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Fishing for Cures:
The Zebrafish as a Powerful Tool to Identify Novel Therapies against Glioblastoma by targeting MTH1 and beyond

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Fishing for Cures:
The Zebrafish as a Powerful Tool to Identify Novel Therapies against Glioblastoma by targeting MTH1 and beyond

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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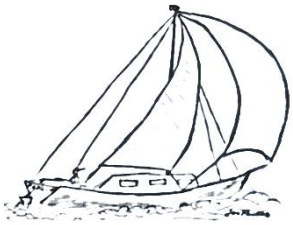

Twenty years from now you will be more disappointed by the things you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails.

Explore. Dream. Discover.

– Mark Twain

To my beloved family

ABSTRACT

Glioblastoma (GBM) is the most aggressive form of brain cancer. Despite today's combinatory therapy consisting of surgery, radio- and chemotherapy, the prognosis remains dismal. Fostered by extensive tumor heterogeneity, cancer cell plasticity and the presence of cancer stem cells, GBM evades almost any therapeutic strategy, leading to high mortality. Thus, the development of novel therapies is of urgent need.

With the identification of the Hallmarks of Cancer several cancer specific characteristics have been described that could serve as promising anti-cancer targets, including the combination of an elevated proliferation rate, crucial changes in cancer metabolism and consequently, an altered redox environment. Cancer cells and GBM in particular depend on effective anti-oxidant defense systems and non-oncogenic addiction enzymes such as MTH1, an enzyme that detoxifies oxidized bases to prevent DNA damage and subsequent cell death.

While potential anti-cancer targets are constantly being identified, the development of novel therapies against GBM is, amongst other reasons, hampered by the lack of orthotopic animal models that support large drug discovery screens. During the last decade, the zebrafish has been introduced as a clinically relevant model for human malignancies including cancer. Owing its biological and technical advantages, the zebrafish is the only vertebrate animal suitable for automated drug discovery screens to facilitate the identification and validation of novel cancer therapies.

In this thesis, we primarily focused on complementing established biochemical and cellular assays with a broad application of the zebrafish model to:

1. **Describe** factors that render cancer cells sensitive to MTH1 inhibitors
2. **Validate** MTH1 as a target in GBM and GBM stem cells
3. **Develop** a new orthotopic *in vivo* model for GBM

In **Paper I** we have demonstrated that the cellular redox environment and activation of the hypoxia signaling axis determine sensitivity to MTH1 inhibition *in vitro* and *in vivo*, thus suggesting that MTH1 inhibition may present a promising approach to treat cancers characterized by deregulated hypoxia signaling and redox imbalance.

In **Paper II** we have tested this hypothesis and showed that depletion or inhibition of MTH1 efficiently reduces viability of patient-derived GBM cultures independent of aggressiveness

in vitro and *in vivo*, thus providing supporting data that MTH1 represents a promising target for GBM therapy in particular.

In **Paper III** we addressed the lack of an orthotopic animal model for GBM which is suitable for large drug discovery screens. We found that GBM cultures transplanted into the blastoderm of zebrafish embryos form a congregated tumor in the central nervous system, fully recapitulating the human disease. As no intracranial transplantation is required, we have developed an orthotopic animal model for GBM that could readily be implemented in fully automatable drug discovery screens in order to accelerate the identification and development of novel therapies against GBM.

LIST OF SCIENTIFIC PAPERS

Thesis publications:

Paper I **Hypoxic Signaling and the Cellular Redox Tumor Environment Determine Sensitivity to MTH1 Inhibition**. Bräutigam L, **Pudelko L**, Jemth A-S, Gad H, Narwal M, Gustafsson R, Karsten S, Carreras Puigvert J, Homan E, Berndt C, Warpman Berglund U, Stenmark P, and Helleday T. *Cancer Research* (2016) 76, 2366-2375.

Paper II **Glioblastoma and glioblastoma stem cells are dependent on functional MTH1. Pudelko L**, Rouhi P, Sanjiv K, Gad H, Kalderén C, Höglund A, Squatrito M, Schuhmacher AJ, Edwards S, Hägerstrand D, Berglund UW, Helleday T, Bräutigam L. *Oncotarget* (2017) 20;8(49):84671-84684.

