB was considered to be part of astrocytomas which were further divided to grades of the disease: pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma (grade IV) (Louis et al. 2007). However, this classification had high variability because grading was done based on human observation, which caused significant variation within the grades in terms of overall survival and clinical response to treatment (Van Den Bent 2010).

Comprehending the molecular mechanism that drive GB tumorigenesis, progression, resistance, and recurrence can provide vital information for patient outcomes via better diagnostics, prognostic and predictive biomarkers, and novel therapies. Therefore, in the last decade there has been multiple large scale molecular studies about the genomic, transcriptomic, epigenomic and metabolic profiling of GB (e.g. Verhaak et al. 2010; Brennan et al. 2013; Q. Wang et al. 2017; TCGA 2008; H. Yan et al. 2009; Killela et al. 2013; Noushmehr et al. 2010). WHO decided to update the classification system in 2016 in collaborative project with neuropathologists and molecular biologists to form clinically relevant subgroups based on tumor morphology and molecular alterations (Louis et al. 2016). Additionally, neural stem cells within the central nervous system (CNS) are now considered to be cells of origin for multiple brain tumors, including GB. In animal models neural stem cells, specially from the subventricular zone, are the origin of GBs (Alcantara Llaguno et al. 2019; J. H. Lee et al. 2018) Therefore, we have gained support towards the notion that classification based only on cell of origin and morphology is not enough to predict the prognosis and treatment of brain tumors.

In 2016, WHO integrated tumor morphology, isocitrate dehydrogenase (IDH) mutation and 1p19q co-deletion status into the classification of diffuse glioma. This classification divides diffuse gliomas into seven different groups: **1)** GB IDH-wild

type or **2)** IDH-mutant, **3)** diffuse or anaplastic astrocytomas IDH-wild type or **4)** IDH-mutant; **5)** oligodendroglioma or anaplastic oligodendroglioma IDH-mutant and 1p19q co-deleted; **6)** diffuse midline glioma H3 K27M-mutant; **7)** malignant glioma not otherwise specified based on histology in the absence of molecular markers (Louis et al. 2016). The new groups show variable ages of diagnosis and prognosis. Patients with GB, **1)** IDH-wild type tend to be older (median age at diagnosis 59 vs 38 years) and have a worse prognosis (median overall survival 1.2 vs 3.6 years) than those with GB **2)** IDH-mutant tumors. Patients with astrocytoma **3)** IDH-wild type are also older (median age at diagnosis 52 vs 36 years) and have a worse prognosis (median overall survival 1.9 vs 9.3 years) than those with astrocytoma **4)** IDH-mutant tumors. IDH-wild type astrocytoma resembles more of IDH-wild type GB in terms of survival and patient age than IDH-mutant astrocytoma. **5)** Patients with oligodendroglioma IDH-mutant and 1p19q co-deleted have a median age 44 years when diagnosed and have the longest median overall survival of 17.5 years (Pekmezci et al. 2017).

Apart from WHO classification, GB has also been further classified into four transcriptomic profiles with classical, neural, proneural, and mesenchymal subtypes (Verhaak et al. 2010). The original classification from Verhaak and colleagues included the transcriptomes of tumor-associated nonmalignant cells. Therefore, further experiment with single cell sequencing with GB cells, GSCs and bulk cells were compared to find GB specific genes. Based on these genes three different GB subtypes were revealed (Q. Wang et al. 2017). Profiles divide GBs into three distinct subtypes: classical, proneural and mesenchymal. **1)** Classical subtype is characterized by loss of PTEN and CDKN2A and EGFR amplification (and point mutations and EGFRvIII); **2)** proneural tumors bear amplifications in PDGFRA, CDK4, CDK6 and MET and loss or mutations in genes IDH1/2 and TP53; **3)** mesenchymal subtype by mutation or loss of TP53, NF1 and CDKN2A. The original neural subtype did not appear anymore with the new classification methods and it is believed to be mainly neuronal lineage cells (Verhaak et al. 2010; Q. Wang et al. 2017).

2.2.2 Molecular alterations in GB

As mentioned, new classification of GB relies on the molecular alterations which have been discovered in the past decade. Now in the era of sequencing the molecular alterations in GB are very well characterized and GB was the first cancer to be sequenced by the TCGA project (TCGA 2008). First alterations in GB were already discovered in the eighties. An EGFR variant EGFRvlll (Libermann et al. 1985) was shown to be a prominent mutation in GB to promote tumorigenesis. After this,

multiple alterations in other genes have been discovered and linked to to invasiveness, resistance, and malignancy of GB.

Primary GB harbors multiple somatic genomic mutations which drive the disease forward. In the last decade, independent researchers have identified three key signaling pathways whose alterations can contribute to the development of GB: upregulation of pro-survival **1)** RTK/PI3K/PTEN pathway or by inactivation of **2)** p53 or **3)** retinoblastoma (RB) tumor suppressor pathways which leads evasion of cell death and altered cell cycle (TCGA 2008; Parsons et al. 2008; Brennan et al. 2013). **1)** RTK/PI3K/PTEN pathway was found to be altered in 90 % cases of GB through amplification or gain-of-function in EGFR (57%), platelet-derived growth factor receptor A (PDGFRA) (13%), MET (1.6%), Fibroblast growth factor receptor 2/3 (3%), phosphoinositide 3-kinase (PI3K) (25%) and deletions or loss-of-function (LOF) of phosphatase and tensin homolog (PTEN) (41%). Interestingly PI3K mutations were mutually exclusive of PTEN deletions. Neurofibromin 1 (NF1) was mutated or deleted in 10%, and it never co-occurred with BRAF mutations (2%). **2)** TP53 pathway is altered in 86% of GB cases through LOF mutations or deletions of tumor protein p53 (TP53) (28%), CDK inhibitors A/B (61%) and amplification of mouse double minute 2-homolog (MDM2) (15%). Alterations in TP53 were mutually exclusive with mutations of MDM family genes and CDKN2A. **3)** RB pathway is altered in 79% of GB cases via by direct LOF mutations or deletions in RB1 (8%) or by amplification of CDK4/6 (16%), and the remainder via CDKN2A/B deletions (61%) (TCGA 2008; Parsons et al. 2008; Brennan et al. 2013).

The new WHO classification of GBs includes molecular analysis of IDH1 and IDH2 mutations as part of GB diagnostics. This mutation has been shown to be more accurate prognostic marker than just histological subgrouping. The prognosis of IDH-mutated GB is better compared to the wild-type, overall survival after therapy being 31 months vs 15 months respectively (Louis et al. 2016). IDH1/2 are metabolic enzymes that transform isocitrate to alfa-ketoglutarate. Mutation in IDH1 codon 132, most commonly being R132H, or IDH2 codon 172 results in impaired IDH1/2 activity (H. Yan et al. 2009; Parsons et al. 2008). This will cause IDH to acquire capability to transform isocitrate into oncometabolite 2-hydroxyglutare that accumulates in cells. This results in impaired activity of epigenetic enzymes such as DNA and histone demethylases causing hypermethylation and therefore affecting gene transcription and cell metabolism (Noushmehr et al. 2010). This causes a change in transcriptional profile as methylation inhibits multiple genes simultaneously. This subgroup is called G-CIMP (glioma CpG Island Methylator Phenotype) which is usually linked to IDH1/2 mutations. G-CIMP positive subgroup is linked to better survival when compared to G-CIMP negative patients. (Noushmehr et al. 2010).

Hypermethylation caused by the IDH mutation in GB, called G-CIMP subtype as discussed above, often correlates with methylation of CpG islands in the promoter region of O_6-methylguanine-DNA methyltransferase (MGMT) which inhibits its transcription (Noushmehr et al. 2010; Turcan et al. 2012). MGMT is a DNA-repair molecule, and it acts by reversing the alkylation of O_6 position guanine at DNA level thus antagonizing apoptosis caused by alkylating agents such as TMZ. Therefore, if MGMT activity is inhibited, it will lead to accumulation of O_6-methylguanine adducts, which in turn lead to incorrect mismatch repair during the DNA replication leading eventually to cell cycle arrest and apoptosis (C. H. Fan et al. 2013). Importance of MGMT promoter methylation as a prognostic marker and predictive biomarker for improved survival is well established in diffuse gliomas (Stupp et al. 2009; Hegi et al. 2005; Wick et al. 2013).

Cancers need to achieve replicative immortalization to survive. It has been shown that this commonly happens through either mutation in TERT promoter or in the α thalassemia/mental retardation syndrome X-linked (ATRX) gene in diffuse glioma. These markers are good prognostic markers and normally their alterations are mutually exclusive because of only needing the other one to achieve immortality (Killela et al. 2013; Wiestler et al. 2013). Analysis of TERT and ATRX mutations within the five WHO 2016 groups have been shown to give additional prognostic value. Out of IDH-WT GB tumors 77% have a TERT mutation, whereas approximately only 3% have an ATRX mutation. On the contrary, IDH-mutant GB subgroup have high proportions of ATRX mutations 63% and low proportions of TERT mutations 6%. TERT and ATRX seem to be linked to IDH-WT and IDH-mut, respectively, therefore ATRX can be seen has a prognostic marker for improved survival (Pekmezci et al. 2017).

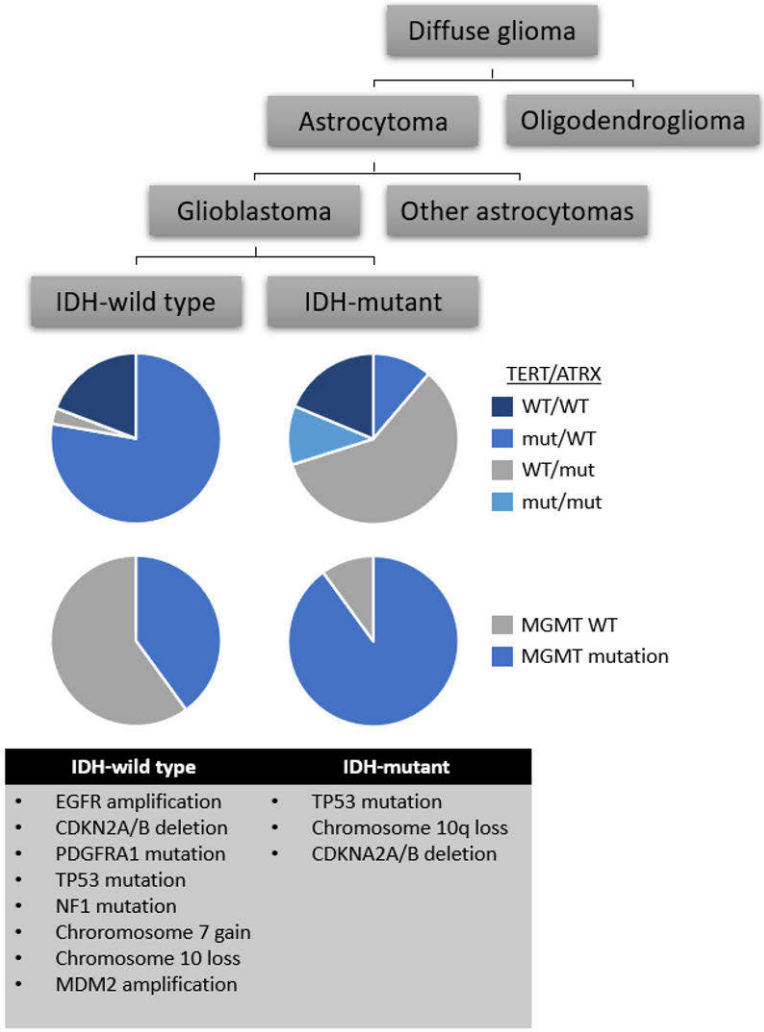


Figure 1. Classification of GB. Diffuse gliomas are divided into astrocytomas and oligodendrogliomas, and then astrocytomas are further divided into GB and other astrocytomas. GBs are categorized in various ways, such as IDH, MGMT or TERT promoter/ATRX gene mutation profile.

2.2.3 Heterogeneity of GB

Translational challenges in GB treatment arise through multiple factors. Even though drug candidates might have superior effect in preclinical studies, including orthotopic mouse models, they often provide little to no benefit in clinical trials (H. Zhang et al. 2019). Possible explanation for this discontinuity between preclinical and clinical findings in GB comes from a combination of tumor heterogeneity, microenvironment, GSCs, and BBB.

Heterogeneity in cancer exhibits on multiple levels: tumors vary between patients, within a patient (intertumoral) and tumor cells vary within the tumor itself (intratumoral). The intratumoral heterogeneity is especially very prevalent in GB. This heterogeneity has been well characterized in the past decade (Brennan et al. 2013; Verhaak et al. 2010; Noushmehr et al. 2010; Q. Wang et al. 2017; Killela et al. 2013; TCGA 2008; H. Yan et al. 2009). Furthermore, recent advancements in single-cell level, using DNA and RNA sequence analysis, have helped to map the heterogeneity (Patel et al. 2014; Q. Wang et al. 2017; Neftel et al. 2019). The intratumoral heterogeneity is an intrinsic quality of GB. It is considered to arise from the alterations, microenvironment and further develop through clonal selection by promoting drug resistant GB cell colonies to survive within the brain parenchyma. The clonal selection and the rewiring caused by intratumoral heterogeneity are one of the main reasons for recurrence of GB. Furthermore, targeted therapies might promote clonal selection, as they are selected based on main mutations, this will induce survival of resistant clones and rewire tumors towards alternative pathways to survive (Johnson et al. 2014).

2.2.3.1 Microenvironment

Another survival mechanism of cancer comes from its ability to modulate the microenvironment for its benefit. The tumor microenvironment in which GB grows is very unique as it is a tumor of a CNS. The various non-cancerous cells, such as stromal, immune, or other glial cells, in CNS are often harnessed to support the growth of GB. Furthermore, there are different microenvironments within the GB which are divided into three categories: perivascular, hypoxic, and invasive. These different microenvironments support tumor cells in their niche by promoting tumor growth, resistance, and phenotypic change to ensure tumor survival (Hambardzumyan and Bergers 2015). The surrounding cells can for example provide growth factor receptor ligands or ECM proteins which induce GB growth and assist in resistance towards targeted therapy (Timothy F. Cloughesy, Cavenee, and Mischel 2014).

In the perivascular niche, GB cells are in close proximity to the abnormally developed vasculature. GBs aberrant vasculature arises by five mechanisms: mechanism that is typical for GB is **1)** vascular co-option in which GB cells seek existing blood vessels (Seano and Jain 2020) followed by **2)** angiogenesis, the development of new blood vessel from pre-existing ones and **3)** vascular mimicry where tumor cells form blood-vessel like networks (Angara, Borin, and Arbab 2017). Additionally, GB supports blood vessel production by two ways **4)** vasculogenesis in which it mobilizes, differentiates, and recruits circulating bone marrow-derived cells, which are endothelial progenitor cells (Hardee and Zagzag 2012), and **5)**

transdifferentiation, in which glioma cells or GSCs differentiate into an endothelial cells (EC), to produce new blood vessel (Ricci-Vitiani et al. 2010). However, this vigorous angiogenesis often leads to disorganized and leaky blood vessels. This is a common phenomenon in of GB, which can also lead to phenotype called glomeruloid microvascular proliferation, in which EC and pericytes are poorly attached leading to dysfunctional vasculature (Dvorak 2015). These abnormalities often cause disruptions in the BBB which will be discussed further in the coming chapter. However, breakdown of the BBB will cause entry of circulating immune cells, such as monocytes, neutrophils and tumor-associated macrophages, to the brain which have been shown to secrete tumor growth inducing chemokines and cytokines (Feng et al. 2015; Ji Liang et al. 2014; Kennedy et al. 2013).

Another microenvironment niche for GB is the hypoxic/necrotic areas. Interestingly, even though reduced nutrient and oxygen delivery and necrosis should slow down tumor growth in the necrotic regions, GB can induce a phenomenon called “pseudopalisading necrosis”, in which tumor cells surround the necrotic area (Brat et al. 2004). This has been shown to predict poor prognosis among diffuse gliomas. The necrotic cell death releases proinflammatory signals into the surrounding tissue, which in turn activates for example tumor-associated macrophages to remove the necrotic debris (Ruffell and Coussens 2015). The third microenvironment in GB is the invasive edge. GB has been shown to migrate as single cells along white matter tracts and blood vessels (Cuddapah et al. 2014). Several matrix metallopeptidase are associated with the invasive microenvironment (Lakka, Gondi, and Rao 2005). Phenotypic shift towards GSCs is common in the invasive tumor niche (Prager et al. 2020). However, as the tumor grows there is a dynamic change constantly happening between the different microenvironments. These changes can further be altered by therapy as the tumor cells travel to more favorable microenvironment to ultimately develop resistance.

2.2.3.2 Glioblastoma stem cells

The evident driver of cellular heterogeneity in GB are GSCs. Over the years it has become evident that GSC are important for GB pathogenesis. As normal stem cells, GSCs have an ability to self-renew, differentiate to multiple cell lineages, pluripotency, angiogenetic capabilities, modulation of immune response and high mobility (Gimple et al. 2019). The GSGs have superior ability to evade and resist drugs, radiation, and the stressful conditions such as oxygen and nutrition deprivation. Reason for these characteristics come from their different genetic or epigenetic attributes, signal rewiring and ability to slow down or even stop and restart proliferation (S. K. Singh et al. 2003). Therefore, GSC resistance to therapy will lead to tumor regrowth, even if treatment was efficient on some population of

GB cells, based on their abilities to reproduce variable cell types needed for tumor maintenance. Classically, GSCs have been detected by multiple transcription factor and structural proteins (SOX2, Nestin, Nanog, Olig2, MYC, BMI1 and Musashi) and cell surface markers (CD15, CD133, CD44, L1CAM and integrin-α6) (Ichiro Nakano 2015). However, these markers are not exclusive to GSC but are found throughout GB cellular populations (Gimple et al. 2019).

Recent single cell RNA-seq efforts have provided additional information about GSC cellular compositions (Neftel et al. 2019; Patel et al. 2014). The model suggests that GSCs primarily consists of four malignant cellular states: Neural progenitor cell-like (NPC-like), Oligodendrocyte-like (OPC-like), Astrocyte-like (AC-like) and mesenchymal-like (MES-like). In GB, proliferation was observed in all four states suggesting that all states induce tumor growth (Neftel et al. 2019). Additionally, MES-like, NPC-like and OPC-like populations have the capacity to propagate tumors in mice (Suvà and Tirosh 2020). The GSC states show also high plasticity as when specific state cells were implanted in mice, they established the whole diversity of the GSC states. Despite this, in GB often one GSC state has higher abundance than others. This is at least partially dictated by tumor genetics as some alterations favor specific GSC state (Neftel et al. 2019). Furthermore, the GSC states showed bias towards different GSC surface or transcription factors. For example, the cell surface markers, CD24 was highest in NPC-like cells, CD133 in OPC-like cells, EGFR in AC-like cells, and CD44 in MES-like cells. Similar bias was also observed with transcription factors (Neftel et al. 2019).

The three microenvironments of GB, perivascular, hypoxic and invasive areas, work as remodelers of GSC activity, which in turn will support the GB microenvironment, heterogeneity, and therapy resistance. The perivascular environment provides GSCs with signals for maintenance of stemness as it induces GSCs ability to migrate and repair DNA damage (Prager et al. 2020). This is done by promoting NOTCH, sonic hedgehog, and nitric oxide signaling pathways, and further by tumor-associated macrophages which release chemokines that promote the GSC proliferation (Zhu et al. 2011; Charles et al. 2010). Consecutively, GSC promote the perivascular niche by producing proangiogenic factors that induce EC activity (Jain et al. 2007). Another common microenvironment of GB, the hypoxic areas, support GSC activity. The hypoxic stress induces GSCs to develop populations that adapt towards survival in the hypoxic and nutrient deprived environment by shifting metabolism towards aerobic glycolysis and glutamine-mediated fatty acid production (Semenza 2013). Additionally, hypoxia promotes quiescent phenotype in GSCs, which is commonly considered to be chemo- and radioresistant (Seidel et al. 2010). The third microenvironment where GSCs thrive is the invasive edge of the GB tumor (L. Cheng et al. 2011). These GSC populations

migrate as the frontline of GB along the vasculature and CNS by utilizing cadherins and integrins and matrix metalloproteinases (Ortensi et al. 2013).

As GSCs often reside in hypoxic or nutrient deprived environment, they need to constantly adjust their metabolic activity to correspond to the current situation. GSCs, as most other cancer cells, have been described mainly to rely on glycolysis for energy metabolism (Garnier et al. 2019). However, in murine models it has been demonstrated that GSCs do not only use non-oxidative glycolysis but that there is also high activity towards mitochondrial oxidative pathways (Marin-Valencia et al. 2012). Similar metabolism has also been seen in surgically resected tumors from GBM patients (Maher et al. 2012). Furthermore, there seems to be a growing evidence that the quiescent GSCs consume less glucose and produce more ATP as their differentiated GB cell counterparts, and therefore are less glycolytic and produce less lactate (Vlashi et al. 2011).

GSCs are highly resistant towards chemo- and radioresistance in GB. As GSCs are often slowly growing ,they have the ability to limit DNA damage through efficient DNA damage response, activate antiapoptotic pathways, and they can further prevent cytotoxicity of compounds through high drug efflux by ABC transporters (G. Liu et al. 2006; Bao et al. 2006). Plasticity between non-GSC and differentiated GB cells can happen as a results of multiple factors. For example, hypoxic conditions within the tumor can promote stem-like phenotypic change, through HIF2a, in the non-GSC population by activating multiple stem cell factors (Heddleston et al. 2009). Alternatively, chemotherapy or radiation have been shown to increase the GSC populations within GB tumors as they trigger phenotypic change towards resistant GSC populations (Auffinger et al. 2014).

2.2.3.3 Blood-brain barrier

For a cancer therapeutics to be effective towards brain tumors they must first cross a barrier between the blood and the CNS. This barrier is better known as the BBB. Another obstacle that hinders drug efficacy in brain is that drug diffusion through brain tissue is low. The role of BBB is to regulate homeostasis of the CNS and ensure normal brain function. However, while the BBB protects the brain from harmful toxins it also hinders the delivery of blood-borne therapies into the CNS. BBB is composed of ECs, which are connected by tight junctions, surrounding the capillaries. This is further surrounded by brain specific basal lamina that is supported by pericytes, astrocyte end feet and neuronal endings and microglia (Abbott 2013). The Basal lamina is an ECM, and it is mainly composed of glycoproteins such as collagen, laminin, fibronectin, nidogens and heparan sulfate proteoglycans. These glycoproteins are often cleaved proteolytically to alternate BBB properties (Thomsen, Routhe, and Moos 2017). ECs surrounding the BBB basal lamina differ

from regular ECs, as they have less pores in their cellular membrane, have limited intracellular trafficking and decreased pinocytosis. Therefore they are considered to be non-fenestrated. (Ayloo and Gu 2019). Pericytes, neurons and astrocytes which are located at the abluminal side of the BBB endothelium are the key regulators of BBB function by controlling the blood vessel production and remodeling (Bell et al. 2010). In addition to this, astrocytes regulate signaling pathways maintaining junctional complexes and produce basal lamina (Giaume et al. 2010). Activation of microglia, which are the most common immune cells in the brain, through inflammation can cause BBB disruption, which is common in cancer (Shemer et al. 2015). This described compartment is also known as neurovascular unit which together determines the structure and physical properties of the BBB as they tightly regulate molecular and cellular transport across it (Abbott 2013).

Transport of molecules through BBB happens via either passive or active transportation. Passive diffusion across the BBB is possible for small molecular weight and highly lipophilic substances such as oxygen. Other molecules need active transportation through the BBB either by vesicle- or carrier-mediated transcytosis. Solute carrier proteins or ATP-binding cassette transporters (ABC transporters), such as multidrug resistant ABC transporters, P-glycoprotein (P-gp), breast cancer resistance protein and multidrug resistance proteins are the master regulators of the transportation through BBB, therefore controlling the homeostasis and safety of CNS by excreting toxins and xenobiotics away from the CNS. However, these transporters affect therapeutic delivery, as many small molecule compounds are substrates of aforementioned efflux transporters, and therefore are ineffective in the CNS (Robey et al. 2018).

In GB BBB is often disrupted. Therefore, BBB is referred in GB as blood-tumor barrier (BTB). As the tumor expands, the nutritional demand increases dramatically. GB cells can alter the properties of neurovascular units by increasing or decreasing blood flow. The BTB is considered to be leakier than normally functioning BBB, which has been shown with magnetic resonance imaging (MRI) and positron emission tomography; there is higher drug accumulation within the GB when compared to healthy brain (Sarkaria et al. 2018). Nevertheless the BTB has very heterogeneous permeability as small molecule drugs can be very unevenly distributed within the tumor (Lockman et al. 2010). Multiple strategies have been developed to bypass or to exploit transporters on BBB to enhance drug delivery. These methods can be categorized into invasive or non-invasive methods. Invasive methods such as direct injection of the drugs to the disease site have had variable results and have been mainly disappointing. Therefore, development of multiple different non-invasive methods has been ongoing for the past decades.

As many therapeutics have affinity towards influx or efflux transporters, their inhibition or utilization for drug transport to CNS have been heavily researched.

To take advantage of influx transporters, drug molecules can be altered to be substrates of influx transporters or an influx transporter receptor ligand can be molecularly linked to the drug molecule thus allowing it to cross the BBB by receptor mediated endocytosis (Lajoie and Shusta 2015). Although this strategy sounds promising, it often results in unwanted toxicity throughout the body because of widespread expression of influx transporter receptors in other tissues. Alternatively, efflux pumps, such as P-gp, which actively pump out pharmacological compounds out of the CNS can be pharmacologically inhibited. One such drug compound is TMZ, which has high affinity towards P-gp. In preclinical studies where P-gp efflux transporter inhibitors were given together with TMZ, its intake was increased by 1.5-fold (de Gooijer et al. 2018) An alternative approach to open up BBB for drug diffusion is to use low-intensity focused ultrasound (FUS) pulses combined with circulating microbubbles that vibrate in response to the ultrasound which will transiently disrupt the BBB (6-24 h) to increase its permeability. Precise mechanism how FUS disrupts the BBB still remains somewhat unclear, but current consensus is that the microbubble vibration, in response to FUS, weakens the BBB integrity and increase the vessel permeability. FUS with microbubbles have been shown to be safe and to increase drug delivery of anticancer agents into CNS (Carpentier et al. 2016).

2.3 Phosphorylation dependent signaling in GB

PTMs are important for cellular homeostasis. However, dysregulated PTM activity promotes malignant transformation of the cells and drives cancer progression. Unbalanced PTM activity is a prevalent phenomenon in GB and they are considered to be important drivers of the disease. Protein phosphorylation is the most common PTM of proteins (Khoury, Baliban, and Floudas 2011). Protein phosphorylation refers to a cellular function where an amino acid residue is phosphorylated by protein kinases by addition of a phosphate group. This results in an alteration in the protein function. This alteration changes the proteins interactions, affinity, and stability, which leads to differentiated role in cellular signaling (V. Singh et al. 2017). However, this reaction can be reversed by PPases which are responsible for removal of phosphate groups from phosphorylated amino acid residues of their substrate molecules. Therefore, they are considered to be negative regulators of phosphorylation signaling. PPase can be classified into classical or atypical and division is done based on the amino acid residues dephosphorylated. They are further classified into serine/threonine phosphatases, tyrosine phosphatases, and dual-specificity phosphatases (Alonso et al. 2004; Y. Shi 2009; Sadatomi et al. 2013). In cancer, common consensus is that PPase suppress oncogenic activity by dephosphorylation of tumor promoting signaling molecules, such as MYC and AKT,

and therefore act as tumor suppressors. However, some PPase can also act as oncogenes. As an example, a PPase SHP-2 binds to Ras and dephosphorylates it, which increases its activity towards Raf and therefore activates the downstream oncogenic proliferation by Ras/ERK/MAPK signaling Kinase signaling pathways in GB (Bunda et al. 2015).

2.3.1.1 The PI3K/AKT/mTOR pathway

The PI3K-Akt-mTOR pathway is one of the most altered molecular pathways in GB, especially in IDH-wildtype. The pathway is responsible for various cellular functions, such as metabolism, proliferation, cell survival and angiogenesis, and its hyperactivity will often lead to cancer. As discussed in the molecular alterations chapter, the pathway is dysregulated almost at every level. The activating kinases such as EGFR, PI3K, AKT or mTOR are often hyperactivated and the negative regulators such as dual-specificity PPase or PTEN are suppressed. However, translating this knowledge for clinical benefit has been troublesome in GB (Le Rhun et al. 2019).

Activation of the pathway begins through PI3-kinase family, which are classified in to three classes varying in their substrate specificity. Class I is considered being the most prominent in tumorigenesis (Engelman, Luo, and Cantley 2006). In short, PI3K is activated through phosphorylation by an extracellular receptors with intracellular tyrosine kinase activity, such as EGFR. After PI3K activation it induces conversion of phosphatidy-linositol-3, 4-bisphosphate (PIP2) into secondary messenger phosphatidylinositol-3, 4, 5-bisphosphate (PIP3) which in turn recruits Akt to inner membrane. However, this can be reversed by a negative regulator of the pathway, PTEN, by dephosphorylating PIP3 back to PIP2 (N. Jiang et al. 2020). After the recruitment to the cell membrane, Akt gets further phosphorylated by protein 3-phosphoinositide-dependent protein kinase-1 (PDPK1) at Thr308 and at Ser473 by mammalian Target of Rapamycin (mTOR) Complex 2 (mTORC2) (Alessi et al. 1996; Sarbassov et al. 2005). After its activation Akt is responsible for regulation of multiple downstream molecules, such as mTOR, p53, CSK3β and antiapoptotic factors (N. Jiang et al. 2020). In the PI3K-Akt-mTOR pathway mTOR acts as both upstream regulator, as mentioned, and as a downstream effector (Guertin and Sabatini 2007). mTOR is responsible for phosphorylation of ribosomal protein S6 kinase, eukaryotic initiation factor 4E and eukaryotic initiation factor binding protein 1, which further activates protein translation, ribosome biogenesis as well as cell growth (Guertin and Sabatini 2007).

As PI3K/Akt/mTOR pathway is one of the most dysregulated pathway in GB, there has been great deal of drug development targeted to the pathway's multiple kinases and phosphatases. As stated, EGFR is altered in approximately 50% of

GBs (Verhaak et al. 2010). This makes EGFR a logical target for GB therapy. Although preclinical studies have shown multiple promising results targeting EGFR, their translation to patients has not been successful (Lassman et al. 2005; Hegi et al. 2011). Traditional small molecule EGFR inhibitors, Erlotinib and Gefitinib have failed in clinical trials as monotherapy (Hegi et al. 2011; Van Den Bent et al. 2009). Furthermore, Erlotinib did not show additional efficacy when combined with TMZ when compared to only TMZ treated patients (Peereboom et al. 2010). However, when recurrent GB patients were selected based on EGFRvIII expression and treated with combination of rindopepimut (EGFRvIII inhibitor) and bevacizumab (VEGF inhibitor) significant increase survival was observed. Nevertheless, the combination failed to show efficacy in newly diagnosed GB (Weller et al. 2017).

PI3K inhibitors are divided into pan-PI3K (targeting all four isoforms of PI3K), isoform-selective (targeting single isoform of PI3K) and dual inhibitors (for example dual PI3K/mTOR inhibitors) (N. Jiang et al. 2020). First generation pan-PI3K inhibitors showed anti-cancer properties *in vivo* and *in vitro* (Vlahos et al. 1994; Guerreiro et al. 2008). Nevertheless, the drugs had high toxicity and poor selectivity and therefore clinical development was halted. Out of the 50 new PI3K inhibitors Buparlisib has shown better stability, selectivity, and lower side effects than others (Rodon et al. 2014). Buparlisib shows pro-apoptotic and anti-proliferative activity *in vitro*, *in vivo* and in cancer patients with PI3K activating mutations. However, Buparlisib had minimal effect in recurrent GB patients with PI3K mutations (Wen et al. 2019). Buparlisib is currently undergoing clinical trials for recurrent GB in combination with radiation (NCT01473901) or bevacizumab (NCT01349660).

Akt isoforms (Akt1, Akt2, Akt3) have variable roles in GB as Akt2 and Akt3 are important for the disease progression while Akt1 does not plays much more minor role. Additionally, Akt3 promotes resistance towards temozolomide as it activates DNA repair pathways (Turner et al. 2015). Inhibitors of Akt can be divided into lipid-based phosphatidyl-inositol analogues, allosteric inhibitors, and ATP-competitive inhibitors. ATP-competitive inhibitors of Akt, such as GDC-0068 and AZD5363, have been shown to have inhibitory effects towards Akt mutated breast cancer cells lines (Brown and Banerji 2017). An allosteric inhibitor of Akt, MK-2206, have been shown to effectively inhibit the expression of Akt and reduce the migration of GB cells (Djuzenova et al. 2019). However, as a monotherapy MK-2206 was not able to reduce the growth on GB cells significantly mainly through alternative activation of mTOR (Djuzenova et al. 2019). In combination with chemotherapeutics or EGFR inhibitors, MK-2206 increases their efficacy in various cancers (Hirai et al. 2010; Holland et al. 2015). In early clinical studies, MK-2206 has been shown to be well tolerated (Yap et al. 2011). However, there are no current

clinical trials with MK-2206 on going for gliomas. Another PI3K and Akt inhibitor perifosine showed promise *in vivo* by reducing Akt phosphorylation and inducing tumor necrosis (Pitter et al. 2011). Later it entered phase II clinical studies for recurrent GB. It was found to be well tolerated nevertheless it was ineffective as monotherapy (Kaley et al. 2019).

Most known mTORC1 inhibitor is rapamycin and its analogues. Rapamycin alters the conformation of mTORC1 and thereby inactivates it which has been shown to work both *in vitro* and *in vivo* (Mecca et al. 2018). However, Rapamycin monotherapy is riddled with resistance as there is often feedback loop through activation of mTORC2 leading to hyperactivation of Akt (Wan et al. 2007). In GB Rapamycin has shown efficacy in PTEN-deficient GB patients (Tim F. Cloughesy et al. 2008). Combination therapies with Rapamycin analogues, such as sirolimus or temsirolimus, with EGFR inhibitors erlotinib have been tested in clinical trials but with ineffective results (Reardon et al. 2010; Wen et al. 2014).

The tumor suppressor PTEN is often inactivated in GB which causes uncontrollable activity of the Akt pathway. Therefore, its activation seems desirable. However, PTEN has been considered to be undruggable in the past. In addition to the mutated forms of PTEN, it is also controlled by PTMs which affect its dimerization, cellular location, and activity (Y. R. Lee, Chen, and Pandolfi 2018). Two potential inhibitors of PTEN have been identified, E3 ubiquitin ligases WWP1 and NEDD4. The inhibitors have been shown co-operate in the regulation PTEN activity (Xinjiang Wang et al. 2007; Y. R. Lee et al. 2019). A natural indolecarbinol compound, Indole-3-carbinol (I3C), has been shown to decrease tumor growth. In clinical trial it showed promise in breast cancer patients with no toxicities (Reed et al. 2005). I3C interacts with both NEDD4 and WWP1 (Y. R. Lee et al. 2019; Aronchik et al. 2014). Inactivation of WWP1 with I3C leads to stabilization of PTEN dimer and restores its ability to transform PIP3 back to PIP2 (Y. R. Lee et al. 2019). I3C inhibits the NEDD4 mediated proteasomal degradation of PTEN in melanoma cells. In wild-type PTEN melanoma cells I3C induces apoptosis, however in mutant PTEN melanoma cells it does not. I3C has been shown to reduce tumor growth in a xenograft model (Aronchik et al. 2014).

2.3.1.2 Pyruvate dehydrogenase kinase

PDK is a key component in regulating the production of acetyl-coenzyme A (Acetyl-CoA) from pyruvate in mitochondria. PDKs can be divided into four different forms PDK 1-4, which have different substrates and tissue specificity. PDK regulates the first part of the pyruvate dehydrogenase complex (PDC), E1, also known as pyruvate dehydrogenase (PDH). Other catalytic components of PDC are dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3). The PDK inactivates

the PDH by phosphorylating it. As PDC regulates the glucose metabolism by generating Acetyl-CoA from pyruvate required for tricarboxylic acid cycle to produce NADH to further generate ATP through oxidative phosphorylation in the mitochondria. Thus, as PDK can inhibit PDH, it can be considered to be a master regulator of glucose metabolism and mitochondrial energy production. The activation of PDK leads to increase in aerobic glycolysis also known as the Warburg effect. This results as PDK phosphorylates and inactivates PDH and shifts pyruvate from mitochondria towards the lactate dehydrogenase in the cytosol to produce lactate through lactic acid fermentation. Lactic acid fermentation produces NAD+ which is required for sustained glycolysis. This phenomenon happens even in the abundance of oxygen in gliomas. The increased glycolysis and lactate production have been shown to contribute towards tumor growth and therapy resistance. Furthermore, the decreased entry of pyruvate into mitochondria limits the mitochondrial activity leading to reduced apoptosis and further contributes towards tumorigenesis. Additionally, increased lactic acid fermentation results in acidosis which increases the breakdown of extra-cellular matrix, and therefore promoting tumor expansion and metastasis (Gatenby and Gillies 2004).

In cancer, PDK activity is regulated in multiple ways. Most commonly PDK is upregulated by hypoxia-inducible factor 1α (HIF) which is hyperactive during hypoxic conditions which are often present in cancer. Additionally, HIF upregulates LDH to increase lactate production (Kim et al. 2006). Akt activates PDK indirectly by for example activating HIF, but there is also evidence that Akt phosphorylates PDK directly in the mitochondria. However, the mechanism for this is not specifically known (Hoxhaj and Manning 2020; Chae et al. 2016). Another Akt target the tumor suppressor P53 has been shown to downregulate PKD activity (Contractor and Harris 2012). In IDH1 mutant GB, PDK is often overexpressed (S. Zhao et al. 2009). Dichloroacetate (DCA), a PDK inhibitor, is a pyruvate mimetic compound which induces mitochondrial function by inhibiting PDK and therefore activating PDH. This effectively shifts the metabolism of cancer cells towards oxidative phosphorylation and simultaneously increasing mitochondrial activity (Stacpoole, Nagaraja, and Hutson 2003). DCA has been shown to effectively induce apoptosis in many cancer cells, including GB, through change in mitochondrial membrane potential and by activating potassium channels (Bonnet et al. 2007; Michelakis et al. 2010).

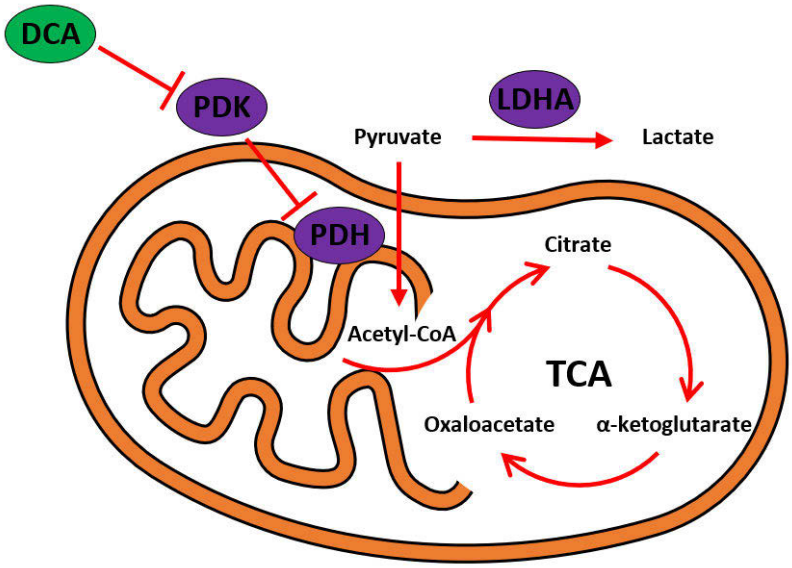


Figure 2. Role of PDK in mitochondrial energy metabolism. Hyperactivated PDK decreases transformation of pyruvate into acetyl-CoA which in turn hinders TCA.

2.3.2 Protein Phosphatase 2A

PP2A is one of the most common serine/threonine phosphatase. PP2A is a master regulator of multiple cellular signaling pathways, such as receptor tyrosine kinase signaling, by dephosphorylating multiple different substrates. Its dysregulation is evident in multiple pathologies such as cardiovascular disorder, diabetes, neurodegenerative disorders, and cancer (Eichhorn, Creyghton, and Bernards 2009).

2.3.2.1 PP2A Structure and subtypes

PP2A complexes are formed by three functional subunits: a scaffolding/structural subunit (PR65) which together with catalytic subunit forms the core dimer. This core complex binds to broad variety of different regulatory B-subunits, which determine the catalytic activity, substrate specificity and physiological function of the PP2A holoenzyme (Eichhorn, Creyghton, and Bernards 2009). The structural subunit is encoded by two different genes, PPP2R1A and PPP2R1B, which have 87% identical sequence (Hemmings et al. 1990). Isoform encoded by PPP2R1A is much more abundant in adult tissues, as PPP2R1B accounts only for 10% of the PP2A-A isoforms. The structural subunits are built from 15 Huntington/elongation/A-subunit/TOR (HEAT) repeats, which determine the binding of the catalytic and regulatory subunits. Interaction between the catalytic subunit and structural subunit happens at four C-terminal (11-15) HEAT repeats, and regulatory subunits bind to

repeats 1-10. (Groves et al. 1999). The catalytic subunits are encoded by two genes PPP2CA and the PPP2CB (Stone, Hofsteenge, and Hemmings 1987). PPP2CA is expressed 10-fold more than PPP2CB due to its more potent promoter region even though they share 97% sequence identity (Khew-Goodall and Hemmings 1988). Active site of the catalytic subunit has two manganese atoms which are responsible for phosphate binding and the hydrolysis of phosphate esters. After the formation of structural and catalytic "AC" core holoenzyme, the structural protein forms a horseshoe like shape structure. This formational change allows the catalytic subunit to have free access to the PP2A substrates, which in turn are recruited by regulatory B-subunits (Cho and Xu 2007). As the catalytic subunit is responsible for AC core holoenzyme formation, it is synthesized in inactive state to avoid unwanted activity immediately after its translation (Fellner et al. 2003).