Paper III **An orthotopic glioblastoma animal model suitable for high-throughput screenings. Pudelko L**, Edwards S, Balan M, Nyqvist D, Al-Saadi J, Dittmer J, Almlöf I, Helleday T, Bräutigam L. *Neuro Oncol* (2018) 20(11):1475-1484

Additional publication, not included in this thesis:

MTH1 promotes mitotic progression to avoid DNA damage in cancer cells Gad H, Rudd S, Mortusewicz O, Stolz A, Amaral N, Bräutigam L, **Pudelko L**, Sanjiv K, Kaldéren C, Jemth A-S, Almlöf I, Visnes T, Schultz N, Boström J, Calderon Montano J, Hagenkort A, Groth P, Loseva O, Gokturk C, Koolmeister T, Wakchaure P, Homan E, Altun M, Ström C, Scobie M, Bastians H, Warpman Berglund U and Helleday T. *Submitted manuscript*.

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LIST OF ABBREVIATIONS

1-10	2-HG	2-hydroxyglutarate
	2-OH-dATP	2-hydroxy-2’-deoxyadenosine-5’-triphosphate
	8-oxo-dGTP	8-oxo-7,8-dihydroxy-2’-deoxyguanosine-5’-triphosphate
	8-oxo-G	8-oxo-guanine
A	A	adenosine
	ALA	5-aminolevulinic acid
	AMP	adenosine monophosphate
	ATP	adenosine triphosphate
B	BBB	blood-brain barrier
	BER	base excision repair
	BTB	blood-tumor barrier
C	C	cytosine
	Cas	CRISPR-associated systems
	CD133	prominin-1
	CDK	cyclin-dependent kinase
	c-kit	receptor tyrosine kinase kit
	CO_2	carbon dioxide
	CML	chronic myeloid leukemia
	CNS	central nervous system
	CRISPR	clustered regularly interspaced short palindrome repeats
	CSC	cancer stem cell
	CYP	cytochrome P450
D	DNA	deoxyribonucleic acid
	DMOG	dimethyloxaloylglycine
	DMSO	dimethyl sulfoxide
	dNTP	deoxyribonucleoside triphosphate
	dpf	days post fertilization
	DSB	double strand break
	DTT	1,4-dithiothreitol
E	eGFP	enhanced green fluorescence protein
	EGFR	epidermal growth factor receptor
	EMT	epithelial-mesenchymal transition
	ENU	ethyl-nitrosourea
	ETC	electron transport chain
F	FDA	Food and Drug Administration
	FGFR	fibroblast growth factor receptor

G	G	guanine
	GBM	glioblastoma
	GEM	genetically engineered mouse
	GLUT1	glucose 1 transporter
	GSC	glioblastoma stem cells
	GSH	glutathione
	GTR	gross total resection
	GRE	glioma-related edema
H	H_2O_2	hydrogen peroxide
	HCMV	human cytomegalovirus
	HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
	HER2	human epidermal growth factor receptor 2
	HIF1α	hypoxia-inducible factor 1α
	hMTH1	human MutT homolog 1
	hpf	hours post fertilization
	HRE	hypoxia response elements
I	IDH-1	isocitrate dehydrogenase-1
	IF	immunofluorescence
	i.v.	intravenous
L	LOH	loss of heterozygosity
	LUC	luciferase
M	MGMT	O^6-methylguanine-DNA-methyltransferase
	MMR	mismatch repair
	MTH1	mutT homolog 1
	MTIC	5-(3-methyltriazen-1-yl)imidazole-4-carboxamide
N	NAC	N-acetyl-L-cysteine
	NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
	Nrf-2	nuclear-factor (erythroid-derived 2)-like 2
	NUDIX	nucleoside diphosphate linked to some other moiety X
	NUDT1	NUDIX type 1
O	O_2	oxygen
P	PDGFRβ	platelet derived growth factor receptor ß
	PDX	patient-derived xenograft
	PI3K	phosphoinositide-3-kinase
	PHD	prolyl hydroxylase domain enzymes
	PPi	diphosphate
	PGE2	prostaglandin E2
	POLβ	polymerase β