The regulatory B subunit has 26 alternative transcripts or splice forms. These are encoded by a total of 15 different genes in the human genome. The B subunits are expressed in a tissue specific manner and are responsible for the localization and substrate specificity of the PP2A holoenzyme (Eichhorn, Creyghton, and Bernards 2009). There are four different B-subunit families which share no sequence similarity expect few conserved amino acids which are responsible for the interaction with the N-terminal HEAT domains of the structural scaffold subunit (X. Li and Virshup 2002). The first B-subunits characterized were B55-family (Mayer et al. 1991) which are encoded by four different genes PPP2R2A, PPP2R2B, PPP2R2C, and PPP2R2D. Some of B55-family proteins can be alternatively spliced resulting in variable isoforms. Isoforms are divided into α, β, γ and δ. B55α and B55δ isoforms are ubiquitously expressed, despite their different cellular localization (Strack et al. 1999). Whereas, B55β, and B55γ are highly enriched in brain (Zolnierowicz et al. 1994). The B55-family is involved in the regulation of multiple signaling pathways, such as mitosis (Wurzenberger and Gerlich 2011), apoptosis (Janssens and Rebollo 2012), DNA-damage signaling (Kalev et al. 2012), tau-protein dephosphorylation (Torrent and Ferrer 2012), extracellular signal-regulated kinases (ERK) signaling pathway (Eichhorn, Creyghton, and Bernards 2009) and transforming growth factor beta signaling pathway (Batut et al. 2008). The B56-family are encoded by five different genes PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, and PPP2R5E. These result in isoforms α, β, γ, δ and ε, respectively. As B55-family, B56-family can be also alternatively spliced which gives differently translated transcripts. B56-family is widely expressed throughout tissues (Eichhorn, Creyghton, and Bernards 2009). However, B56β and B56δ have higher expression in brain and B56α and B56γ are more abundant in heart. The B56-family is involved in mitosis (Wurzenberger and Gerlich 2011), apoptosis (Janssens and Rebollo 2012), dopaminergic signaling (Walaas et al. 2011), Akt, Wnt, and c-Myc signaling (Eichhorn, Creyghton, and

Bernards 2009). Therefore, they are considered to be the main tumor suppressive PP2A subunits (Westermarck and Hahn 2008).

Third B-subunit family, PR72, are encoded by three different genes PPP2R3A, PPP2R3B, and PPP2R3C. PPP2R3A gives rise to two alternative splicing variants PR130 and PR72, and PPP2R3B gives rise to two alternative splicing variants PR48 and PR70 which all share the same C-terminal tail. The PR72-family has an important role in noncanonical Wnt signaling (Eichhorn, Creyghton, and Bernards 2009), EGFR signaling (Zwaenepoel et al. 2010) and neuronal signaling (Walaas et al. 2011). Furthermore, the PR70 was found to interact with tumor suppressor RB (Magenta et al. 2008) while the other splice variant of PPP2R3B the PR48 interacts with cdc6 (Z. Yan et al. 2000). Least well-established B-subunit family called striatins is formed by PR110 (striatin), PR93 (SG2NA) and zinedin, which are encoded by three genes STRN, STRN3 and STRN4. SG2NA is ubiquitously expressed but striatin and zinedin are mainly expressed in brain (Benoist, Gaillard, and Castets 2006). The striatin family proteins are calmodulin-binding proteins which have a role in Hippo signaling pathway (Ribeiro et al. 2010), Golgi polarization (Kean et al. 2011) and cytokinesis (Hyodo et al. 2012). Furthermore, PP2A-STRN3/4 complexes have been shown to have oncogenic properties. As PP2A-STRN3/4 inactivates the hippo signaling pathway through MST1/2 dephosphorylation, it results in YAP activation which is a transcriptional regulator and a known oncogene (Tang et al. 2020).

In total this predicts for over 90 different combinations of the PP2A which are responsible for PP2A localization, activity, and substrate specificity. This repertoire of forms shows that PP2A is a master regulator of cellular activity through controlling of phosphorylation.

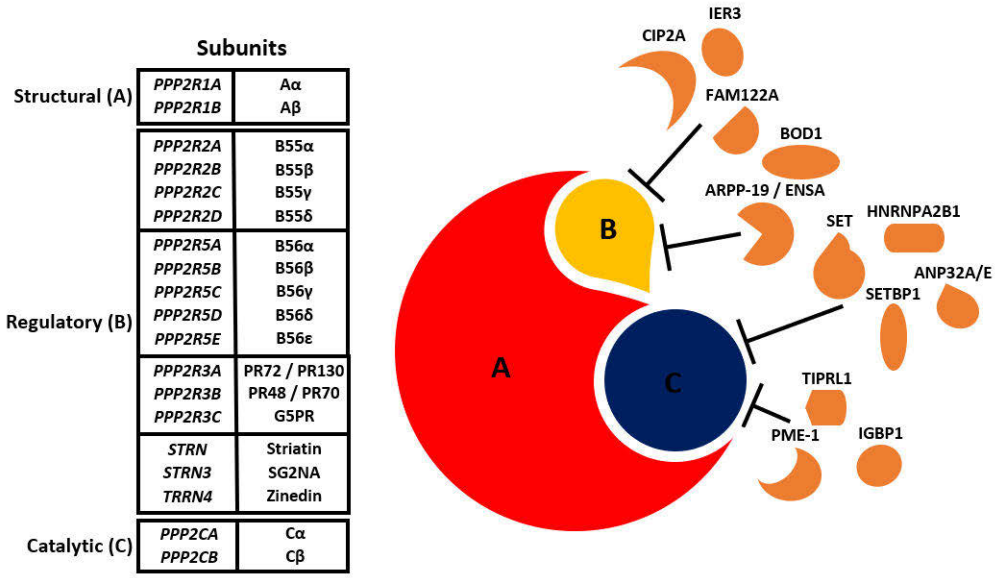


Figure 3. PP2A subunits and its endogenous inhibitors which control the holoenzyme composition and activity thus regulate PP2As substrate specificity.

2.3.2.2 PP2A in cancer

PP2A holoenzyme function is regulated through multiple factors including PTMs, endogenous inhibitors and viral proteins which affect the stability of the holoenzyme. For example, phosphorylation of the regulatory B subunits affects the substrate specificity of PP2A and sub-cellular localization by influencing their ability to bind to the catalytic core of the PP2A protein. Therefore, having proper PP2A activity within cells is important to ensure normal cellular function. Variations in activity can promote tumorigenesis, because PP2A functions as an inhibitory protein on several growth promoting signaling pathways (Janssens and Rebollo 2012; Westermarck and Hahn 2008). PP2As involvement in tumorigenesis was first found after transformation of cells with okadaic acid, which is an inhibitor of PP2A (Pallas et al. 1990; Fujiki, Sueoka, and Suganuma 2013). Furthermore, PP2A was found to be target of multiple DNA tumor virus proteins, such as simian virus 40 small-t, adenovirus E4orf4 and polyomavirus small/middle tumor antigens (Pallas et al. 1990; Shtrichman et al. 1999; Walter et al. 1990). The viral proteins affect the B-subunits by removing them from the holoenzyme and therefore changing the substrate specificity of PP2A (Fujiki, Sueoka, and Suganuma 2013). Different PP2A subunits have been linked to tumorigenesis. As PP2A-Aβ mediates cell differentiation through AKT and inactivates transformative function of RAS GTPase RaIA, disruption in PP2A-Aβ activity can lead to tumorigenesis, as RAS and AKT

hyperactivity can lead to cancer (Sablina et al. 2007; Hwang et al. 2013; Shirakawa and Horiuchi 2015). In addition, inhibition of PP2A through specific PP2A B-subunits B56α, B56γ and PR72/PR130 contribute to tumorigenesis by activation of PI3K/AKT, Myc and WNT pathways (Sablina et al. 2010).

2.3.2.2.1 PP2A subunits and cancer

Multiple mutations in PP2A subunits have been found in cancer (Sangodkar et al. 2016). The most commonly mutated subunit is the structural subunit PPP2R1A gene, which mutations has identified mutations in breast, lung, melanoma, ovarian, endometrial, uterine and colon cancers (Ruediger, Pham, and Walter 2001a; Calin et al. 2000; McConechy et al. 2011; Shih and Wang 2011). Most point mutations in Aα subunit arise within HEAT repeat 5, which disrupts the regulatory subunit binding (Ruediger, Pham, and Walter 2001b). Point mutations E64D and E64G in PPP2R1A remove its ability to bind B56 subunits (Ruediger, Ruiz, and Walter 2011). In glioma, reduced expression of PPP2R1A has been reported (Colella et al. 2001). Another isoform Aβ encoded by PPP2R1B is mutated in multiple cancers such as breast, lung, colon, melanoma, ovarian, cervical, HCC, NHL, CLL and B-CLL. Mutations in PPP2R1B are mainly missense mutations: G8R, P65S, G90D, L101P, K343E, D504G, V545A, V448A, a double mutant L101P/V448A or an in-frame deletion DE344–E388 (Ruediger, Pham, and Walter 2001a; Sangodkar et al. 2016; Sablina et al. 2007). Additionally, aberrant RNA splicing of Aβ can cause ineffective transcripts of PPP2R1B (Kalla et al. 2007). As Aα abnormalities, Aβ changes also cause ineffective binding to B- and C subunits.

Regulatory subunits also harbor mutations but in much lower frequency than A-subunits. In lung cancer, PPP2R5C often carries F395C mutation which causes aberrant interaction between the B56γ and p53 (Shouse, Nobumori, and Liu 2010). Gene encoding B55α (PPP2R2A) was deleted in 67.1% of cases in prostate cancer, however only 2.1% of the cases had a homozygous deletion (Y. Cheng et al. 2011) It has also been reported to be deleted in breast cancer and myeloma (Curtis et al. 2012; Mosca et al. 2013). Regulatory B subunits are more often altered through abnormal expression than mutations. In acute myeloid leukemia (AML), B55α expression was decreased in a 231-patient cohort and inhibition of B55α was highly associated with AKT phosphorylation (P. P. Ruvolo et al. 2011). Additionally, decreased expression of PPP2R5A and PPP2R5C has been reported in metastatic melanoma patients (Deichmann et al. 2002). Furthermore, regulatory subunits are often regulated through epigenetic alterations. For example in colorectal cancer, ductal carcinoma in situ and breast cancer, PPP2R2B is inactivated through methylation which has caused increased MYC signaling (Muggerud et al. 2010). Catalytic C subunit is rarely mutated. Some mutations in PPP2CA occur in prostate

cancer and AML. Additionally, in androgen insensitive prostate cancer cell lines PPP2ACA is downregulated when compared to androgen sensitive cell lines (Bhardwaj et al. 2011).

Binding of regulatory B-subunits to the core holoenzyme is regulated by methylation and phosphorylation of the C-terminal tail of the catalytic subunit. For the binding of B-subunits to AC holoenzyme, a methylation of carboxyl group Leu_{309} in catalytic subunit by leucine carboxyl methyltransferase 1 (LCMT1) is required (Longin et al. 2007). This connection can be disrupted by phosphatase methylesterase (PME-1) by demethylation of the C-terminal tail (Ogris et al. 1999). Demethylation by PME-1 is often linked to cancer progression, because methylation by LCMT1 increases holoenzyme stability (Eichhorn, Creyghton, and Bernards 2009). Phosphorylation of multiple C-terminal tail residues of C-subunit also modulates the interaction between the AC holoenzyme and B-subunits. Phosphorylation of Y307 and T304 inhibits binding of B-subunits, especially PR55, to the holoenzyme. Furthermore, phosphorylation of Y307 blocks the methylation of L309 by LCMT-1, and therefore additionally reducing the holoenzyme stability (Longin et al. 2007).

2.3.2.2.2 Endogenous inhibitors of PP2A

PP2A has multiple endogenous inhibitors which limit its tumor suppressive activity. One such is PME-1, which role in PP2A suppression was already discussed as it demethylates the catalytic subunits C-terminal Leu-309 residue, the methylation of which is required for PP2A activation. In addition, demethylation by PME-1 stabilizes PP2A in an inactive form by removing Mn^{2+} ions from the catalytic subunit which is required for its activity (Xing et al. 2008). This inactive forms accounts for approximately 25 % of total cellular PP2A and can be reactivated by ATP/Mg2+-dependent phosphatase two a phosphatase activator (Longin et al. 2004). PME-1 activity is increased in approximately 50% of glioma tumor samples. PME-1 overexpression correlates well with MEK/ERK pathway activation and Ki67 proliferation marker activity in glioma. Furthermore, there's correlation between PME-1 activity and glioma tumor grade in patients (Puustinen et al. 2009).

Another highly expressed endogenous inhibitor of PP2A is CIP2A. CIP2A inhibits PP2A by binding to B56-family proteins and blocks them from the holoenzyme (Junttila et al. 2007). Inhibition of PP2A through CIP2A results in activation and stabilization of c-MYC and activation of AKT, which leads to cancer cell proliferation. Furthermore, CIP2A stabilizes other survival and growth inducing proteins such as E2F1, mTOR and DAPK through PP2A inactivation which promote inhibition of autophagy, senescence and apoptosis pathways (Laine et al. 2013; Puustinen et al. 2014; Guenebeaud et al. 2010). CIP2A is encoded by gene

KIAA1524, which is overexpressed in many malignancies such as gastric, lung, colon, esophageal, tongue, breast, prostate, ovary, head and neck squamous cell carcinoma, astrocytic gliomas, and leukemia (Haesen et al. 2012; Khanna, Pimanda, and Westermarck 2013).

ARPP and ENSA are part of cAMP-regulated phosphoprotein family. ARPP further has two splice variants: ARPP-16 and -19 (Haesen et al. 2012). ARPP and ENSA are considered to be mitotic PP2A inhibitors, as their binding to B55α/δ increases PP2As activity towards CDK1 and promotes mitotic entry (Gharbi-Ayachi et al. 2010; Mochida et al. 2010). Activation of ARPP and ENSA requires phosphorylation of certain serine residues. This phosphorylation is done by greatwall kinase (Gharbi-Ayachi et al. 2010). ARPP-19 is overexpressed in hepatocellular carcinoma (HCC) and it was shown to inhibit PP2A regulated cell cycle arrest and promote tumor cell proliferation (H. Song et al. 2014).

SET is one of the most overexpressed endogenous inhibitors of PP2A in cancers. SET inhibits PP2A complex by binding to the PP2A catalytic subunit (M. Li, Makkinje, and Damuni 1996; Arnaud et al. 2011). Furthermore, SET has been shown to interact with PP2A complexes with a regulatory subunit B56 in gastric cancer cells (Enjoji et al. 2018). High activity of SET has been shown in multiple hematological malignancies, such as AML, chronic myelogenous leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia (CLL) (Haesen et al. 2012; Westermarck and Hahn 2008), and in colorectal, breast and lung cancer (H. Liu et al. 2015; Cristóbal et al. 2015; C. Y. Liu et al. 2019), which is often caused by overexpression.

In addition to SET, acidic leucine-rich nuclear phosphoprotein-32A (ANP32a) also inhibits PP2A by direct binding to catalytic subunit when its non-phosphorylated (Reilly et al. 2014). Phosphorylation of ANP32a causes it to be removed from PP2A. Increased expression of ANP32A has been shown in oral squamous cell carcinoma (Velmurugan et al. 2016), HCC (C. Li et al. 2012), colorectal cancer (H. Shi et al. 2011), pancreatic tumor (Williams et al. 2010) and hepatocellular cancer (C. Li et al. 2012). Counter intuitively ANP32a also has some tumor suppressive and pro-apoptotic activity separate from PP2A (Haesen et al. 2012). Another ANP32 family member ANP32e has been shown to regulate PP2A activity during synaptogenesis in brain tissue. For PP2A-ANP32e interaction, phosphorylation of ANP32e is required (Costanzo et al. 2006). Two other endogenous PP2A inhibitors related to SET are SET-binding protein (SETBP1) and HNRNPA2B1. For SETBP1 to inhibit PP2A it requires binding to SET (Cristóbal et al. 2010) and HNRNPA2B1 PP2A inhibition is enhanced when binding to SET (Vera et al. 2006). SETBP1 protects SET from protease cleavage which increases the amount of full-length SET protein. This interaction between SETBP1 and SET does not affect ability of SET to bind to PP2A (Cristóbal et al. 2010). SETBP1 is

overexpressed in AML (Van Waalwijk Van Doorn-Khosrovani et al. 2005) and mutated in malignant lymphoma, acute leukemia, pancreatic carcinoma, and colorectal cancer (Minakuchi et al. 2001).

Other endogenous inhibitors of PP2A: 1) TIPRL1, also known as TIP, is a ubiquitously expressed inhibitor of PP2A which binds to C-subunit and inhibits PP2A activity. TIPRL1 shifts PP2A activity towards ATM/ATR regulated DNA damage response pathway (McConnell et al. 2007). TIPRL1 is overexpressed in HCC (I. S. Song et al. 2012). 2) BOD1 and 3) IER3 interact with B56-family and have a role in mitosis and ERK activation, respectively (Porter et al. 2013; Letourneux, Rocher, and Porteu 2006). 4) FAM122A interacts with Aα and B55α to inhibit PP2A (L. Fan et al. 2016). 5) PPP1R17 also known as G-substrate has been shown to inhibit PP2A activity (Chee et al. 2007). 6) Immunoglobulin-binding protein 1 (IGBP1) interacts with catalytic subunit of PP2A which alters PP2As activity and substrate specificity by displacing the catalytic subunit from the holoenzyme (Grech et al. 2016). Deletion of IGBP1 causes apoptosis as a results of PP2A activity increase. Furthermore, it has been shown that IGBP1 binding to catalytic subunit of PP2A inhibit its activation by blocking methylation (Grech et al. 2016).

In summary, given the critical role the endogenous inhibitors play in determining the substrate specificity of the PP2A complex it is important to understand their functionality and role in PP2A activity.

2.3.2.2.3 PP2A in brain tumors

One of the hallmarks of GB are its dysregulated phosphorylation pathways (Dunn et al. 2012). However, inhibition of kinase pathways has not led to any clinical therapies in the recent years (Tomiyama et al. 2019). Recently, the critical role of PP2A in GB has been identified in regulation of various oncogenic signaling pathways (Tomiyama et al. 2019; Kaur et al. 2016) as its downregulation can induce cellular proliferation, formation of resistance against drug or irradiation, or impairment of tumor immunity in cancer (Kauko et al. 2018; Kaur et al. 2016; Allen-Petersen et al. 2019; Peter P. Ruvolo 2016). Furthermore, downregulation of PP2A expression has been observed in glioma tissues in multiple data sets (Colella et al. 2001; Y. L. Fan et al. 2013). However, this does not often happen through genetic alterations in the subunit-encoding genes of PP2A in GB (Brennan et al. 2013; Kaur et al. 2016). Instead, in GB PP2A is often inhibited by endogenous inhibitor proteins of PP2A, such as PME-1, CIP2A and ARPP19 (Kaur et al. 2016; Qin et al. 2018; T. Jiang et al. 2016). Inhibition of these PP2A inhibitors results in suppressed growth of GB and induces cell death alone or when combined with kinase inhibition (Kaur et al. 2016; Qin et al. 2018; T. Jiang et al. 2016). PME-1 has been shown to mediate

widespread multikinase inhibitor resistance in GB. The PME-1 mediated resistance was associated with high cytoplasmic HDAC4 activity which was decreased after PME-1 inhibition. The synthetic lethality induced by PME-1 or HDAC4 inhibition together with multikinase inhibitors was dependent on proapoptotic protein BAD (Kaur et al. 2016). PME-1 is overexpressed in 50% of astrocytic gliomas and its levels correlate with the disease progression (Puustinen et al. 2009). In GB cells lines, CIP2A protein levels have been reported to be overexpressed (Merisaari et al. 2020; Qin et al. 2018). *In vitro* CIP2A promotes proliferation and invasiveness of GB cells. Inhibition of CIP2A in GB cell lines leads to induced apoptosis, decreased invasion and proliferation, and downregulation of PP2A downstream signaling such as Akt (Qin et al. 2018). Furthermore, CIP2A expression correlates with worse survival of GB patients (Qin et al. 2018). As PP2A activity is modulated by inhibitor proteins and not genetically, it is a suitable target for therapy as discussed later (Westermarck 2018).

2.3.2.3 Therapeutics targeting PP2A

Molecules directly activating PP2A have been under heavy investigation after the discovery of PP2A activating role of antipsychotic tricyclic compound family called phenothiazines (PPZ) (Gutierrez et al. 2014). These compounds also display anti-tumoral effects (Gutierrez et al. 2014). Nevertheless, as these compounds are antipsychotics, they exhibit extrapyramidal and anti-cholinergic effects through dopaminergic antagonism, which limits their potential as cancer therapy (Sudeshna and Parimal 2010). However, there are reengineered derivates of these PPZ where the dopaminergic activity has been removed and the anti-proliferative properties are kept (Kastrinsky et al. 2015; Sangodkar et al. 2017).

A series of Small Molecule Activators of PP2A (SMAPs) have been developed from PPZ derivatives. Out of SMAPs, the most characterized is the compound DT-061 (Sangodkar et al. 2017). It has been shown that DT-061 interacts with PP2A holoenzymes B56α (Leonard et al. 2020). DT-061 increased the number of PP2A/B56α complexes by interacting with all three subunits. PP2A/B56α was further stabilized when the holoenzyme contained methylated C-subunit (Leonard et al. 2020). DT-061 also promotes the interaction with the other tumor suppressive B subunit PP2A-B55α (Morita et al. 2020). DT-061 has also been shown to activate PP2A without any B-subunits by only binding to the A-C complex (Sangodkar et al. 2017). DT-061 and other SMAPs have been shown to induce apoptosis and decrease phosphorylation of PP2A targets such as ERK. Furthermore, they have high antitumoral activity in multiple *in vivo* xenograft models (Sangodkar et al. 2017; Farrington et al. 2020; Kauko et al. 2018). Simultaneously with SMAP development, there has been development of an alternative PPZ derivatives called iHAPs

(improved heterocyclic PP2A activators) (Morita et al. 2020). iHAPs have been shown to induce the formation of the PP2A holoenzyme containing the tumor suppressive B56ε subunit (Morita et al. 2020). Additionally, iHAPs do not require a methylated PP2A C-subunit for the holoenzyme assembly (Morita et al. 2020). iHAPs have been shown to significantly reduces tumor growth in acute lymphocytic leukemia (ALL) xenograft models (Morita et al. 2020). Both of these PPZ derivatives do not show any toxic effects even with high doses (Sangodkar et al. 2017; Leonard et al. 2020; Kauko et al. 2018; Morita et al. 2020).

Alternative way of PP2A activation is to inhibit some of its endogenous inhibitors, such as SET, PME-1 or CIP2A. Sphingolipids, such as ceramide and its derivatives, have been shown to disrupt the PP2A/SET interaction with antitumoral effects (Perry et al. 2012). An FDA approved small molecule drug FTY720 (Fingollimod) is an analogue of sphingosine. FTY720 prevents SET dimerization, which is required for PP2A binding, by binding to the N-terminals residues of SET. FTY720 can also bind to the globular region of SET. This causes SET to be released from the PP2A-C-subunit due to conformational change (De Palma et al. 2019). FTY720 has been shown to decrease activity of multiple PP2A dependent pathways, such as AKT and ERK (Neviani et al. 2007). Furthermore, it has been shown to be effective in multiple *in vivo* cancer models, such as colon cancer, lung cancer, breast cancer leukemia, HCC and prostate cancer (Saddoughi et al. 2013; Neviani et al. 2013, 2007; L. Chen et al. 2014; Jie Liang et al. 2013). Two potent derivates of FTY720 have also been developed called C11 and CM-1231. Both are highly effective in disrupting the PP2A-SET complex and are non-phosphorylatable which reduces their undesirable anti-inflammatory effects (Pagano et al. 2019; Vicente et al. 2020).

Some compounds have also been reported to inhibit endogenous PP2A inhibitor CIP2A. An FDA approved proteasome inhibitor, Bortezomib, which is used to treat multiple myeloma, have been shown to mediate PP2A (Niesvizky et al. 2015; K. F. Chen et al. 2010). Independent of PP2A, Bortezomib induces cell cycle arrest and apoptosis through inhibition the 26S subunit of the proteasome (Adams and Kauffman 2004). However, Bortezomib derivatives kept their antitumoral effect even when proteasomal inhibition capabilities were removed (Hou et al. 2013). It is suggested that this antitumoral effect is enhanced through CIP2A, as Bortezomib reduced CIP2A levels in multiple cancers (K. F. Chen et al. 2010; Lin et al. 2012). Another known drug, Erlotinib, which is an EGFR kinase inhibitor (Y.-L. Wu et al. 2015), have been shown to effect CIP2A (Kuen Feng Chen et al. 2012). Furthermore, derivatives of Erlotinib which have their EGFR activity removed kept the antitumoral effect. This happened through transcriptional downregulation CIP2A expression (Kuen Feng Chen et al. 2012). Other natural compounds, such as Celastrol and cucurbitacin B, have also been shown to inhibit CIP2A (Qin et al.

2018; Z. Liu et al. 2014). Cucurbitacin B have been shown to induce antitumoral effects in preclinical GB models through CIP2A mediated downregulation of AKT pathway (Qin et al. 2018).

Normally, reactivation of PP2A has been considered to be the desirable goal due to its tumor suppressive activity. However, its inhibition has also shown promise as a cancer therapy. Most studied PP2A inhibitor is a small molecule drug LB100 which is a Cantharidin derivative (D. Wei et al. 2013). LB100 has been shown to be effective in combination with chemo- or radiotherapy in preclinical models of GB and pancreatic cancer (D. Wei et al. 2013; Gordon et al. 2015; Hong et al. 2015). Furthermore, it has been shown to enhance the effect of immune checkpoint inhibitors in preclinical models of colon cancer and melanoma (Ho et al. 2018). In clinical trials (Phase I) LB100 has been shown to have combinatory effect with Docetaxel in solid tumors (Chung et al. 2017). Furthermore, currently LB100 is in phase II clinical trial for treatment of GB and astrocytoma.

2.3.2.4 Combination therapies with PP2A

Even though PP2A reactivation alone has shown promise in early studies (Kastrinsky et al. 2015; Sangodkar et al. 2017), there is also evidence that it might be a potential target to reduce resistance for other compounds such as kinase inhibitors (Kaur et al. 2016; Kauko et al. 2018; Allen-Petersen et al. 2019).

In primary AML cell lines, combination of SET inhibitor (OP449) and kinase inhibitors resulted in synergistic increase in cell death *in vitro*. Specifically, synergistic effect was achieved in AML cell lines CMK (JAK3A527V driven) and MOLM-14 (FLT3-ITD driven), which were targeted with either JAK or FLT3 inhibitors respectively in combination with PP2A reactivation by OP449 (Agarwal et al. 2014). Similar results in FLT3-ITD-driven AML cell lines have been received with another SET inhibitor (FTY720) in combination with a FLT3 inhibitor PKC412 (Midostaurin) (Smith et al. 2016). However, in addition to inhibiting FLT3 midostaurin has been shown to inhibit multiple other kinases (Gallogly, Lazarus, and Cooper 2017). Furthermore, in CLL cell lines, where PP2A-C subunit is hyperphosphorylated by LYN (a SRC family kinase), there is strong combinatory effect in induction of apoptosis and dephosphorylation of oncogenic kinases, such as AKT, when combining SRC family kinase inhibitors (dasatinib) and PP2A reactivating agent (FTY720) (Zonta et al. 2015).

PP2As inhibition has also been shown to be responsible for resistance to BET bromodomain inhibitor (BBI) JQ1. It has been demonstrated in triple negative breast cancer that by activating PP2A with perphenazine the resistance towards BBIs can be eliminated (Shu et al. 2016). In non-small cell lung carcinoma (NSCLC), there is often hyperactivation of KRAS and overexpression endogenous PP2A inhibitors

SET and CIP2A. In a screen with KRAS driven cell lines PP2A inhibition was combined with 230 different kinase inhibitors to find potential resistance mechanisms when PP2A activity is downregulated. PP2A inhibition increased resistance to inhibitors in the MAPK pathway (MEK and ERK inhibitors). *In vitro* MEK inhibitor (trametinib) was shown greatly to synergize with small molecule activators of PP2A (SMAPs) and *in vivo* another MEK inhibitor (selumetinib) caused significant tumor regression in combination with SMAPs (Kauko et al. 2018). Furthermore, in pancreatic ductal adenocarcinoma, which is considered to be highly resistant towards kinase inhibitors, combination of SMAPs together with mTOR inhibitor (INK128) achieved synthetic lethality both *in vitro* and *in vivo*. Additionally, the combination suppressed the AKT pathway signaling and reduced expression of c-MYC (Allen-Petersen et al. 2019). Moreover, advanced lung adenocarcinoma patients often developed resistance towards EGFR inhibitors. This is thought to happen through abnormal activation of the PI3K and MAPK oncogenic pathways downstream of EGFR. As these pathways are substrates of PP2A, it has been shown that by combining SMAPs with an EGFR inhibitor (afatinib) in an EGFR-resistant xenograft, tumor growth inhibition was achieved with the combination (Tohmé et al. 2019).

PP2A reactivation has been combined with chemotherapeutics in multiple studies. In breast cancer combination of doxorubicin (DOX) and FTY720 has been shown to have antitumoral effect (Rincón et al. 2015). Similar results have been achieved also in melanoma, hepatoblastoma, colorectal, ovarian and lung cancer cells with a combination of FTY720 with either cisplatin, oxaliplatin or 5-Fluorouracil (Cristóbal et al. 2015; N. Zhang et al. 2013; Ishitsuka et al. 2014; Y. Li et al. 2018; Stafman et al. 2019).

2.4 Nanoparticles

Nanoparticles have gained attention during recent years for their potential to be used for drug delivery, diagnosis, or therapy. Nanoparticles that can act as delivery platforms for various pharmaceutical agents are the most predominant within the nanomedicine research covering approximately 75% of the field (Ragelle et al. 2017). Nanoparticles offer multiple benefits in drug delivery. Nanoparticles can be guided towards area of interest with specific coating, the release speed of compounds can be controlled, and nanoparticles can have improved properties to pass through barriers, such as BBB. With these benefits use of nanoparticles as drug carriers can decrease toxicity, increase drug solubility, change drug metabolism, and modify pharmacokinetic profiles of therapeutics (Manzano and Vallet-Regí 2018). Depending on the intended use, variable materials can be used to produce nanoparticles. Most common ones are inorganic particles, dendrimers, polymeric

micelles, carbon nanotubes, liposomes, silica-based materials (Maleki Dizaj et al. 2015; Chengyuan Zhang et al. 2014; Talelli et al. 2015; H. Wu et al. 2014; R. Mo, Jiang, and Gu 2014; Vallet-Regí et al. 2018). In this thesis I will focus on MSNs.

2.4.1 Mesoporous silica nanoparticles

Mesoporous silica nanoparticles (MSNs) have shown their potential as biocompatible drug delivers in the recent years in many pathologies and specifically in cancer (Jafari et al. 2019). Their unique properties make them efficient for multiple purposes. MSNs are very robust and therefore are mechanically, thermally, and chemically very stable. Because of their porous structure and large surface (1000 m^2/g), they are able to absorb various molecules in high capacities. The pores in MSNs are between 2-50mm, hence being the medium sized silica-nanoparticle, when compared to microporous (<2nm) and macroporous (>50nm) nanoparticles (Jafari et al. 2019). On top of their loading capabilities, MSNs possess good biocompatibility, biodegradability, and little to no toxicities. MSNs protect their loading well and do not prematurely release their contents. Furthermore, their surface is easily modifiable because of the silanol groups, which are present at the surface (Jafari et al. 2019).

MSNs are very tolerable. *In vitro* cells tolerate the MSNs up to 100 μg/ml (Hudson et al. 2008) and in animal models MSNs show no toxicities up to 200 mg/kg (Jie et al. 2010). These doses are much higher than the ones that are used for therapy modalities. Cytotoxicity of MSNs is often dependent on particle size and surface charge. For example, smaller (15 nm) MSNs induce more cytotoxicity than bigger MSN particles (100nm) in ECs (Napierska et al. 2009). Furthermore, positively charged MSNs have a higher and faster cellular uptake than anionic particles. However, cationic MSNs are also more toxic (Oh et al. 2010).

2.4.2 Nanoparticle targeting

One benefit of using MSNs as drug carriers comes from the possibility of tissue targeting. This reduces drugs causing unwanted side effects or damaging healthy tissues. In cancer, the targeting of MSNs against tumor tissue can be done either through passive or active targeting. Passive targeting of MSNs towards solid tumors happens naturally through so called enhanced permeability and retention effect (Nakamura, Jun, and Maeda 2015). This phenomenon is caused by tumors aberrant vasculature. As the tumors vasculature grows rapidly the blood vessels develop abnormally leading to wider fenestrations between the ECs which allows easier extravasation of MSNs to tumor cells and therefore leading to accumulation of MSNs (Nakamura, Jun, and Maeda 2015). Furthermore, MSNs do not diffuse back

out to the blood flow through the capillary endothelium (Iturrioz-Rodríguez, Correa-Duarte, and Fanarraga 2019). As an example, MSNs coated with polyethyleneimine / polyethylene glycol (PEI/PEG) and loaded with DOX and P-gp siRNA showed increase in accumulation to tumor tissue compared to healthy tissue (Meng et al. 2013).

Active targeting of tumors with MSNs is based on overexpression of specific receptors on tumor cells. Such targets can be for example HER2 (Milgroom et al. 2014), EGFR (Reda et al. 2019), or VEGFR (Goel et al. 2014). MSNs can be functionalized with high affinity ligands against these receptors. This will increase the uptake of loaded MSNs into tumor cells and therefore reduce peripheral toxicities observed with MSNs and the compounds. In active targeting, MSNs will enter the cells through endocytosis, leading them to endosomes and lysosomes which might lead to effect called proton sponge, which is discussed later (Iturrioz-Rodríguez, Correa-Duarte, and Fanarraga 2019). A study demonstrated that MSNs loaded with DOX and labeled with αv-β3 integrin ligands increased the MSN intake into GBM cells and enhanced the BBB penetration (J. Mo et al. 2016).

However, translation of functionalized MSNs to clinics has not been straightforward (Iturrioz-Rodríguez, Correa-Duarte, and Fanarraga 2019). When functionalized MSNs are injected into bloodstream their surface will often be covered with proteins forming a so-called “corona” on top of the MSN. The proteins masking the nanoparticle differs the targeting and biodistribution of the nanoparticles (Richtering, Alberg, and Zentel 2020). Additional problem for cancer targeting is that nanoparticles poorly penetrate deep into the tumor mass. This is caused by high density of collagen in tumors ECM. However, this barrier can potentially be tackled by labeling the MSNs with collagenase, which would proteolytically digest the collagen and allow MSNs to dive deeper into tumor tissue (Villegas, Baeza, and Vallet-Regí 2015).

2.4.3 Drug release methods from nanoparticles

Problems associated with drugs are often related to their pharmacokinetic and -dynamic properties, such as degradation too early or at the wrong place causing ineffective therapy or potential side-effects. Therefore, advantage of nanoparticles is their easily modifiable surface which can be reshaped to not release its contents until it is desired (Vallet-Regí et al. 2018). The signal for release can be either internal or external. Internal stimuli can come from the local tissue, such as the tumor, where MSNs are desired to have their cargo released. Such stimuli can be pH, redox or enzyme activity which can be specific or hyperactivated in the target tissue (Manzano and Vallet-Regí 2018). As tumors are much more acidic compared to normal tissue, because of “Warburg effect” causing increased acidosis, MSNs

functionalized with pH sensitive polymers can be used to release the drug directly at the tumor site (Men et al. 2020). Enzyme activity is dysregulated in cancer cells, and therefore can also be used to trigger MSNs. For example, increased matrix-metalloproteinase activity in some cancers can be utilized to activate drug release (Y. Liu et al. 2015). Aside from internal signals, external activation of release can also be used. The benefit when comparing external signal to internal signal comes from reproducibility (Iturrioz-Rodríguez, Correa-Duarte, and Fanarraga 2019). The most common external signaling methods are magnetic and light activation. Superparamagnetic iron oxide nanoparticles can be activated by magnetic stimuli to produce heat leading to release of compounds (Torres-lugo and Rinaldi 2013) or gold-nanoparticles can be activated through light stimuli because of their photosensitivity (Fazio et al. 2019).

2.4.4 Nanoparticles in GB

Nanoparticles present interesting potential in GB due to their modifiability. As discussed, nanoparticles can be used to preserve drugs through avoidance of deterioration in blood circulation before reaching the site of interest or to guide the drugs to their specific target. Specifically, in GB treatment, nanoparticles can be also used to assist in passing through the BBB. Therefore, nanoparticles have been highly investigated in regards of GB in the past decades. There are multiple different delivery methods for nanoparticles into CNS. Intracranial administration of nanoparticles is one potential method. Even though it is quite invasive, dosing of nanoparticles can be done at the same time as surgery of the tumor. It has been shown that nanoparticles remain longer in the tumor than non-nano-drugs (Alphandéry et al. 2017). Another suitable method for CNS drug delivery is intranasal route. As intracranial, this route also circumvents the problematic BBB allowing high nanoparticle concentration in the CNS (Sukumar et al. 2019).

To enhance nanoparticle permeation through BBB, they can be coated with various surfactants to allow them to be transferred by receptor mediated transcytosis. Already in 1995, nanoparticles were successfully coated with surfactants to enhance the delivery across the BBB *in vivo* (Kreuter et al. 1995). They used polysorbate 80 (PS80) as surfactant which they compared to multiple other surfactants. However, PS80 was superior to all other tested surfactants (Bickel, Yoshikawa, and Pardridge 2001). Furthermore, it has been shown that nanoparticles coated with PS80 and then overcoated with apolipoproteins show further enhanced uptake (Kreuter et al. 2002). The intake of beforementioned nanoparticles happens through receptor mediated transcytosis by interacting with LDL receptors on BBB ECs (Kreuter et al. 2002). Still today, PS80 is considered to one of the golden standards for increasing the BBB permeability of nanoparticles as has been shown even in other studies (Gelperina et al.

2010). There are multiple other surfactants to promote BBB permeability, such as angiopep-2, a ligand of low-density lipoprotein receptor-related protein 1, or transferrin receptor ligands (Ulbrich et al. 2009; Guo et al. 2019; Xuan Wang et al. 2018). Another way is to conjugate nanoparticles with positive charged groups. This can be achieved with some proteins such as chitosan. This allows them to be transported through BBB with adsorptive-mediated transcytosis (Z. H. Wang et al. 2010). As discussed, nanoparticles can be functionalized to target specific proteins. In the case of GB, there are multiple potential targets, such as CD133, CD33, CD15 or L1CAM. Additionally, as GSCs are often the cause for recurrence and resistance, their targeting is feasible. It has been shown that gold nanoparticles functionalized with GB stem cell marker nestin selectively targeted GB stem cells (Alphandéry 2020).

Nanoparticles can be used to assist in the maximal resection of GB. GB cell targeting functionalized nanoparticles can be loaded with both MRI contrast agent and a fluorescent dye (5-ALA) to enhance the visualization and imaging of the GB tumors (Ni et al. 2014). Another way to define GB tumor is by targeting tumor-associated macrophages with near-infrared fluorescent silica coated iron oxide nanoparticles (C. Lee et al. 2018). Drug loaded nanoparticles can improve the efficacy of different therapy modalities in GB. Most commonly used therapy for GB after surgery is chemotherapy. Loading TMZ into nanoparticles has been shown to increase its efficacy and cytotoxicity towards GB cells when compared to normal TMZ (Fang et al. 2015). Two other chemotherapies carboplatin and cisplatin, when loaded into nanoparticles, showed increased tumor cytotoxicity, decreased overall toxicity, better penetration and stability in GB tissue (Clark Zhang et al. 2017; Arshad et al. 2015).

2.4.5 Proton sponge effect of nanoparticles

As discussed earlier, MSNs often enter cells by receptor-mediated endocytosis. Hence, they will be in endo- or lysosomes. Cells will try to expel MSNs through exocytosis and if the MSNs load is not released during this time into cytoplasm the loaded drug will not have any effect. Therefore, nanoparticles will need to escape the endo-/lysosomal space (Iturrioz-Rodríguez, Correa-Duarte, and Fanarraga 2019).

Currently there are three different strategies to achieve endosomal escape for nanoparticles. 1) nanoparticles can induce degradation of endosomal membrane by interacting with it, and so escaping back to cytoplasm. The mechanism works by an increase in hydrophobicity of nanoparticles which leads to interaction with membrane lipids causing disruption of the membrane. For example, hydrophobic surface can be achieved in nanoparticles by functionalizing them with carboxylic acid monomers which become hydrophobic in endo-/lysosomes after decrease in pH (Jones et al. 2003). 2) Another new way is to have swelling nanoparticles which

eventually will break the endo-/lysosomal membrane (You and Auguste 2009). 3) Third and most common technique for endosomal escape of nanoparticles is the proton sponge effect. The effect is caused by cationic surface of nanoparticle. As positively charged nanoparticles, for example PEI functionalized, bind to lipid groups on the cell membrane they are endocytosed into cells. When PEI-nanoparticles are in the endo- or lysosomes their unsaturated amino groups work as a sponge as they are able to intake protons pumped in by the proton pumps. This phenomenon keeps the proton pumps active and leads to excessive intake of Cl^- ions through chloride channels and H_2O molecules to balance the acidification and pH of the endo-/lysosomes. This results in swelling of the endo-/lysosomes which eventually ruptures the membrane and leads to burst of nanoparticles into cytoplasm (Cupic et al. 2018; Iturrioz-Rodríguez, Correa-Duarte, and Fanarraga 2019). As mentioned, PEI functionalized nanoparticles are one way to induce the proton sponge effect through their amino groups. MSNs coated with PEI have been shown to deliver siRNAs to mouse xenografts and reduce tumor growth (Shen et al. 2014). However, the proton sponge is still somewhat controversial and there is open discussion whether the effect causes irreversible membrane rupture or just temporary pore formation (Cupic et al. 2018).