	POLκ	polymerase κ
	PTEN	phosphatase and tensin homolog
	pVHL	van Hippel-Lindau tumor suppressor protein
	PVP	polyvinylpyrrolidone
R	RB1	retinoblastoma 1
	redox	reduction-oxidation
	ROS	reactive oxygen species
	RPE	retinal pigmented epithelial
S	siRNA	small interfering ribonucleic acid
	SOD	superoxide dismutase
	SOX2	SRY (sex determining region Y)-box 2
	STR	subtotal resection
T	T	thymine
	TALEN	transcription activator-like effector nucleases
	TKI	tyrosine kinase inhibitor
	TL	tupfel long-fin
	TMZ	Temozolomide
	TXN	thioredoxin
V	VEGFR	vascular endothelial growth factor receptor
W	WHO	World Health Organization
	WT	wildtype
Z	zf	zebrafish
	zfMTH1	zebrafish MutT homolog

1 INTRODUCTION

Despite intensive research for novel anti-cancer therapies, cancer remains one of the leading causes of mortality worldwide. Although the incidence of cancer increases with behavioral risk factors and age, specific cancer types predominantly arise in children and young adults of a certain age, or dependent on ethnicity, gender, and region – showing that cancer can affect anyone. According to the World Health Organization (WHO), annual cancer cases are expected to rise from 14 million in 2012 to 22 million within the next two decades, underlining the urge to develop successful anti-cancer therapies[1].

1.1 THE NATURE OF CANCER

Cancer summarizes a large group of diseases that can affect any part of the human body. It is generally thought that virtually all cells can transform into cancer cells. This transformation is a highly complex multistep process involving numerous alterations in molecular control mechanisms. Under physiological conditions, the cells in our body ensure tissue integrity and organ function by accurately balancing between cell proliferation and programmed cell death, so called apoptosis. However, genetic predisposition[1] as well as environmental factors[2], such as the exposure to radiation or toxins may facilitate the acquisition of mutations in our genetic code, also known as deoxyribonucleic acid (DNA), as well as changes in the epigenetic landscape. Some of these alterations induce the activation of oncogenes or the inactivation of tumor suppressor genes, which consequently disrupt the distinct balance between proliferation and apoptosis to promote abnormal tissue growth, or tumors. Generally, tumors can be benign or malignant. Benign tumors are localized and noninvasive, while malignant tumors are highly invasive as they grow beyond boundaries and spread through the circulatory system to invade distant organs and consequently, impair their function by forming metastases, the most deadly feature of cancer.

All of the features a cell may gain upon malignant transformation by the activation of oncogenes and the inactivation of tumor suppressor genes are summarized as the Hallmarks of Cancer, including self-sufficiency in growth signals and limitless replicative potential, resistance to growth suppressors and apoptosis, genome instability and mutation, induction of angiogenesis, capability of tissue invasion and metastasis, alteration in energy metabolism and evasion of immune detection[3] **(Figure 1)**. These features clearly distinguish cancer cells from normal tissue and consequently affect the development of how we treat cancer.

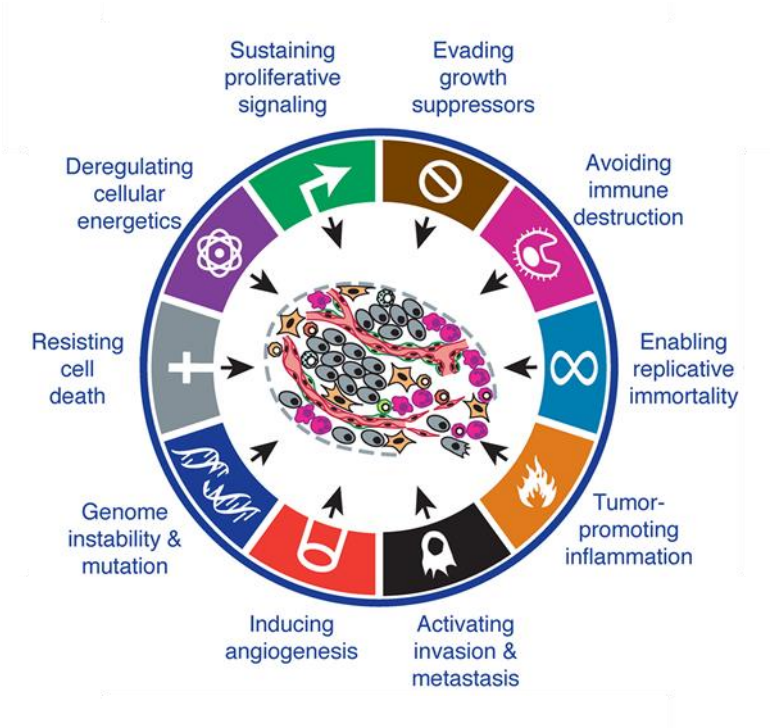


Figure 1: The Hallmarks of Cancer
This illustration presents an updated version of the Hallmarks of Cancer[4]. Due to a remarkable progress in cancer research the originally proposed hallmarks (sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death) have been complemented with enabling characteristics (avoiding immune destruction, tumor-promoting inflammation, genome instability and mutation, deregulating cellular energetics), thus offering a variety of molecular targets for the development of anti-cancer drugs. Reprinted with permission from Elsevier, figure adapted from Hanahan and Weinberg, 2011.