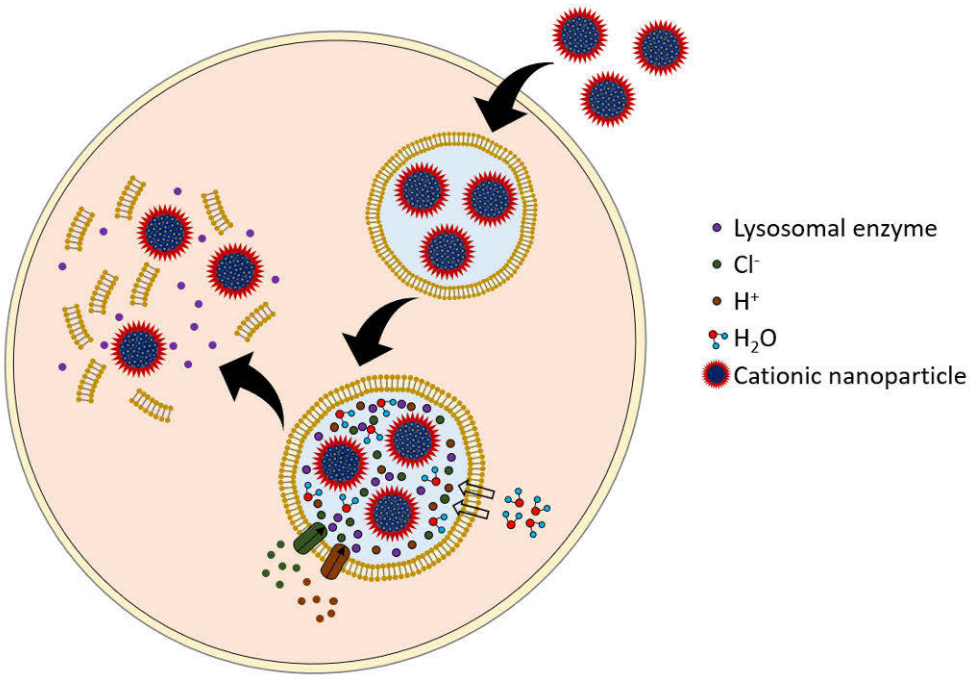


Figure 4. Induction of the proton sponge effect after intake of positively charged nanoparticles into lysosomes (Modified from (Iturrioz-Rodríguez, Correa-Duarte, and Fanarraga 2019)).

3 Aims

The purpose of this thesis was to explore novel therapy targets in GB. The importance of PP2A and its target pathways have been earlier demonstrated in cancer, and further in GB. Inhibition of PP2A, through its endogenous inhibitor PME-1, drives resistance towards multikinase inhibitors in GB. Novel derivatives of PPZs (SMAPs) have shown to have efficacy in multiple cancers, however, their potency in GB is unknown. Additionally, although potential use of nanoparticles is an emerging field in GB, further validation is required to determine optimal BBB permeability, drug release and possible toxicities.

The specific aims of this study:

I. Confirming the BBB permeability of SMAPs *in vivo* and evaluation of the efficacy in heterogenous GB cell lines and GSCs.

II. Identification of kinases inhibited by UCN-01 which are required for the synthetic lethality together with PP2A reactivation in GB cells.

III. To determine the BBB permeability of PEI-MSNs loaded with drugs, evaluate their potential to release the drugs, test efficacy on GB cells, and observe for possible toxicities.

4 Materials and Methods

4.1 Western blotting and antibodies

Treated or siRNA transfected cells were lysed in 2x Laemmly buffer (4% SDS, 20% glycerol, 120mM Tris), prepared with 6X SDS loading buffer and boiled. Samples were resolved by 4-20% SDS-PAGE gel (BioRad). Proteins were transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% milk-TBS for 30 minutes in room temperature (RT) and then incubated with primary antibodies in a required dilution overnight at 4°C. Secondary antibodies (1:5000) were created in 5% Milk-TBS-Tween 20 and incubated for 1 hour in RT. Samples were visualized with Odyssey (LI-COR Biosciences, Nebraska, USA). Primary antibodies used in the studies were PARP-1 (sc-7150, 1:1000), P62 (sc-28359, 1:500), PME-1 (sc-20086, 1:1000), CIP2A (sc-80659, 1:500), SET (sc133138, 1:1000), β-actin (sc-47778, 1:10000) from Santa Cruz Biotechnology, cPARP (ab32064, 1:1000) from Abcam, LC3-β (2775s, 1:1000), SOX2 (3579p, 1:500), AKT (9272S, 1:1000), anti-phospho Akt S473 (9271, 1:1000) from Cell Signaling, Nestin (MAB5326, 1:1000) from Merck, anti-phospho PDHE1-A type I (S300) (ABS194, 1:1000) from Millipore, ARPP-19 (11678-1-AP, 1:250) from Proteintech and secondary antibodies were purchased from LI-COR, mouse (926-32212, 1:5000), and rabbit (926-68021, 1:5000).

4.2 Cell culture

All cell cultures were maintained in a humified atmosphere of 5% CO_2 at 37°C. Established human GB cell lines U87MG (gift from Ari Hinkkanen, University of Eastern Finland, Joensuu, Finland), A172, U118, U251 (gift from Pirjo Laakkonen, University of Helsinki, Helsinki), E98-FM-Cherry (gift from William Leenders, Radboud Institute for Molecular Life Sciences, Nijmegen, The Netherlands) and human fibroblasts (gift from Johanna Ivaska, Turku Bioscience, Turku, Finland) were cultured in DMEM (Sigma-Aldrich) and T98G (VTT Technical Research Centre, Turku, Finland) were cultured in Eagle MEM (Sigma-Aldrich). All growth mediums were supplemented with 10% (except fibroblasts were supplemented with

20%) of heat-inactivated fetal bovine serum (FBS) (Biowest), 2 mM L-glutamine and penicillin (50 U/mL) / streptomycin (50 µg/mL).

The patient-derived GSCs BT-3-CD133$^+$, BT-12 and BT-13 (Kuopio University Hospital, Finland (Le Joncour et al. 2019)) were cultured in DMEM/F12 (Gibco) and supplemented with 2 mM L-glutamine, 2% B27-supplement (Gibco), penicillin (50 U/mL) / streptomycin (50 µg/mL), 0.01 µg/mL hFGF-β (Peprotech), 0.02 µg/mL hEGF (Peprotech) and 15 mM HEPES-buffer. Patient-derived primary glioma stem cells (U3008, U3013, U3017, U3054, U3137, U3179 and U3213) cultures were obtained from HGCC biobank (Xie et al. 2015). They were cultured as adherent in serum-free neural stem cell media containing 1:1 mix of DMEM/F12 with glutamax (Fisher Scientific) and neurobasal media (Fisher Scientific) with 1X B-27-supplement (Fisher Scientific), 1X N-2-supplement (Fisher Scientific), 1% penicillin-streptomycin (Sigma-Aldrich), 0.01 µg/mL hFGF-β (Peprotech) and 0.01 µg/mL hEGF (Peprotech). Primary GB cells were cultured on Primaria vessels (Fisher Scientific) which were coated with 1% laminin solution (Sigma-Aldrich). For assays requiring adherent cell, such as colony growth and microscopy, GSC populations were cultured on Matrigel (Becton Dickinson) coated dishes. All cell lines were cultured under standard conditions of 5% CO_2 at 37°C.

Mouse ECs from brain microvessels (bEND3) were maintained in DMEM (Lonza) supplemented with 10% decomplemented FBS (Lonza), 2 mM L-glutamine (Sigma) and penicillin:streptomycin (50 U/mL and 50 µg/mL respectively). Mouse immortalized astrocytes (HIFko) were maintained in Basal Eagle Medium 1 (BME-1, Sigma) supplemented with 5% decomplemented FBS (Lonza), 1 M HEPES (Sigma), 2 mM L-glutamine (Sigma) 100 mM sodium pyruvate (Sigma), 3 g D-glucose and penicillin:streptomycin (50 U/mL and 50 µg/mL respectively).

4.3 Compounds

For *in vitro* work, all compounds were dissolved in dimethyl sulfoxide (DMSO) unless otherwise stated and stored in -20 °C. DCA was dissolved in mQ water and stored in -20°C. For *in vivo* work, SMAPs (DT-061, DBK-1154 and DBK-1160) were dissolved in 10% N,N-dimethylacetamide (Sigma), 10% Kolliphor HS 15 (Sigma) and 80% sterile water, and kept in RT and protected from light. The SMAP synthesis can be found in detail in a public patent application PCT/US2015/019674. For *in vivo*, UCN-01 was dissolved in 2% w/v sodium citrate, pH 3.5 buffer and DCA was dissolved in PBS. Compounds were obtained from the following suppliers: AKT1/2 inhibitor, staurosporine, CEP-701, UCN-01, PKC412, Z-VAD-FMK, sodium salt of DCA and lipoic acid from Sigma-Aldrich. FRAX486, OSU-0301 and Vemurafenib from SelleckChem. K252a and rebeccamycin from Enzo Life Sciences. K252c from Tocris Bioscience. MK-2206 from MedChemExpress.

SMAPs (DT-061, DBT-1154, DBK-1160, DT-766) were kindly supplied by Prof. Michael Ohlmeyer (Icahn School of Medicine at the Mount Sinai, NY, USA). DT-061 was also purchased from ProbeChem.

4.4 Colony formation assay

An optimized number of cells were seeded in either 12-well or 24-well plates (Sigma-Aldrich) and allowed to adhere. Matrigel matrix (BD Biosciences) coated plates were used for patient-derived glioma stem cells. After approximately 24 hours cells were treated with PEI-MSNs, drugs, or siRNAs. After 72h incubation PEI-MSNs, drugs, or siRNAs containing media was replaced with non-drug containing medium and incubated until the control wells were confluent. Cells were fixed with ice cold methanol and stained with 0.2% crystal violet solution in 10% ethanol for 15 min at RT. Plates were dried and scanned with Epson Perfection V700 Photo scanner. Quantification of colonies were done with ImageJ by using the Colony area plugin (Guzmán et al. 2014). Colony formation assays at hypoxic conditions were performed in InvivO2 400 incubator (Ruskinn Technology Ltd, Bridgend, UK) at following conditions 1% O2, 5% CO2 and 90% humidity.

4.5 Cell viability assay

Optimized number of cells were seeded in non-transparent 96-well plates (Perkin Elmer) and allowed to adhere. After 24 h, medium was removed, and cells were treated with drugs for 24 h – 72 h. For established GB cell lines CellTitre Glo assay (Promega) was used to measure the cell viability according to the instructions provided by the manufacturer. Bioluminescence was measured from the 96-wellplates with BioTek Synergy H1 plate reader (BioTek). For glioma stem cells medium was changed into fresh medium containing 10% of Alamar blue solution (10099022, Fisher Scientific). Fluorescent signal was read after 210 min of incubation in 37°C using Wallac Victor 1420 plate reader (Perkin Elmer).

4.6 Caspase-3 and -7 activity

Optimized number of cells were seeded in non-transparent 96-well plates (Perkin Elmer) and allowed to adhere. After 24, medium was removed, and cells were treated with drugs for 24 h – 72 h. In experiments whrer Pan-caspase inhibitor Z-VAD-FMK (10 mM, Promega) was used, it was added at the same time as drugs. Bioluminescent Caspase-Glo® 3/7 Assay (Promega) was used to measure Caspase-3 and -7 activity, which utilizes a substrate containing Caspase-3 and -7 target peptide DEVD-amino

luciferin. Bioluminescence was measured from the 96-wellplates with BioTek Synergy H1 plate reader (Biotek).

4.7 Cancer cell line encyclopedia and drug sensitivity profiling

Copy number variations and mutations data for U87MG, U118, A172 and T98G GB cell lines were gathered from CCLE (https://portals.broadinstitute.org/ccle). Data for E98 was collected from publications (Claes et al. 2008; Navis et al. 2015). Drug sensitivity data for primary patient derived glioma cell lines was collected from a publication (Johansson et al. 2020) and sensitivity data for U87MG, U118, A172 and T98G was collected from (https://www.cancerrxgene.org/).

4.8 *In vitro* BBB assays

In vitro BBB assay is described in detail in the original publication (Le Joncour, Karaman, and Laakkonen 2019). In brief, transwell inserts representing BBB were created by co-culturing mouse brain micro vessel ECs (bEND3) (blood side) and immortalized mouse astrocytes (HIFko) (brain side) for 6 days. Next steps varied between experiments which are explained next.

4.8.1 *In vitro* BBB damage / permeability measurements

To measure the damage caused to the *in vitro* BBB by different compounds movement of a small-molecular-weight fluorescent dye, sodium-fluorescein (Na-Fl), was followed as it diffused through the *in vitro* BBB over time. In the experiment altered diffusion of Na-FI was followed after introduction of drugs NZ-8-061/DT-061 and DBK-1154. 24 hours after the introduction of drugs Na-FI solution (50 µM) in medium was added into inserts. Samples were collected from both the side (blood and brain) of the inserts and transferred to 96-well plate for quantification. The media was always replaced with containing Na-Fl media. Results were quantified with fluorescent plate reader (480/560 nm).

4.8.2 Passage through *in vitro* BTB

Inserts were placed on GSC (BT-12) organoids on glass coverslips to complete the BTB and 100 ng of PEI-MSI were added on the endothelial side. After 24h, BTB dishes were stained with LysoTracker Red DND-99 according to the manufacturer's recommendations (Invitrogen) before fixation with ice-cold 4% PFA (10 min) and nuclear counterstaining with DAPI (Sigma). BT-12 coverslips and Transwell

membranes containing both bEND3 and HIFko cells were cut and mounted on Mowiol 4-88 (Sigma) and imaged on a Zeiss LMS880 confocal microscope.

To quantify the cell viability of the bEND3, astrocytes, and BT-12 cells from the BTB, cells were gently detached with accutase (Sigma) collected, counted and $5x10^5$ cells/mL were transferred in a 96-well plate. 10 µL of 3-(4,5- Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT; 5 mg/ml in PBS) was added on the cells before incubating for 2h at 37°C. Eventually, cells were lysed 10% SDS, 10 mM HCl) o/n and the absorbance was measured at 540 nm using Multiskan Ascent software version 2.6 (Thermo Labsystems). Results were expressed as the % of absorbance relative to the control, untreated BTB cells.

4.8.3 Measuring SMAP concentration after diffusion through the *in vitro* BBB with HPLC-MS/MS

Samples of NZ-8-061/DT-061 and DBK-1154 were collected from both sides of the *in vitro* BBB inserts at 1, 6, 12 and 24 hours. The media was removed and replaced with fresh media containing drugs after each timepoints. NZ-8-061/DT-061 and DBK-1154 were introduced in 15 µM concentration. Glipizide (Sigma-Aldrich) was used as an internal standard. All working and stock solutions were prepared in DMSO. All calibration standards and quality control samples were prepared in Basal Medium Eagle medium. For concentration measurements of DBK-1154 and NZ-8-061/DT-061 HPLCMS/MS was used. Samples were prepared by mixing a 25 µl of standard or quality control sample or study sample with 35 µl of internal standard (15 µM) and 440 µl of acetonitrile. Injection volume was 10 µl for the HPLCMS/MS. After separation with a Waters SunFireTM C18 column (2.1 x 150 mm, 3.5 µm) with a gradient flow system (0.1 % formic acid in water and acetonitrile), quantitative detections were conducted in multi-reaction monitoring mode (MRM) with an AB Sciex API 4000TM triple quadrupole mass spectrometer connected to a Shimadzu Prominence HPLC system. For the internal standard, NZ-8-061/DT-061 and DBK-1154, the respective precursor ions (m/z) were 533.2, 521.1 and 446.1. The fragment ions (m/z) monitored and used for quantitation were 196.1 for DBK-1154, 184.0 for NZ-8-061/DT-061 and 321.1 for the internal standard. The chromatograms were processed using AB Sciex Analyst® 1.6.3 software. The fit-for-purpose validated concentration range was from 15 nM to 15 µM using quadratic regression and 1/x2 weighting. The study samples were analyzed and calculated using range from 150 nM to 15 µM. Inter-assay accuracies of six quality control samples [two parallel quality control samples at three different concentration levels (450 nM, 4.5 µM and 12 µM)] ranged from 84.9 % to 112 % for DBK-1154 and from 81.9 % to 109 % for DT-061.

4.9 Preparation and characterization of hyperbranched PEI functionalized MSNs (PEI-MSNs)

MSNs were prepared according to a protocol from previously published work (J. Zhang et al. 2014). The MSNs were prepared by co-condensation of tetraethyl orthosilicate (TEOS) (Sigma-Aldrich) and 3-aminopropyltriethoxysilane (APTES) (Sigma-Aldrich) as silica sources. Briefly, a mixed solution was prepared by dissolving and heating Cetylmethylammonium bromide CTAB (Fluka) (0.45 g) in a mixture of DI water (150 mL) and ethylene glycol (Sigma-Aldrich) (30 mL) at 70 °C in a reflux-coupled round flask reactor. Ammonium hydroxide (30 wt%, 2.5 mL) was introduced to the reaction solution as the base catalyst before TEOS (1.5 mL) and APTES (0.3 mL) was added to initiate the reaction. Decane (Alfa Aesar) (2,1 mL) and 1,3,5-Trimethyl-benzene (TMB) (ACROS) (0.51 mL) were used as swelling agents before the addition of the silica sources, decane was added 30 min before TMB and after the addition of TBM, the synthesis solution was mixed for 1.5 h. The molar ratio of used reagents in the synthesis of MSN was 1TEOS : 0.19APTES : 0.18CTAB : 0.55TMB : 1.6 decane : 5.9NH3 : 88.5 ethylene glycol : 1249H2O . For inherent fluorophore labeling of the MSNs, TRITC was pre-reacted with APTES in a molar ratio of (APTES:TRITC) 3:1 in ethanol (0.5 mL) under vacuum for 2 h. Subsequently, the pre-reaction solution was added to the synthesis solution before the addition of TEOS. The reaction was allowed to proceed for 3 h at 70 °C. Then, the heating was stopped where after the as-synthesized colloidal suspension was aged at 70 °C without stirring for 24 h. After the suspension was cooled to RT, the suspension was separated by centrifugation. After collecting the particle precipitate, the template removal was carried out by the ion-exchange method. Briefly, the collected particles were extracted three times in ethanolic NH_4NO_3 solution, washed with ethanol (Baghirov et al. 2016), and resuspended in DMF for long-term storage. The surface modification of MSNs with hyperbranched PEI by surface-initiated polymerization was carried out according to an inhouse-established protocol (Rosenholm, Penninkangas, and Lindén 2006). To initiate PEI polymerization from the MSNs surfaces, aziridine (Menadiona S.L.Pol.) was used as a monomer with toluene as solvent, in which the MSN substrate was suspended in the presence of catalytic amounts of acetic acid. The suspension was refluxed under atmospheric pressure overnight at RT, filtered, washed with toluene, and dried under vacuum at 313 K. Henceforth, the obtained nanoparticles are abbreviated as PEI-MSNs. Full redispersibility of dried, extracted, and surface-functionalized MSN was confirmed by redispersion of dry particles in HEPES buffer at pH 7.2 and subsequent dynamic light scattering (DLS) measurements (Malvern ZetaSizer NanoZS). The fine architecture of the nanoparticles was further confirmed by transmission electron microscopy (TEM) (Jeol JEM-1200EX electron microscope)

operated at 80 kV. The success of surface polymerization was confirmed by zeta potential measurements (Malvern ZetaSizer NanoZS).

4.10 Light microscopy

GSCs were grown on as monolayers on Matrigel (Becton Dickinson) coated glass coverslips and further treated with 10 μg/mL of PEI-MSNs were conjugated with either TRITC (Tetramethylrhodamine-isothiocyanate) for early endosome and lysosome staining or with FITC (Fluorescein isothiocyanate) for mitochondrial staining, for 48h. For endosome and lysosome staining GSCs were fixed with 4% Paraformaldehyde (PFA) for 10 min. The cells were permeabilized using 0.1% Triton X-100 for 10 min and blocked with horse serum. The 1° anti-EEA1 (goat) antibody for recognition of early endosomes (Santa Cruz Biotechnology) was prepared (1:100) in PBS (10% horse serum). The 1° anti-LAMP-1 (mouse) antibody for recognition of lysosomes (Abcam, UK) was prepared (1:100) in PBS (10% horse serum). Antibody incubation was performed overnight at +4 °C. The cells were washed three times with PBS; Alexa 488 secondary (Anti-goat and anti-mouse) antibodies (Sigma-Aldrich) in PBS were added to the cells at RT for 1h. The cells were mounted on coverslips using VECTASHIELD (4′,6-diamidino-2-phenylindole). For mitochondrial staining, cell medium (0.5 mL) was collected from the plate and mixed with 0.2 μL of Mitotracker Orange® (Thermo Fisher Scientific Inc) returned to the cells drop-by-drop. The cells were finally incubated for 20 min at 37 °C. The cells were washed 3x with PBS, fixed for 10 min with 4% PFA, and mounted using VECTASHIELD (4′,6-diamidino-2-phenylindole) on glass slides for microscopy.

The microscopy setup consisted of Zeiss 780 (Zeiss, Germany) confocal microscope, PMT, and 100X oil objective. DAPI was excited by 405 lasers and emission was collected in the blue channel. Alexa 488 (early endosomes and lysosomes) was excited with 488nm argon laser and emission was collected by green channel (510-550 nm). The TRITC labeled PEI-MSNs were excited by 561 nm laser and emission were collected (575-610 nm). FITC-conjugated PEI-MSNs were excited with 488 nm argon laser and emission was collected by green channel (510-550 nm). The Mitotracker Orange® was excited by 561 nm laser and emission were collected at 575-610 nm.