1.1.1 Cancer heterogeneity

Recent advances in sequencing technologies and molecular diagnostics have helped researchers to understand the highly dynamic nature of cancer. Cancer cells display distinct genotypic profiles that determine cellular properties such as morphology, metabolism, proliferation, metastatic potential and sensitivity to therapy[5]. These genotypic profiles vary between different cancer types (inter-tumor heterogeneity) as well as among cancer cells within the same tumor (intra-tumor heterogeneity)[6]. Inter-tumor heterogeneity is closely related to intra-tumor heterogeneity, which can be explained by two different, but not mutually exclusive models.

The "Clonal Evolution Model" implies that all cells in a tumor are biologically equivalent and potentially tumor initiating. As first described by Peter C. Nowell in 1976, cancers develop in a reiterative multistep process of clonal expansion by following the rules of Darwinian evolution[7]. The underlying mechanism of clonal expansion is the interplay of selectively advantageous "driver" mutations and selectively neutral "passenger" mutations

with changes in the microenvironment[8]. While most genetic and epigenetic alterations are deleterious for cells, selectively advantageous genetic and epigenetic alterations induce critical phenotypical changes that enhance survival and allow for tumor growth initiation by occupying distinct tissue habitats[9]. However, due to increased genomic instability, these phenotypes are highly unstable and therefore prone to accumulate further genetic and epigenetic alterations. Consequently, new sub-clones with a selective advantage arise resulting in successive clonal expansions and hence, polyclonal outgrowth[7] **(Figure 2a)**.

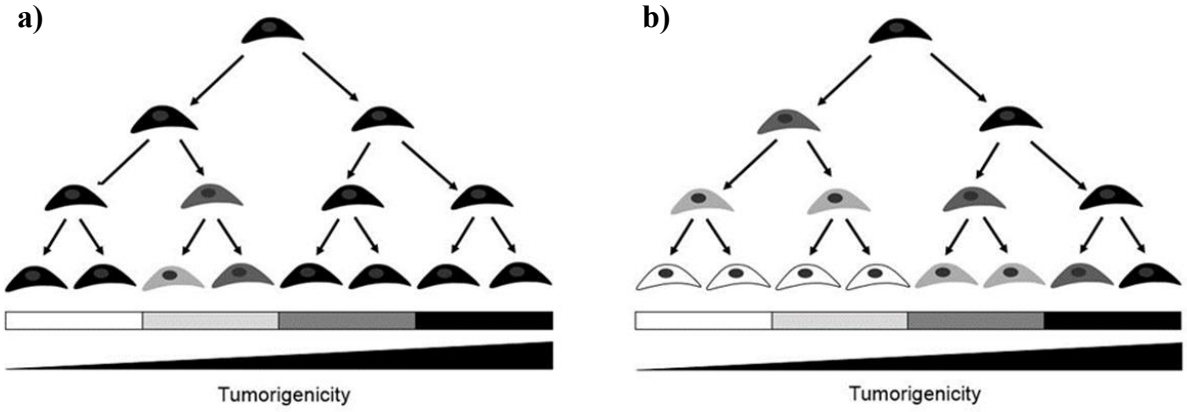


Figure 2: Models of tumor heterogeneity
According to the "Clonal Evolution Model" most of the cells residing in a tumor acquire selectively advantageous mutations that enhance survival and promote tumor growth initiation (a). The "Cancer Stem Cell Model" indicates that aberrant growth seen in tumors originates from and is sustained by a mutated population of stem cells, which have the ability to self-renew and differentiate. Differentiated cancer cells gradually lose tumorigenicity (b).