4.11 Transmission electron microscopy

GSCs were grown on as monolayers on Matrigel (Becton Dickinson) coated glass coverslips glass coverslips and further, treated with 10 μg/mL of PEI-MSNs 24 and 72h. The BT-12 GSCs were fixed with 5% glutaraldehyde s-collidine buffer, post-

fixed with 2% OsO4 containing 3% potassium ferrocyanide, dehydrated with ethanol, and flat embedded in a 45359 Fluka Epoxy Embedding Medium kit. Thin sections were cut using an ultramicrotome to a thickness of 100 nm. The sections were stained using uranyl acetate and lead citrate to enable detection with TEM. The sections were examined using a JEOL JEM-1400 Plus transmission electron microscope operated at 80 kV acceleration voltage (Prabhakar et al. 2017).

4.12 Animal experiments

The animal experiments for this thesis were carried out according to the Animal Experiment Board in Finland (ELLA) for the care and use of animals under the licenses 4161/04.10.07/2015 and 9241/2018. The animals were kept under specific pathogen-free conditions in individually ventilated cages in the animal care facility. Mice were kept on a 12-h light/dark cycle with access to the autoclaved water and irradiated chow ad libitum. Animals were monitored closely and upon showing discomfort or weight loss (10% reduction from the highest weight), they were sacrificed with CO_2 and cervical dislocation. The mice used for this study were female Athymic Nude-Foxn1nu or Balb/cOlaHsd-Foxn1nu/nu mice and they were purchased from Envigo France.

Mice were subcutaneously injected on both dorsal side flanks with 5 * 106 viable U87MG-luc cells in 150 µl PBS. After palpable tumors (200 mm3) mice were randomized into group with a web-based program (Laajala et al., 2016). Tumors were palpated three times a week during the study and mice weights were recorded regularly. Mice were treated with either vehicle, NZ-8-061/DT-061 (30 mg/kg), UCN-01 (3 mg/kg) or the combination of both drugs for two weeks. On the final day of the experiments, mice were sacrificed by cervical dislocation and tumors were isolated, imaged, weighted and stored by snap freezing in liquid nitrogen or fixing in formalin. Formalin-fixed samples were embedded in paraffin, sectioned for future immunohistochemical staining.

The intracranial GB mouse models were done by preparing cell suspensions of $1.5 * 10^5$ cells in 5 µl of PBS which was inoculated into the brain of anaesthetized (isoflurane) mice. GB cell lines E98 and BT3-CD133$^+$ were used. Injection was done as followed. A small incision (0.5 mm) was made to skin middle of the skull and a small hole (Ø 0.2 mm) was drilled above the injection site. Coordinates for the injection site from bregma were either 1 mm posterior (BT-CD133$^+$) or 1 mm (E98) anterior depending on the model, 2 mm to right, 3 mm depth from the skull. Injection was done slowly within 2 min and the needle was allowed to stay in the injection site for 2 min. After the injection, wound was sutured, and mice were allowed to wake up in a controlled environment. Drug treatment was started after the tumors were visible with bioluminescence and the mice were randomized into

equal groups based on the signal. Randomization was done with web-based program (Laajala et al. 2016). In the experiments, tumor growth was followed twice a week by bioluminescence imaging using Xenogen IVIS Spectrum (Caliper Life Sciences). For the bioluminescence imaging, mice were injected with 150 mg/kg mouse weight XenoLight D-luciferin substrate (Caliper Life Sciences). The imaging was performed under isoflurane gas anesthesia. Images were quantified with Living imageVR. After the intracranial experiment's brains were collected and either fixed in formalin or frozen in isopentane on dry ice. Formalin-fixed samples were embedded in paraffin, sectioned and histologically or immunohistochemically stained. Antibodies used were HE (Fluka), Vimentin (Dako), Ki67 (Dako). Ki67 staining's were quantified with ImmunoRatio program (jvsmicroscope.uta.fi/immunoratio/). In the intracranial drug treatment studies there were approximately 10 mice per group. In the monotherapy studies, NZ-8-061/DT-061 was given in 30 mg/kg and DBK-1154 100 mg/kg and dosed twice a day. In the combination studies mice were given oral dosage of DBK-1160 (100 mg/kg, twice per day) with DCA (100 mg/kg, twice per day) and MK-2206 (100 mg/kg, three times per week) for 3-4 weeks. Higher dose for DBK-1154/DBK-1160 is explained because of its lower bioavailability through oral dosing.

For the dosing studies of PEI-MSNs intracranial implantation of U87MG-GFP or BT-12 cells as described above. After 20 days of tumor growth, 100 µg of PEI-MSN in PBS were injected in the caudal vein (100 µl) or intranasally (3 dosages of 5 µl given every two hours). Intranasal delivery of PEI-MSNs (35 µg in 5 µL of PBS) was distributed drop by drop, alternating between the nostrils. The procedure was repeated 3 times every two hours. Animals were euthanized 2 hours after completing the last intranasal dosage and eight hours after IV injections of the PEI-MSNs and brains were snap-frozen in -50°C isopentane (Honeywell). Brain cryosections (9 µm) were cut using a cryotome (ThermoFisher), collected on Superfrost Ultra slides (ThermoFisher), and fixed in an ice-cold 4% PFA bath. Brain microvessels were stained overnight using a rat anti-mouse PECAM-1/CD31 (1:400, 553370, BD Pharmingen). Cell nuclei were counterstained with DAPI (1 µg/mL, Sigma), samples were mounted with Mowiol 4-88, and imaged on a Zeiss LMS880 confocal microscope.

4.13 siRNA Screens

A custom human kinase siRNA library containing three non-overlapping siRNAs targeting 37 kinase were purchased from Qiagen (FlexiPlate 100 pmol SO3056885, Qiagen). All together two independent siRNA screens were done in T98G cells. AllStars negative and AllStars Death (Qiagen) were used as negative and positive controls, respectively. In the first screen kinase siRNA library in combination with

the control (scrambled) and PME-1 siRNAs (three variants) was dispensed in black clear bottom tissue-culture treated 384-well plates (Corning 384) using an Echo acoustic dispenser. The assay plates were used right away or used later in which case they were kept sealed in -20°C until used. For transfection, Opti-MEM medium (Gibco) containing Lipofectamine RNAiMAX (Invitrogen) was added (5 µl per well) using Multidrop Combi (Thermo Scientific) and plates were mixed for 15-30 min at RT. After that, T98G (500 cells per well) were added in 20 µl of culture medium (EMEM, 10% FBS, Pen/Strep, L-Glutamine) using Multidrop Combi. Final siRNA concentration was 12 nM. After transfection, cells were be incubated at +37°C for next 72 hours in the presence of 5% CO_2. Cell proliferation was measured by adding 25 µl per well of CellTiter-Glo (Promega), and luminescence was detected using Pherastar FS plate reader (BMG Labtech, Baden-Württemberg, Germany). In the second screen, the kinase siRNA library was dispensed first as previously described, T98G cell seeded and incubated at +37°C for next 48 hours. Then cells were treated with DT-061 and DT-766 (5 µM) for the next 24 hours and cell proliferation was measured by CellTiter-Glo. Using collected data for each plate, the following calculations were performed to obtain % inhibition values for all wells (% inhibition=100*((average$_{neg}$–average$_{sample}$)/(average$_{neg}$–average$_{pos}$))). The most effective combinations of kinase siRNAs with DT-061 or PME-1 siRNAs (inhibition greater that 40%) were selected as hits for further validation. For each kinase siRNA GARP was calculated and synergy scores between siRNA kinases and DT-061 or siPME1 were explored using the Highest Single Agent and Bliss models (L. Zhao, Wientjes, and Au 2004; Lehár et al. 2007).

4.14 Mitochondrial respiration measurement

Mitochondrial respiration in T98G cells was characterized as an indicator of cellular metabolism and fitness in response to the exposure to DCA, MK-2206 alone or in combination with DT-061 by extracellular flux analysis using Agilent Seahorse XF Analyzer (Agilent Seahorse Bioscience, Santa Clara, CA, USA). For this purpose, Agilent Seahorse XF Cell Mito Stress Test was applied to T98G cells and oxygen consumption rate (OCR) was measured in function of time and added respiration modulators according the manufacture instructions. In short, T98G cells were seeded at 10,000 cells per well in a Seahorse 96-well XF Cell Culture microplate in 100 µL of the growing medium and were allowed to adhere for 24 h. One day prior to the assay, cells were treated with DCA (10 mM), MK-2206 (7 µM) alone or in combination with DT-061 (10 µM). In addition, the Seahorse XF Sensor Cartridge was hydrated the day before running the XF Assay by filling each well of the XF Utility Plate with 100 ul mQ in a non-CO2 37 ◦C incubator for 24 h to remove CO2 from the media that would otherwise interfere with measurements that are pH

sensitive. On the day of analysis, unbuffered XF Assay Media was used for extracellular flux measurements. For this reason, the growth media was replased with 120 µL·well of XF assay media (non-buffered DMEM supplemented with 10 mM glucose, 1 mM sodium pyruvate and 2 mM glutamine (adjusted to pH 7.4)) and incubated at 37 ◦C in a non-CO2 incubator for 1 h before running assay. Mitochondrial function of the cells was analyzed by sequential injections of modulators (with shown final concentration in the wells): oligomycin (1.5 µM) was used to block ATP synthase, carbonyl-cyanide-4-(trifluoromethoxy) phenyhydrazone (FCCP, 1 µM) was used to make the inner mitochondrial membrane permeable for protons and allow maximum electron flux through the electron transport chain, and rotenone/antimycin A mix (0.5 µM) were used together to inhibit complexes I and III, respectively. These compounds were suspended in pre-warmed XF Assay Medium and loaded into the designated injection ports of the hydrated sensor cartridge corresponding to the order of injection. Three basal OCR measurements were performed before the addition of modulators, followed by the sequential addition of oligomycin, FCCP, and rotenone/antimycin A. Measurement cycles were performed after each addition of given compounds. The Seahorse XF Mito Stress Test Report Generator was used to calculate the Seahorse XF Cell Mito Stress Test parameters from Wave data. The exported to Excel data were normolised to total protein per well using BCA assay.

4.15 Statistical analyses

All of the experiments were carried out at least twice on different occasions. Data are presented as mean ± SD and statistical analyses were carried out using a two-tailed Student's t-test assuming unequal variances unless otherwise mentioned. Ki67 comparison was determined with a two-tailed Mann−Whitney test. Survival was determined by Gehan−Breslow−Wilcoxon test. Differences with probability value $P<0.05$ were described as statistically significant.

4.16 LUMIER BACON assay

LUMIER (LUminescence-based Mammalian IntERactome) BACON (bait control) assay is well described by Taipale et al. in the following articles (Taipale et al. 2014; Taipale 2018). In short, 3xFLAG-tagged bait proteins are transfected into 293T cells expressing the Chaperone-Renilla (prey) luciferase in a 96-well plate. After transfection, the cell lysates expressing each bait protein are applied to anti-FLAG coated 384-well plates, which captures the bait protein. The amount of luminescence in the well, after washing off nonspecifically binding proteins, indicates the interaction between the bait protein with the prey protein. After the luminescence

measurement, the amount of bait protein is measured with ELISA, using a different, polyclonal anti-FLAG antibody conjugated to horseradish peroxidase.

4.17 Bioinformatics analysis

Cytoscape (https://cytoscape.org/) was used to visualize the STRING interactive map analysis of hit kinases. Synergy score was calculated with a SynergyFinder web application (https://synergyfinder.fimm.fi/).

5 Results

5.1 Monotherapy efficacy of BBB permeable SMAPs in GB (I)

5.1.1 Development of BBB permeable SMAPs (I)

Earlier members of the SMAP series, RTC-5, and RTC-30, have shown antitumoral activity (Kastrinsky et al. 2015). However, we aimed to improve their oral bioavailability and potency by constraint of the linear spacer moiety between the tricyclic and sulfonamide, which resulted in drug structures DBK-1154 and NZ-8-061 (I: Fig. 1A, S1). Even though original SMAPs were derivatives of tricyclic compounds known for their good BBB permeability we were unsure whether the polarity introduced to tricyclics by the sulfonamide and hydroxyl moieties (I: Fig. 1A) would alter this. Therefore, we wanted to confirm the BBB passage capabilities of NZ-8-061 (a.k.a. DT-061) that has been used in multiple cancers outside the CNS (Sangodkar et al. 2017; Kauko et al. 2018; McClinch et al. 2018). First, we used an *in vitro* BBB model, which is made by co-culturing murine brain microcapillary ECs and astrocytes in specific transwell inserts (Le Joncour, Karaman, and Laakkonen 2019). Quantification by HPLC-MS/MS showed that NZ-8-061 is able to cross this artificial BBB (I: Fig. 1B-C). Furthermore, NZ-8-061 did not harm the *in vitro* BBB itself as diffusion of low-molecular weight fluorescent probe, the sodium fluorescein (Na-Fl), did not change after NZ-8-061 pre-treatment (I: Fig. 1D). Hence it can be concluded that the NZ-8-061 passes through the *in vitro* BBB without damaging it and causing it to become leaky. Next, we wanted to study the BBB penetration of NZ-8-061 *in vivo*. Two delivery methods were used administering 1 mg/kg i.v. or a bolus oral dose of 100 mg/kg. NZ-8-061 showed complete 100% bioavailability with oral dosing based on dose-adjusted fraction absorbed (%F) and moderately quick clearance (I.V. = 3.3 h; P.O. = 4.4 h) based on half-life (T1/2) of the drug. After oral dose peak plasma concentration is around 14 mM, which combined with good clearance and high area under curve shows good and stable systemic exposure. In addition, orally dosed NZ-8-061 partitions into brain with a plasma/brain ratio of 1:1 based on HPLC-MS/MS analysis from the whole-brain homogenate 6 h after drug administration (I: Fig. 1E). This proof of principle data shows that NZ-8-061 is an

orally bioavailable, BBB-permeating drug and a potential candidate for GB treatment.

5.1.2 NZ-8-061 potently inhibits the viability of GB cells with heterogenous genetic background (I)

As discussed earlier in the literature review, PP2A harbors very few mutations or deletions in GB (Kaur et al. 2016). However, we found that in GB PP2A is inhibited through its inhibitory proteins, PME-1, CIP2A, SET and ARPP-19, which are all highly expressed in multiple GB cell lines (I: Fig. 2A). In GB genomic heterogeneity and GSCs are known factors which affect the therapy responses. Therefore, we examined our GB cell lines (U87MG, A172, U118, T98G and E98) for their genomic characteristics and stemness properties (Barretina et al. 2012; Claes et al. 2008; Navis et al. 2015). The cell lines exhibited heterogenous genomic alterations which is common for GB. The only shared genomic alteration throughout the cell lines was a full copy number loss of CDKN2A (I: Fig. S2A). Out of the tested cell lines only E98 expressed known GSC markers SOX2 or Nestin (I: Fig. 2B). We further examined the resistance profiles of GB cell lines for kinase inhibitors. We have previously shown kinase inhibitor resistance in T98G GB cell line (Kaur et al. 2016). However, to further determine kinase inhibitor resistance in our GB cell lines, we used IC_{50} data from Genomics of Drug Sensitivity in Cancer database (https://www.cancerrxgene.org/). As expected, based on the heterogenous mutation profiles (I: Fig. S2A), all cell lines have variable response towards kinase inhibitors (I: Fig. S2B). These data offer insight into a potential non-genetic mechanism for the PP2A deregulation, characterize GBs high heterogeneity and resistance profiles in GB cells.

The GB cell lines exhibiting diverse genetic background and variable drug sensitivities were then screened for their sensitivity towards PP2A reactivating therapy. All tested GB cell lines show dose-dependent reduction in cell viability and in colony formation towards PP2A reactivator NZ-8-061 (I: Fig. 2C-E). Contrary to the kinase inhibitor responses, no clear differences in IC_{50}s or correlation to genetic background was found. As an inactive control, we used SMAP derivative TRC-766, which has a similar structure but is biologically inactive and unable to active PP2A even in high concentrations (Sangodkar et al. 2017). As expected, TRC-766 did not affect the cell viability of GB cell lines even at 40 mM concentrations (I: Fig. S3B). This fact supports PP2A reactivation mediated GB cell killing activity of NZ-8-061. To further test the therapeutic potential of NZ-8-061, we validated its potency in patient-derived primary glioma cells (Xie et al. 2015), which represent all three different molecular subtypes of GB (Q. Wang et al. 2017) (I: Fig. S4A-B). The patient-derived glioma cell lines were first shown to express Nestin and Sox2, as

well as PAIPs. They also have diverse genetic background and kinase inhibitor responses (I: Fig. S4C, S5A-B, S6, 2F). After treated with NZ-8-061, all primary glioma cell lines had nearly complete inhibition of cell viability (I: Fig. 2G). The lower NZ-8-061 concentration, when comparing to standard GB cell lines, is explained by culture medium as there is lack of serum in NSC media. These observations show NZ-8-061 potency across multiple GB cell lines with heterogenous genetic background, stemness properties and disease subtype.

5.1.3 Preclinical activity of NZ-8-061 in an infiltrative intracranial GB model (I)

To determine *in vivo* therapeutic efficacy of orally dosed NZ-8-061, we used previously published orthotopic model of GB (Claes et al. 2008). We selected E98 GB cell line due to its human GB-like infiltrative growth (I: Fig. 3A) (Claes et al. 2008), GSC properties (I: Fig. 2B), and its response to NZ-8-061 *in vitro* (I: Fig. 2C, E). NZ-8-061 was dosed twice a day at 30 mg/kg based on pharmacokinetic and tissue distribution data (I: Fig. 1E). That exposed the brain to approximately 10 μM of NZ-8-061, which is roughly the same concentration in which IC_{50} was achieved *in vitro* (I: Fig. 2E). NZ-8-061 achieved tumor growth stasis at day 12 and significantly reduced the tumor size until the end of the experiment (I: Fig. 3B). The therapeutic response was confirmed by a significant inhibition of proliferation in NZ-8-061 treated mice, through reduced Ki67 expression, at the endpoint tumor samples (I: Fig. 3C-D). Consistent with previously published data (Sangodkar et al. 2017; Kauko et al. 2018), no apparent systemic or CNS toxicities were observed in the NZ-8-061 treated mice (I: Fig. S7, S8A).

5.1.4 DBK-1154, with higher degree of brain/blood distribution, and more efficient in vitro activity, increases survival of mice bearing orthotopic GB tumors (I)

Although, NZ-8-061 was able to achieve significant size reduction in intracranial tumors, it failed to improve the survival of the mice (I: Fig. S8B). Therefore, we tested an alternative SMAP, DBK-1154, for its GB killing properties and BBB permeability. DBK-1154 can be considered to be more lipophilic because its dibenzoazepine tricyclic has a hydrocarbon bridge versus an oxygen bridge in NZ-8-061 (I: Fig. 1A). DBK-1154 (cLogP 7.0) has higher LogP (log octanol-water partition coefficient) than NZ-8-061 (cLogP 6.6), which is a measurement of lipophilicity. Furthermore, DBK-1154 (79 A^2) has lower total polar surface area versus NZ-8-061 (88 A^2). Higher cLogP value and lower polar surface are generally

correlate with better CNS distribution (Kelder et al. 1999). DBK-1154 shows significantly lower plasma concentration in mice when compared with NZ-8-061 after oral dosing (I: Fig. 4A, S8C). However, as can be expected, DBK-1154 has better *in vitro* BBB permeability (I: Fig. S9A-B) and *in vivo* it distributes to CNS better than NZ-8-061 as concentration in brain tissue is 2.3-fold higher than in plasma (I: Fig. 4A).

DBK-1154 inhibited cell viability with lower concentrations than NZ-8-061 (I: Fig. S9C), it also inhibited colony formation with almost 2-fold lower IC_{50}-values than NZ-8-061 (I: Fig. 4D) in GB cell lines. In addition, DBK-1154 induced apoptosis with a single 13 μM dose, but NZ-8-061 did not, at 48h shown by Caspase-3/7 cleavage (I: Fig. 4B). Similarly, DBK-1154's potency was seen across the patient-derived primary glioma cells (I: Fig. 4E). *In vivo* validation of DBK-1154 treatment was done in the same model as previously used with NZ-8-061. As DBK-1154 has lower oral bioavailability, we dosed it at 100 mg/kg, twice a day, which gave us approximately 8 μM CNS exposure based on the pharmacokinetic data. This again is roughly the same as IC_{50} of DBK-1154 *in vitro* (I: Fig. 4D). DBK-1154 therapy began reducing tumor growth after 5 days after the beginning of treatment (I: Fig. S9D) and reached a significant difference at day 14 (I: Fig. 4F). Furthermore, the higher *in vivo* brain penetrance and *in vitro* efficiency of DBK-1154 translated to almost 2-fold longer overall mouse survival. Significantly increased median survival for the DBK-1154 treated was 26 days and for the vehicle treated mice 15 days (I: Fig. 4G). These data indicate that DBK-1154 has great potential as a therapy candidate for GB.

5.2 Identification of kinases responsible for synthetic lethality in combination with PP2A activation in GB (II)

5.2.1 Pharmacological activation of PP2A robustly synergizes with a multikinase inhibitor UCN-01 (II)

We have previously shown that we can achieve synthetic lethality in GB cells by depleting an endogenous PP2A inhibitor PME-1 to reactivate PP2A in combination with multikinase inhibition (Kaur et al. 2016). Furthermore, we had already demonstrated that SMAP monotherapy increases the survival of an orthotopic GB mouse model (I). Based on these results we hypothesized that SMAPs can mimic PME-1 inhibition to induce synthetic lethality together with multikinase inhibition (II: Fig. 1A).