However, over the past decade, an increasing amount of data argued for the existence of tumor-initiating cells, often referred to as cancer stem cells (CSCs). According to the "Cancer Stem Cell Model" cancers are derived and maintained by a subset of highly tumorigenic CSCs, which are defined by their ability to self-renew and to differentiate into non-tumorigenic progeny[10]. While inter-tumor heterogeneity depends on differences in the transformed stem cell of origin, intra-tumor heterogeneity reflects phenotypic differences induced by epigenetic alterations[11] **(Figure 2b)**.

On a conceptual basis, it is more likely that aberrant growth seen in cancer originates from a mutated population of stem cells, which has lost normal homeostatic control on tissue development. Compared to other cell types, CSCs are considered to be suitable candidates for malignant transformation as they share similar features as normal stem cells such as self-renewal capacity and a long life span. Since CSCs are able to escape the limits of

proliferation, they can accumulate genetic mutations over a long time[12]. These considerations suggest that malignant transformation arises due to oncogenic mutations in the stem cell compartment of normal tissues, resulting in unlimited proliferation and cancer. Evidence for the existence of CSCs and their tumorigenic potential was first demonstrated in leukemia [13,14] and subsequently in solid tumors including breast cancer[15] and glioblastoma[16].

The highly dynamic and unique genetic composition of individual cancers offers clinically significant challenges in terms of identifying suitable anti-cancer targets, thereby hampering the generation of effective treatment strategies[6]. Therefore, it is inevitable to understand the underlying mechanism of cancer cell propagation and the extent of its clonal architecture as early as possible. Taken together, both models offer valuable approaches to understand and characterize cancer heterogeneity leading to the development of refined treatment strategies.

1.1.2 Hypoxia in cancer

Generally, hypoxia describes a state of reduced oxygen tension that is potentially detrimental to aerobic organisms. In the context of cancer it refers to the situation where rapidly proliferating cancer cells outgrow their blood supply, consequently leading to the generation of areas with significantly lower oxygen concentrations than present in healthy tissues[17]. However, cells that reside in such hypoxic niches adjust their gene expression profile to the low oxygen supply through the pVHL-HIF1 system, the key mediator of oxygen homeostasis.

The van Hippel-Lindau tumor suppressor protein (pVHL), an E3 ubiquitin protein ligase is responsible for regulating the oxygen-dependent stability of hypoxia-inducible factor 1 (HIF1). HIF1 is a transcription factor regulating the expression of genes involved in angiogenesis, proliferation and survival as a response to low oxygen levels. It is a heterodimer consisting of two subunits, HIF1α and HIF1β. Subunit HIF1α is constitutively expressed under both normoxic and hypoxic conditions and localized in the cytoplasm[18,19].

In the presence of oxygen, HIF1α is rapidly degraded by oxygen and iron dependent prolyl hydroxylase domain enzymes (PHDs) which hydroxylate HIF1α at two prolyl residues. The hydroxylated form of HIF1α is recognized and bound by pVHL, which directs the attachment of a polyubiquitin chain and induces proteasomal degradation of the HIF1α subunit, thereby inhibiting its transcriptional activity[20] **(Figure 3**).

However, in the absence of oxygen, PHDs are inactive and the hydroxylation of HIF1α proline residues is suspended. Consequently, subunit HIF1α is stabilized and translocates to the nucleus, where it forms the heterodimer HIF1 together with subunit HIF1β[18,20,21]. Upon binding to so called hypoxia response elements (HRE), which represent specific DNA binding sites of target genes, HIF1 activates the expression of hypoxia-inducible genes in order to adapt to hypoxic conditions[22] **(Figure 3)**.

Based on the oxygen-dependent regulation of multiple essential signaling pathways involved in cell proliferation, survival as well as angiogenesis, hypoxic niches play a crucial role in the development and progression of cancer. It has been shown that hypoxia furthermore promotes cancer cell mobility, metastasis and therapy resistance by inducing epithelial-mesenchymal transition (EMT)[17] as well as maintenance of cell quiescence and self-renewal capacity as observed in CSCs[23]. In addition to that, increased HIF1 activity alters cellular metabolism by inducing a shift from normal energy metabolism to aerobic glycolysis[24], which is further elaborated on in the following section.