Our results confirm that PP2A reactivation by SMAPs (DT-061) sensitizes GB cells to multikinase inhibitor UCN-01 (II: Fig. 1B). This happens in a dose dependent

manner which is visualized in a synergy matrix (II: Fig. 1C). Moreover, the combination of UCN-01 and DT-061 significantly increased caspase 3/7 activity indicating increased apoptosis. Furthermore, pan-caspase inhibitor Z-VAD-FMK fully inhibited the apoptosis (II: Fig. 1D). Apoptosis was further confirmed with PARP cleavage (II: Fig. S1A). This synergistic phenomenon was further validated in multiple different GB cell lines (II: Fig. 1F, S1B). However, in non-cancerous fibroblasts we did not observe significant reduction in cell viability (II: Fig. S1C). The combinatory effect was also seen in hypoxic environment, which is a common resistance mechanism in GB (II: Fig. 1E, S1D, S1F). To assess efficacy of PP2A reactivation by DT-061 in combination with multikinase inhibition by UCN-01 *in vivo* we used same subcutaneous GB model as previously (Kaur et al. 2016). The combination treatment significantly reduced tumor growth when compared to monotherapies or the vehicle treated mice (II: Fig. 1G). These preliminary results greatly show the potential of PP2A reactivation by SMAPs to synergize with multikinase inhibition both *in vitro* and *in vivo* in GB.

5.2.2 Strategy for Characterization of Actionable Target kinase of multikinase inhibitors (SCAT)

UCN-01 is a nonspecific multi-targeted kinase inhibitor (Klaeger et al. 2017; Sato, Fujita, and Tsuruo 2002). In order to identify the specific kinases together with PP2A reactivation required for the SL in GB cells we developed a combination of screens: 1) LUMIER Beacon assay (Taipale 2018), which compares kinase binding of staurosporine derivatives 2) siRNA screen of potential kinase hits from the LUMIER Beacon assay together with PP2A reactivation 3) bioinformatics analysis of steps 1) and 2), and 4) identification of selective small molecule inhibitors for small molecule kinase inhibitor validation experiments (Fig. 2).

5.2.3 SCAT screening for sensitive kinases under PP2A reactivation (II)

In addition to UCN-01, we screened with SCAT different staurosporine analogues (CEP-701, K252a, rebeccamycin and K252c) together with DT-061 to see if they induce similar synthetic lethality as UCN-01. Out of the staurosporine analogues, K252a and CEP-701 induced synthetic lethality together with DT-061 in GB cells. However, K252c and rebeccamycin did not have as potent effect (II: Fig. S2A). Considering that UCN-01 and other staurosporine analogues are broad-spectrum kinase inhibitors, we set out to screen kinase selectivity profiles of them in an LUMIER Beacon assay (II: Fig. 2A, S2B). Binding of the staurosporines against kinases were measured as percentage of control treated samples. 28 kinases were

identified to interact with CEP-701, K252a, and UCN-01 but not with rebeccamycin or K252c after using a filter of -0.5-fold change as threshold for interaction (II: Supl. table S2).

In order to screen for kinases responsible for the synthetic lethality of GB cells together with PP2A reactivation, we decided to increase the discovered 28 kinase list to 37 kinases to include known altered kinases of GB (Brennan et al. 2013; Verhaak et al. 2010). The screen was conducted with a custom human kinase siRNA library (Qiagen) which had three non-overlapping siRNAs targeting each of the kinase genes in T98G cells together PP2A reactivation by either DT-061 treatment or PME-1 depletion (II: Fig. 2B-D, S2C). For each kinase, siRNA Gene Activity Ranking Profile (GARP) was calculated and synergy scores between siRNA kinases and DT-061 or siPME1 were explored using the Highest Single Agent and Bliss models. The best combinations of kinase siRNAs and PP2A reactivation were ranked (II: Fig. 2C) and selected for further validation (synergy score greater than 20). Additionally, a STRING interactive map analysis of hit kinases revealed Ras/Raf/MAPK and PI3K/AKT/mTOR pathways (II: Fig. 2E).

For target validation, we chose inhibitors targeting Ras/Raf/MAPK, PI3K/AKT/mTOR pathways as well as mitochondrial energy metabolism (Figure 5.). Requirements for the drugs were that they had to be orally bioavailable and cross the BBB. To confirm the specificity of the selected compounds we used kinase selectivity database (II: Supl. table S5) (http://129.187.44.58:7575/) (Klaeger et al. 2017). The selected kinase inhibitors were screened in GB cell line T98G together with different SMAPs, DT-061 and DBK-1154. As a PP2A reactivation control, we used an inactivate SMAP, DBK-766, to show that reactivation of PP2A is needed for the synthetic lethality (Sangodkar et al. 2017). The results indicate that SMAPs sensitize GB cells to MK-2206, AKT1/2i and OSU-03012 (AKT signaling), DCA (PDK1-4 inhibitor) and mubritinib (MINK1 inhibitor). Similar results were not achieved with the inactive SMAP (DBK-766) (II: Fig. 2F). This was further confirmed with a colony growth assay (II: Fig. S3D). However, RAF inhibitors Ly3009120 and Vemurafenib or PI3K inhibitor (Ly294002) did not achieve same combinatory effect with PP2A reactivation as was seen in the screen (II: Fig. 2F). Therefore, inhibitors of AKT, PDK1-4, and MINK1 kinases were selected for further study.

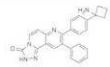
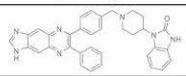
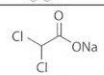
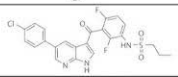
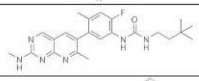
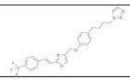
Drug	Target	Structure
MK-2206	Akt1-3	
AKT1/2i	Akt1/2	
LY294002	PI3Kα/β/δ	
OSU-03012	PDPK1	
DCA	PDK1-4	
Vemurafenib	B-Raf^{V600E}	
Ly3009120	A/B/C-Raf	
Mubritinib	MINK1	

Figure 5. Used kinase inhibitors, their targets and structure.

5.2.4 Hit inhibitors validation in heterogeneous GB cell lines (II)

As GB is known for its intratumoral heterogeneity and high intrinsic therapy resistance, we wanted to validate our kinase hits further. To achieve this, we selected multiple GSCs (BT3-CD133^{+}, BT12 and BT13) and established GB cell lines (U87MG and E98) to screen the potential combinations of kinase inhibitors in combination with SMAPs (Le Joncour et al. 2019). The sensitivity to direct AKT inhibition (MK-2206 and AKT1/2i) in combination with SMAPs varied throughout the GB cell lines. However, by targeting PDPK1 (OSU-03012) or PDK1-4 (DCA) in combination with SMAPs we observed a clear response in all the cell lines. Again, human fibroblasts were used as a control cells to show the GB cell specificity of the combinations (II: Fig. 3A-F). Similar results with the combinations were obtained in colony formation assay where combinations significantly inhibited the growth of GB cell lines and the GSCs when compared to human fibroblasts (II: Fig. S3D). Thus, we can conclude that under PP2A reactivation heterogenous GB cell lines are vulnerable for AKT pathway and PDK1-4 inhibition. Another hit from the screen, a MINK1 inhibitor mubritinib, sensitized T98G GB cells to SMAPs in nanomolar doses (II: Fig. 2G). However, other GB cell lines showed high resistance towards

mubritinib treatment (II: Fig. S3D). However, since mubritinib was not efficient in other GB cell lines we excluded the compound from further studies.

5.2.5 Triple targeted therapy advantage for heterogeneous GB (II)

As seen in previous results in (II: Fig. 3) not a single combination was efficient in all of the GB cell lines. This is a common problem in GB as very rarely compounds or combinations are universally effective against all the GB cell populations (Gimple et al. 2019). Therefore, we wanted to combine both AKT pathway and PDK1-4 inhibition together with PP2A reaction as a triple therapy. An advantage in targeting more than one kinase or pathway is increased potency. This is due to potential synergism of the inhibited pathways and shutdown of rewiring of cellular signaling. By combining PDK1-4 (DCA) and AKT (MK-2206) inhibition together with PP2A reactivation (DT-061) we were able to fully eradicate heterogenous GB cell lines and GSCs (II: Fig. 4A-B). This hints that there may have been rewiring and resistance mechanisms developing when only treating with one of the compounds in combination with PP2A reactivation.

After promising *in vitro* results, we proceeded to *in vivo* studies with two different orthotopic glioma models. The GSC (BT3-CD133$^+$) (Le Joncour et al. 2019) and GB cell line E98 (Claes et al. 2008; Merisaari et al. 2020) models were both used due to their variable growth patterns. As BT3-CD133$^+$ grows more as a bulk and E98 has a very infiltrative growth pattern which both in combination resemble the human GB very well. In short, mice were treated with either vehicle, PP2A reactivator (DBK-1160) or with the triple therapy combination of DBK-1160, DCA and MK-2206. We observed a significant growth difference between the vehicle and the triple therapy in orthotopic E98 mice model (II: Fig. 4D). Similar effect was not seen with GSC cells (II: Fig. 4C). However, these results did not translate in to increased survival in either of the mouse models (II: Fig S4). These data potentially indicate that *in vivo* there is even further rewiring happening to promote resistance in the GB tumors within the mice CNS.

5.2.6 Abolishment of the rewiring mechanism in heterogenous GBs

Fully consistent with our rewiring hypothesis, we found that MK-2206 fully inhibited the AKT S473 phosphorylation, but interestingly MK-2206 enhanced phosphorylation of PDHE1α S300, which indicates upregulation of PDH activity, in all the tested GB cell lines (II: Fig. 5A-C). In contrast, DCA-treatment completely removed phosphorylation of PDHE1α S300 but enhanced phosphorylation of AKT

S473 in T98G and BT3-CD133$^+$ cells (II: Fig. 5A-C). However, combination of MK-2206 and DCA shut-down phosphorylation of both phosphosites across all cell lines (II: Fig. 5A-C). DT-061 treatment altered AKT and PDK1-4 signaling in various ways. In all cell lines except for T98G, DCA+DT-061 combination inhibited AKT S473 phosphorylation but resulted in less efficient PDHE1α S300 inhibition than with DCA alone (II: Fig. 5C). DT-061 did rescue the compensatory PDHE1α S300 phosphorylation induced by MK-2206. Nonetheless, when GB cells were treated with triple therapy, both AKT S473 and PDHE1α S300 were efficiently inhibited, except for PDHE1α S300 signal in BT12 cells (II: Fig. 5A-C). To confirm apoptotic potential of the combination therapies, we observed induction of cleaved PARP after the triple therapy in all GB cell lines (II: Fig. 5A, D). Additionally, the combination of DCA and DT-061 were able to induce apoptosis in T98G (II: Fig. 5A, D). These observations confirm that there is clear signal rewiring in the GB cells and that there is a crosstalk between mitochondrial metabolism and growth signaling pathways. The data also supports the fact that increase in PP2A activity is required to achieve cytotoxicity across heterogenous GB cells after inhibition of both AKT and PDK1-4 signaling pathways.

5.2.7 SMAPs modulate mitochondrial functions (II)

We further evaluated how the drugs affect the cellular metabolism in mitochondria. The cells were exposed to either MK-2206 or DCA or in combination with DT-061 and analyzed by Seahorse Real-Time XF Analyzer. As excepted, DCA alone increases ATP production as it reactivates the oxidative phosphorylation in the mitochondria, which is commonly inhibited in cancer (II: Fig. 6H). On the contrary, MK-2206 seems to reduce ATP production (II: Fig. 6H) which is most likely due to its role in downregulating PDH activity (II: Fig. 5A, C), which in turn leads to reduced change of pyruvate to acetyl-CoA, hence reducing mitochondrial ATP production. Additionally, MK-2206 seems to disrupt mitochondrial-linked respiration and on the contrary, there is some indication that DCA might enhance the mitochondrial-linked respiration (II: Fig. 6B, F, J). Surprisingly, DT-061 had a broad-spectrum effect on mitochondrial metabolism. It seems to decrease the basal, maximal, and spare respiratory induced by DCA (II: Fig. 6B, F, J). Furthermore, alone and in combination with DCA or MK-2206, DT-061 causes increased proton leak (II: Fig. 6C). This data provides a potential explanation for DT-061 ability to lower the apoptotic threshold in combination with DCA and MK-2206.

5.3 Cationic polymer, PEI, functionalized MSNs exhibit selective inhibition of GSCs (III)

5.3.1 PEI-MSNs exhibit specific toxicity towards GSCs without induction of apoptosis or autophagy (III)

GSCs BT3-CD133^{+}, BT12, and BT13 (III: Fig. S2) and an established GB cell line T98G were exposed to PEI-MSNs (1-50µg/ml) in a colony formation assay (III: Fig. 1A-B). GSCs exhibited clear inhibition of colony growth after treatment with PEI-MSNs, even at the lowest (1µg/ml) concentration. However, similar effect was not observed in established GB cell lines T98G, A172, U87MG, or in MDA-MB-231 or HeLa (III: Fig. 1A-B, S3, S5). Although, some reduced colony growth was observed with the highest concentration in T98G, U87MG, and A172 (III: Fig. 1A-B, S3, S5). However, this observation has been established already in the literature since PEI can induce some non-specific toxicities in high concentrations (Vancha et al. 2004; Hunter 2006; Moghimi et al. 2005; Florea et al. 2002; Omidi and Kafil 2011). We further confirmed that PEI was required for the cytotoxicity as MSNs without PEI did not cause cell death at the concentration range of 1-50µg/ml (III: Fig. S4). Cell death mechanism for GSC was further investigated. BT12 was selected for the analysis as it was highly sensitive for PEI-MSN induced cell death. BT12 cells were exposed to PEI-MSNs for 24h and 48 hours in a concentration of 10 µg/ml. However, BT12 cells did not show any increase in apoptotic (cPARP) or autophagic (P62 & LC3B) activity in either of the time points when compared to control treated BT12 cells (III: Fig. 2A-B).

5.3.2 PEI-MSNs localize within the lysosomes and induce LMP (III)

As it is known that nanoparticles enter cell through endocytosis (S. Zhang, Gao, and Bao 2015), we wanted to confirm if this is also the case with PEI-MSNs in BT12 GSC. We selected early endosomes (EEA1), nucleus (DAPI), mitochondria (Mitotracker), and lysosomes (LAMP-1) to visualize cells and PEI-MSN localization within intracellular organelles (III: Fig. 3). After 48h treatment of BT12 cells with PEI-MSNs, particles were not observed within the nucleus, mitochondria or in the early endosomes (III: Fig. 3). However, PEI-MSNs were abundantly co-localized within the lysosomes (III: Fig. 3, S6). Nonetheless, with confocal microscopy we cannot detect individual particles because of its limited resolution as PEI-MSNs have only a 50 nm diameter (Hell and Wichmann 1994). Therefore, we were unable to identify any endosomal escape of PEI-MSNs and to confirm a proton sponge effect potentially induced by PEI-MSNs via lysosomal membrane destabilization.

To gain more insight into intracellular effects and localization of PEI-MSNs, we further used TEM. With TEM, we were able to see the widespread dissemination of single PEI-MSNs throughout the cytoplasm (III: Fig. 4). We observed three different types of vesicles with TEM within the BT12 cells: 1) vesicles still filled with PEI-MSNs (III: Fig. 4A) 2) completely empty vesicles with PEI-MSNs localized close to the vesicular membrane (III: Fig. 4B) 3) vesicles which are partially filled with PEI-MSNs (III: Fig. 4C-D). TEM images indicate that endosomal escape of PEI-MSNs has happened after LMP (III: yellow arrows in Fig. 4A-D). This specific membrane permeabilization of lysosomes in GSCs can potentially have been caused by an effect called proton sponge.

5.3.3 PEI-MSNs cause morphological abnormalities in GSCs (III)

We further observed multiple abnormalities within the cells caused by PEI-MSNs in the BT12 GSCs (III: Fig. 5). PEI-MSNs caused the widespread structural damage of mitochondria, such as swelling and rupture of cristae (III: Fig. 5C, D). Other abnormalities observed were loss of vesicular integrity which caused the high prevalence of PEI-MSNs in the cytoplasm (III: Fig. 5A-B). None on these were seen in the untreated control cells (III: Fig. S8A-D). However, PEI-MSNs were not observed to penetrate the nuclear space (III: Fig. S7A-B).

5.3.4 PEI-MSNs cross the neurovascular unit *in vitro* and *in vivo* (III)

To test whether PEI-MSNs could be potentially be used *in vivo* to target GSCs, we validated their permeability capabilities in an *in vitro* model of BTB (Le Joncour, Karaman, and Laakkonen 2019). In brief, the BTB model is established by co-culturing mouse brain microvascular ECs and astrocytes in Transwell inserts. When the cells have adhered and formed a BBB-like tight layer the inserts were placed on top of GSCs, after which PEI-MSNs were introduced to the endothelial side of the *in vitro* BBB. After 24h incubation of BTB with PEI-MSNs, some PEI-MSNs were still observed in the ECs and astrocytes. Mostly co-localized with lysosomes (III: Fig. 6A-B). To confirm the passage of PEI-MSNs through the *in vitro* BBB, we saw that PEI-MSNs were also abundantly detected in the BT12 GSCs on the other side of the *in vitro* BBB (III: Fig. 6C). We confirmed the integrity of the *in vitro* BBB after three days of PEI-MSN incubation by showing that the cell viability of the ECs or astrocytes was not reduced (only -3% and -6% respectively, when compared to non-treated). Interestingly, similarly as in colony growth assays, cell viability of

BT12 cells was reduced by 31% (III: Fig. 6D) suggesting specific toxicity against GSCs.

In vivo BBB permeability tests of PEI-MSNs were conducted in mice which had BT12 or U87MG GB cells orthotopically implanted. We evaluated two alternative delivery methods: through injection into caudal vein (IV) or intranasal dosage route. After IV injections, PEI-MSNs were observed in close proximity with brain blood capillaries in different regions of the cerebral cortex (III: Fig. 7A, C). With intranasal administration distribution close to blood vessels was not observed, but PEI-MSNs could still be observed all around the brain parenchyma, for example in the posterior parts of the encephalon (III: Fig. 7B-C). As expected, following intranasal dosage, PEI-MSNs were observed in the olfactory bulbs in very high density (III: Fig. 7D). Similar accumulation was not observed with IV injection. However, PEI-MSNs were still observed in the olfactory bulbs in similar densities as in the rest of the brain (III: Fig. 7D). Lastly, we confirmed that the PEI-MSNs penetrate into the intracranial tumors. With both delivery methods, we observed tumoral distribution of PEI-MSNs. However, IV injected mice seemed to exhibit better PEI-MSN accumulation at the tumor site (III: Fig 7E-F). Both delivery methods showed heterogenous and widespread distribution of PEI-MSNs throughout the brain. However, intranasally administrated PEI-MSNs showed very high concentration at the entrance point in the olfactory bulb. IV injected PEI-MSNs were more widespread in the distant brain structures such as hippocampus and the tumor than intranasally administrated PEI-MSNs. Nevertheless, this study provides a proof of principle that the brain tumors can be reached with non-invasive delivery methods. Furthermore, longer timepoints could show better distribution and intake of PEI-MSNs in the brain, as travelling PEI-MSNs are endocytosed at the nerve endings is slower than the blood flow.

6 Discussion

6.1 SMAPs inhibit growth of heterogenous GB cells and GSCs

GB has been heavily studied in the past decades and these efforts have resulted in crucial information from the genomic, transcriptomic, post-translational and metabolic landscape of GB. This knowledge has led into discovery of multiple driver mutations of GB and the disease itself is now classified into various subtypes (TCGA 2008; H. Yan et al. 2009; Noushmehr et al. 2010; Verhaak et al. 2010; Brennan et al. 2013; Q. Wang et al. 2017). Therapies for clinical studies of GB are often selected based on the most prominent mutation. However, because of high intratumoral heterogeneity in GB and RTK mutations often being subclonal, the efficacy of any single therapeutics is limited. This limitation is further obstructed by cellular rewiring which induces alternative pathways for survival and proliferation after therapeutic intervention (Johnson et al. 2014). Thus, there is still an unmet need for efficient therapeutics to improve the long-term survival of GB patients.

An alternative method to suppress oncogenic activity is to increase dephosphorylation of pathways, such as AKT or MAPK pathway (Westermarck 2018). We showed that increase in PP2A activity by SMAPs, DBK-1154 and NZ-8-061 caused efficient GB cell death *in vitro* (I). It has been previously indicated that increase in PP2A activity decreases activity of AKT and MAPK pathways (Puustinen et al. 2009). This phenomenon was also observed with SMAPs (II). SMAPs demonstrated efficacy in all the heterogenous GB cell and GSC populations with variable backgrounds (I). The data further shows that kinase inhibitors do not have similar universal effect in all the cells lines, as their efficacy was mainly specific in certain GB cell lines (I). In our study, SMAPs were dosed in micromolar concentration which is much higher than the normal concentrations for kinase inhibitors. However, this is explained by the amount of its molecular target, the PP2A, in cells (Y. Shi 2009). Furthermore, SMAPs are known to bind to serum, hence the potency is often hindered by the cell culture medium or by plasma proteins *in vivo*. Oral dosing of mice with SMAPs, with exposure that corresponded approximately the IC_{50}s of *in vitro* models, was able generate significant antitumor effects (I) in an infiltrative and aggressive intracranial GB model (Claes et al. 2008).

With DBK-1154, we were also able to demonstrate a significant, almost 2-fold, prolongation in survival of mice (I). These wide-spectrum effects of PP2A reactivation in heterogenous GB cell lines and GSCs can potentially be explained by PP2As role in multiple cancer driver pathways (Eichhorn, Creyghton, and Bernards 2009). Perhaps a broad PP2A reactivation could challenge the present paradigm of targeting genetically altered cancer drivers with specific inhibitors (Verhaak et al. 2010; Barretina et al. 2012; Brennan et al. 2013).

6.2 SMAPs exhibit good safety profile and high BBB permeability

There are multiple compounds to activate PP2A directly or indirectly. Natural compounds, such as celastrol and cucurbitacin B which inhibit CIP2A, have a lot of non-specific effects (Qin et al. 2018; Z. Liu et al. 2014). Additionally, the SET inhibitor, FTY720, which has been shown to be potent anti-leukemic drug has cardiotoxicity at the dose required for therapeutic levels (Vicente et al. 2020). The discovery that PPZ directly activates PP2A and have an anti-tumoral effect led to development of multiple derivatives in which the extrapyramidal and anti-cholinergic effects have been removed (Kastrinsky et al. 2015; Sangodkar et al. 2017; Gutierrez et al. 2014). Currently, most potent derivatives are SMAPs and iHAPs. Neither of them shows any toxic effects even at high doses. Both derivative families have potential for development and should not exclude each other as they activate different holoenzymes of PP2A. iHAPs mainly target B56ε and SMAPs B56α and B55α (Sangodkar et al. 2017; Leonard et al. 2020; Kauko et al. 2018; Morita et al. 2020). SMAPs are non-toxic when used on therapeutic levels. DBK-1154 has been shown to be non-toxic up to 800 mg/kg daily dosages with no observed body weight loss, adverse behavioral or neurological effects were observed (Sangodkar et al. 2017; Kauko et al. 2018; McClinch et al. 2018). For comparison, the therapeutic doses for intracranial GB models are 100 mg/kg. However, DBK-1154 induces hepatocellular hypertrophy (panlobular), in a dose dependent manner. This hypertrophy is likely due to the compound specific pregnane X receptor agonist activity (Y. Jiang et al. 2019). Nevertheless, this effect was adaptive rather than a permanent toxic effect (I). Due to the fact that SMAPs are not toxic they could be considered to be added to multiple combination treatments as a “background” therapy as PP2A inactivation has been shown to be a common resistance mechanism in GB (Kaur et al. 2016; Tomiyama et al. 2019).

Another limiting obstacle for the development of GB therapies is the BBB which limits the drug molecules access to the tumor site (Harder et al. 2018). Most small molecule drugs are small, lipid soluble molecules that cross the BBB by transmembrane diffusion. However, some drugs require active transportation

through the BBB, and therefore, penetration can be limited (Banks 2009). To limit unwanted side-effect, drug molecules developed for disease outside of the CNS often have poor BBB permeability Hence, BBB permeability is undesired if the target disease is not located within the CNS. This limits repurposing of drugs towards GB therapy. However, we demonstrated that SMAPs kept their original BBB penetration properties as they are derivatives of tricyclic neuroleptics (I). Structure of SMAPs is significantly different from the tricyclics, as the tertiary amine salt of tricyclics was replaced with an aryl sulfonamide and addition of a hydroxyl moiety. As can be expected from the total polar surface area and cLopP differences between DBK-1154 and NZ-8-061, the DBK-1154 preferentially partitions into the brain. Potentially this higher brain accumulation of DBK-1154 might explain its better potency in intracranial GB model when compared to NZ-8-061.

In conclusion, our results suggest that the chemical structure of DBK-1154 could be used as an example for further development of BBB permeable SMAPs. Altogether, the data from the first study (I) demonstrates sufficient preclinical proof-of-principle evidence for SMAPs as a novel therapy for GB. The SMAPs, NZ-8-061 and DBK-1154, exhibit *in vivo* efficacy, adequate safety profile and penetrate the problematic BBB in infiltrative intracranial model of GB. However, a clear limitation of our study (1) was that the *in vivo* studies were only done with one GB cell model. Therefore, therapeutic effects of SMAPs need to be addressed in the future in GB cell models representing all major GB subtypes.

6.3 Multikinase inhibitor resistance in GB is eliminated by PP2A reactivation

Even though SMAPs as a monotherapy already increased the survival of the mice, it is very likely that rewiring of cellular signaling would occur to develop resistance as was demonstrated in the study (I). Similar efficiency has been observed with multiple other monotherapies, as promising preclinical results with targeted single agent often have limited efficacy when they have translated into clinical trials of GB (Tomiyama et al. 2019; Kaley et al. 2019; Zanders, Svensson, and Bailey 2019). One possible reason for this discontinuity, for the targeted therapies, could be that information from genomic studies do not fully represent the tumor activity as they do not always predict PTMs and protein activities. Recent studies where genomic and phosphoproteomic data were gathered from same tumor samples demonstrated that genomic alterations rarely correspond with the PTMs. Therefore, to fully understand cancer cell signaling pathways, and potential resistance mechanisms, it is important to interpret both the genomic and the PTMs within tumor (Mertins et al. 2016; W. Wei et al. 2016). Hence, to fully inhibit tumor growth the concept of combination therapies seems reasonable for GB therapy.

Multikinase inhibitors have been proven to be effective in multiple malignancies (Gallogly, Lazarus, and Cooper 2017; T. Li et al. 2012; Rock et al. 2007). However, multikinase inhibitors induce unwanted side-effects as they have various off targets. Therefore, it is important to discover which are the specific kinases that are required for the antitumoral response. Earlier we have demonstrated that by combining PP2A reactivation with multikinase inhibition we can achieve synthetic lethality in GB cells (Kaur et al. 2016). However, this study had limitations as PP2A was reactivated through inhibition of an endogenous inhibitor of PP2A, PME-1, with in siRNA. Hence, we repeated the experiment with similar settings expect that we replaced PME-1 siRNA with a SMAP to reactivate PP2A. Thus, we demonstrated that PP2A reactivation by SMAP (DT-061) sensitized GB cells to UCN-01 both *in vitro* and *in vivo* (II). Furthermore, we achieved same synergistic phenomenon in multiple heterogenous GB cell lines through apoptotic cell death *in vitro* (II). The combinatory effect was also seen in hypoxic environment, which is a common resistance mechanism in GB (II) (Huang, Chen, and Zhang 2016). These preliminary results by us greatly reproduce the fact that PP2A is known to modulate kinase inhibitor responses in multiple hematological malignancies, NSCLC and GB (Neviani et al. 2013; Kaur et al. 2016; Kauko et al. 2018). However, our study was limited due to the fact that we used a subcutaneous *in vivo* model which lacks the CNS microenvironment which has an important role in GB development (Lenting et al. 2017).

6.4 Dissection of UCN-01 target kinases

In order to have more clinically relevant combination with PP2A reactivation for GB, we searched for alternatives for the multikinase inhibitor UCN-01. Even though UCN-01 has clinical transability, it does not cross the problematic BBB as it is a derivative of staurosporine (Jimeno et al. 2008). Furthermore, it is important to know which specific kinases need to be inhibited together with PP2A reactivation to achieve the synthetic lethality and to avoid the adverse effects through multikinase inhibition.

Considering that UCN-01 is a broad-spectrum kinase inhibitor, we set out to screen its kinase selectivity profile to find which kinases it potentially interacts with (II). In total we found 28 kinases to interact with UCN-01 (II). To increase the relevance towards GB, we included most commonly altered kinases of GB to the screen, in which we screened for the kinases responsible together with PP2A reactivation for the synthetic lethality of GB cells (Brennan et al. 2013; Verhaak et al. 2010). The screen identified MAPK and AKT pathways, mitochondrial metabolism (PDK1) and MINK1 as potential targets together with PP2A reactivation. Most of the hits from the screen, which synergize best with PP2A

reactivation, well resemble the common hyperactivated pathways in GB. AKT pathway, which is one of the most dysregulated pathways in GB (Brennan et al. 2013), was well presented in the screen as AKT1&3, PIK3CA and PDPK1 inhibition synergized with PP2A reactivation. Interestingly, monotherapies of these inhibitors have failed in clinical trials for GB (Wen et al. 2019; Kaley et al. 2019). Furthermore, inhibition of a common oncogene RAF1, which is also represented in a subset of GB patients (Schreck, Grossman, and Pratilas 2019), was found to synergize with PP2A reactivation (II). The third pathway identified was mitochondrial metabolism, as PDK1 inhibition synergized with SMAPs. PDK1 hyperactivity increases glycolysis in cells and decreases oxidative phosphorylation which is very common phenomenon in GB to promote growth and resistance to therapies (Michelakis et al. 2010). Furthermore, PDK1 inhibition, with a small molecule drug DCA, has been shown to induce apoptosis in GB cell lines (Michelakis et al. 2010). New identified target to synergize with PP2A reactivation in the screen was MINK1.

To confirm the screen results we chose inhibitors targeting MAPK, AKT pathways as well as mitochondrial energy metabolism and MINK1. Requirements for the drugs were that they had to be orally bioavailable and cross the BBB. The results indicated that SMAPs sensitized heterogenous GB cell lines to MK-2206, AKT1/2i and OSU-03012 (AKT signaling), DCA (PDK1-4 inhibitor) and mubritinib (MINK1 inhibitor) (II). However, RAF inhibitors Ly3009120 and Vemurafenib, or PI3K inhibitor Ly294002 did not achieve same combinatory effect with PP2A reactivation. Resistance to RAF inhibitor Vemurafenib could be caused by GB cell lines used not harboring the V600E mutant of BRAF which is required for the drug to work (Maverakis et al. 2015). However, Ly3009120 should work even in the absence of V600E mutation (Sullivan et al. 2020). The PI3K inhibitor, Ly294002, is a first-generation pan-PI3K inhibitors which is considered to have quite poor selectivity. Explanation for the ineffectiveness of Ly294002 could arise from PTEN downregulation, which is a common resistance mechanism in GB (Rodon et al. 2014; Brennan et al. 2013). Even though MINK1 inhibitor, mubritinib, had high potency towards T98G GB cell line in nanomolar doses together with SMAPs it failed to show similar efficacy in other GB cell lines. This difference in response can potentially be explained by the fact that modulation of MINK1 leads to multinucleated cells and inhibition of competition of abscission (Hyodo et al. 2012). This fact might make already multinucleated T98G cells more sensitive to this treatment under PP2A activation.

6.5 Crosstalk between the Akt pathway and mitochondrial metabolism

Nevertheless, none of the proposed double combinations achieved universal efficacy throughout all tested GB cell lines or GSCs (II). This result well presents the problem of clinical GB therapy. As GB is very heterogenous disease, often some subpopulations develop resistance towards the used treatment (Zanders, Svensson, and Bailey 2019). Furthermore, among the tested cell lines, GSCs were the most resistant towards the combinations (II). This resembles their role in the development of post-treatment resistance and recurrence of GB (Gimple et al. 2019).

With this, we hypothesized that by combining the inhibition of AKT pathway and activation of mitochondrial metabolism together with PP2A reactivation as a triple therapy, we would increase the potential of the therapy combination by blocking both pathways. The triple combination was able to fully eradicate heterogenous GB cell lines and GSCs through apoptotic cell death (II). However, the triple therapy did not achieve similar effect *in vivo* as it did *in vitro* (II). This discontinuity between *in vitro* and *in vivo* is most likely a result of many variables. Potentially the lack of in vivo efficacy could be explained by misleading data generated in 2D cell culture *in vitro*, in which cells undergo phenotypical changes (Benton et al. 2009; Gomez-Roman et al. 2017). For example, in GB cell lines it has been demonstrated that 3D culture induces resistance towards EGFR inhibitor erlotinib and radiation either alone or in combination (Gomez-Roman et al. 2017). Similarly, colorectal cancer cell lines cultured in 3D conditions have alterations in cellular morphology, phenotype, gene expression and were resistant to EGFR inhibition when compared to 2D culture (Poschau et al. 2015). Furthermore, microenvironment in brain also plays an important role in resistance towards therapies. Astrocytes have been shown to decrease sensitivity of GB cells to therapies *in vivo* and in 3D cell cultures (Pustchi et al. 2020; H. Zhang et al. 2020). Additionally, the compounds might not penetrate the tumor tissue in sufficient concentrations to achieve a stable therapeutic window and only induce cell death in the invading niche of GB cells. Hence, the bulk of the tumor grows and ultimately causes the death of mice due to neuronal damage and intracranial pressure.

Consistent with our rewiring hypothesis, we found that MK-2206 and DCA alter mitochondrial activity and growth signaling pathways. As can be excepted, DCA enhances mitochondrial-linked respiration and ATP production as it reactivates the oxidative phosphorylation in the mitochondria, which is commonly inhibited in GB (II) (Michelakis et al. 2010). On the contrary, MK-2206 downregulated PDH activity which in turn led to disrupted mitochondrial-linked respiration and reduce ATP production, which promote glycolysis and lactic acidosis, both being common mechanisms of therapy resistance in GB (Gatenby and Gillies 2004). However, we found an alternative resistance mechanism towards DCA-treatment, as DCA

enhanced the phosphorylation of AKT in some of the GB cell lines leading to increased activity of AKT pathway. The connection between AKT and PDK has been observed earlier, as AKT has been shown to directly decrease the activity of PDK (Hoxhaj and Manning 2020). However, the phenomenon most likely does not happen directly by AKT in this case. Therefore, PDK hyperactivity is possibly induced by via upregulation of HIF or p53 activity (Hoxhaj and Manning 2020; Contractor and Harris 2012). This signal rewiring between AKT and PDK pathways was completely abolished when MK-2206 and DCA were combined (II).

Interestingly, PP2A reactivation alone or in combination with MK-2206 or DCA altered GB cells in various ways. DT-061 was able to reduce the AKT S473 phosphorylation induced by DCA in all GB cell lines except T98G. This observation goes well together with the knowledge that PP2A inhibits AKT signaling (Sangodkar et al. 2016). In addition, DT-061 did downregulate the signal rewiring induced by MK-2206 by inhibiting the PDHE1α S300 phosphorylation. However, the triple combination of MK-2206, DCA and DT-061 completely inhibited both pathways *in vitro*. None of the compounds induced apoptosis as a monotherapy in the different GB cell lines. However, the triple combination induced robust cytotoxicity through apoptosis in all the GB cell lines. The results demonstrated that inhibition of both AKT and PDK1-4 pathways is required to inhibit the compensatory signaling and to achieve cell death, PP2A reactivation is required. Additionally, PP2A reactivation alone decreased basal, spare, and maximal respiratory capacity and inhibited the DCA-induced respiration.

Staurosporines induce the release of cytochrome c (Cyt c) and decrease mitochondrial membrane potential leading to apoptosis (Ly, Grubb, and Lawen 2003; Rego, Vesce, and Nicholls 2001). However, Bcl-2 overexpression can protect cells from apoptosis after staurosporine therapy as Bcl-2 hinders both the Cyt-c release and the decrease in mitochondrial membrane potential (Rego, Vesce, and Nicholls 2001). Therefore, the demonstrated synthetic lethality together with DT-061 and UCN-01 towards GB cell lines might be due to PP2As interaction with Bcl-2. It has been demonstrated that PP2A downregulates Bcl-2 activity (Deng, Gao, and May 2009) hence promoting Cyt-c release and the decrease in mitochondrial membrane potential. Furthermore, we demonstrated that DT-061 causes increased proton leak either directly, or through decrease in Bcl-2 activity, which has been reported to increase proton leak (Aharoni-Simon et al. 2016). However, the increased proton leak results in further decrease in mitochondrial membrane potential, leading to higher probability of apoptosis (Ly, Grubb, and Lawen 2003; Rego, Vesce, and Nicholls 2001).

In summary, we demonstrated that there is a clear crosstalk between the Akt and PDK signaling pathways which results in therapy induced resistance (II). Furthermore, we discovered that PP2A alters mitochondrial function by increasing

proton leak and inhibiting spare respiratory capacity, thus, reducing the apoptotic threshold of GB cells. As a result, we propose an alternative therapy for GB which mimics the multikinase inhibition, by combining AKT and PKD1 inhibition with PP2A reactivation.

6.6 GSCs exhibit specific vulnerability towards LMP

In the study (III), we originally aimed to develop PEI-MSNs for the delivery of siRNA (RNAi therapy) and small molecule drugs, such as UCN-01 into the CNS to treat GB tumors. Overall goal was to solve the problems with BBB permeability and to increase the accumulation of the compounds to the tumor site. However, in the preliminary studies of the PEI-MSNs in GB cells and GSCs, we discovered a specific toxicity towards the GSCs, as PEI-MSNs even without loading (drugs or siRNA) exhibited high lethality towards the GSCs. Thus, we decided to pursue this phenomenon.

During recent years MSNs have gained attention due to their good biocompatibility, biodegradability, and little to no toxicities to be used for drug delivery, diagnosis, or therapy (Ragelle et al. 2017). On the contrary, our results demonstrated that PEI-MSNs inhibited the growth of GSC even at 1µg/ml concentration (III), which is considered to be very low concentration as previously *in vitro* cells have been shown to tolerate MSNs up to 100 µg/ml (Hudson et al. 2008) and in animal models MSNs show no toxicities up to 200 mg/kg (Jie et al. 2010). Similar lethality was not observed in other established cancer cell lines even up to 50 µg/ml (III). We further discovered that the toxic effect was due to the PEI functionalizing of the MSNs as MSNs without PEI did not induce similar lethality in the GSC. Similar, PEI inducible non-specific toxicities have been earlier observed in high concentrations (Florea et al. 2002; Vancha et al. 2004; Hunter 2006; Moghimi et al. 2005; Omidi and Kafil 2011). The addition of PEI to the MSNs positively charges them, which is potentially the reason behind increased toxicity (Oh et al. 2010). This vulnerability of the GSCs contradicts the common consensus that GSCs are considered to be the most resistant population within the GB tumor (Gimple et al. 2019).

We found PEI-MSNs were abundantly localized within the lysosomes (III), which is normal as MSNs often enter cells by receptor-mediated endocytosis (Iturrioz-Rodríguez, Correa-Duarte, and Fanarraga 2019). Furthermore, we found with intracellular microscopic analysis that PEI-MSN disrupt the lysosomal membrane leading to eruption of lysosomal enzymes into the cytoplasmic space via LMP (III). We hypothesized that this phenomenon occurred due to an effect called “proton sponge” (Vermeulen et al. 2018), as PEI is believed to promote lysosomal

escape via the proton sponge effect (Nel et al. 2009). The LMP caused by proton sponge effect often leads to cell death though apoptosis or necrosis depending on the number of lysosomal enzymes discharged into the cytoplasm (F. Wang, Gómez-Sintes, and Boya 2018). We did not find any indications of apoptotic cell death in the GSCs after PEI-MSN treatment (III) which might predict that cells died acutely by necrosis. Similar vulnerability to LMP has been observed in the same GSCs by cationic amphiphilic antihistamine class drug, clemastine (Le Joncour et al. 2019).

To test whether the PEI-MSNs could be clinically relevant for the treatment of GB we demonstrated that they sufficiently cross the BBB of mice (III). Both delivery methods, intranasal and intravenous, exhibited widespread distribution of PEI-MSNs throughout the brain. However, intravenously injected PEI-MSNs were more spread into the distant brain structures and intranasally administrated PEI-MSNs accumulated into olfactory bulbs (III). Limitations of intranasal administration is that it only enters the brain through olfactory bulbs, as on the contrary intravenous injections has more comprehensive administration through the BBB. Earlier data has also shown that PEI-MSNs are considered to cross the BBB at ease (Baghirov et al. 2016), thus, making it a superior dosing regimen.

Therefore, our data strengthens the theory of the lysosomal vulnerability of GSCs as our data suggests that PEI functionalization of MSNs induces selective cell death of resistant GSCs by proton sponge effect. Nevertheless, a limitation of the study was that we only tested our hypothesis in three different GSCs. Thus, it would be important to find if this phenomenon occurs throughout different GSC populations.

7 Summary

We have previously demonstrated that PP2A is often inhibited in GB and acts as a modulator of kinase resistance. In this thesis, we confirmed PP2As non-mutational inhibition by its endogenous inhibitors throughout heterogenous GB cell lines. We established that a series of novel BBB permeable PP2A reactivators, SMAPs, exhibit robust cytotoxicity towards these heterogenous GB cell lines both *in vitro* and *in vivo*. We further revealed the specific kinases which to inhibit under PP2A reactivation to replicate PP2As combinatory effect together with UCN-01. The thesis presented a triple combination therapy, as AKT and PDK inhibition was highly effective against GB cell lines together with PP2A reactivation. In summary, results from these studies provide proof-of-principle data for the preclinical efficacy of SMAPs in GB together with potential combinatory therapies. However, these studies had limitations as only few orthotopic models of GB were used, thus, in the future efficacy of SMAPs needs to be evaluated in additional models representing all major GB subclasses.

In another study of this thesis, we demonstrated a novel use for nanoparticles as we observed that MSN functionalized with PEI induced specific cell death in GSCs through LMP. We hypothesized that this phenomenon occurred through the proton sponge effect. Taken together, the results reveal novel vulnerabilities in lysosome-associated pathways in GSCs. Furthermore, similar results were also obtained in another independent study. Additionally, the study indicates a therapeutic potential of the proton sponge effect by PEI functionalized MSNs.

Altogether, my thesis work reveals novel cellular vulnerabilities of GB to be used as potential therapy modalities in the future.

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