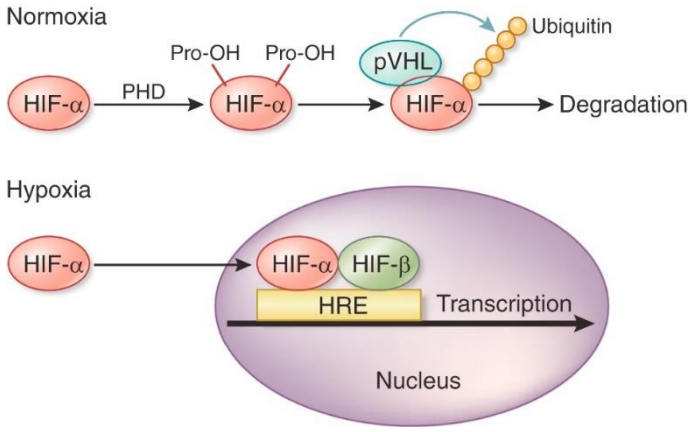


Figure 3: Prolyl hydroxylase domain-hypoxia-inducible factor (PHD-HIF) oxygen-sensing system
In the presence of oxygen, HIF1α is hydroxylated at specific proline residues by PHD proteins, and thus recognized by pVHL which induces HIF1α ubiquitination and degradation by the proteasome. In the absence of oxygen, HIF1α is stabilized and translocates to the nucleus where it dimerizes with HIF1β to bind HREs, hence inducing the transcription of target genes. HIF1, hypoxia-inducible factor 1; PHD, prolyl hydroxylase domain; pVHL, von Hippel-Lindau protein; HRE, hypoxia response elements. Reprinted with permission from Elsevier, from Sugahara *et al.*, 2017.

1.1.3 Metabolic changes in cancer

In order to sustain essential molecular processes and biological functions, normal cells that experience aerobic conditions primarily produce energy upon full oxidation of glucose via oxidative phosphorylation in mitochondria. Under hypoxic conditions normal cells are limited to produce energy by breaking down glucose into lactate in the cytosol via (anaerobic) glycolysis[24], which is also the main energy source in brain[25], liver[26] and muscle cells[27,28]. However, this process requires far more glucose while being energetically unfavorable as it produces only 5 % of glucose's energy potential.

In 1924, Otto Warburg discovered that cancer cells predominantly produce energy by an excessive conversion of glucose to lactate even in the presence of oxygen. This process is called aerobic glycolysis, or the "Warburg effect"[29]. Due to the fact that aerobic glycolysis is less efficient than oxidative phosphorylation in terms of adenosine triphosphate (ATP) production, cancer cells' preference for aerobic glycolysis remained questionable. While Warburg himself postulated that the change in metabolism is the fundamental *cause* of cancer[30], current explanations rather indicate that it is the ultimate *consequence* of malignant transformation such as an adaptation to deficient oxidative phosphorylation in damaged mitochondria or to hypoxic areas, as mentioned earlier. However, as this does not explain why cancer cells rely on aerobic glycolysis in the presence of oxygen, the metabolic change may also be associated with rapid cell growth as glycolysis increases the generation of metabolites required in proliferating cells[31]. Therefore, it has been postulated that the metabolism of cancer cells, similarly to proliferating cells in general, has adapted to rapid cell growth by facilitating both uptake and incorporation of nutrients into the biomass[32]. Along with this hypothesis, it has been shown that numerous oncogenes which are activated in cancer cells such as phosphoinositide-3-kinase (PI3K), Myc and Ras are linked to both growth control and glucose metabolism *i.e.* by regulating the expression of glucose 1 transporter (GLUT1) on the cell membrane and hence, the influx of glucose that is subsequently converted via aerobic glycolysis[33].

While the precise biological reason for the Warburg effect remains unknown, the most reasonable explanation for the metabolic change seen in cancer cells is the constant generation of building blocks for new cancer cells. However, the price cancer cells have to pay for this metabolic switch in order to maintain constantly high growth rates is reflected in an altered redox balance[34], which is discussed in the following section.

1.2 REDOX HOMEOSTASIS

1.2.1 Redox balance and redox signaling

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_{2-}), which can be produced unintentionally as unavoidable side products of aerobic metabolism or intentionally by various enzymatic systems, constitute the redox environment of a cell. Under physiological conditions, cells have comprised highly efficient scavenging systems to balance the production and elimination of ROS and thereby, maintain redox homeostasis.

When present in low levels, these reactive molecules regulate cellular signaling pathways that are involved in cell proliferation, differentiation and survival[35]. Amongst others, central transcription factors including anti-oxidant major regular Nrf-2 (nuclear-factor (erythroid-derived 2)-like 2)[36], HIF1α[21] and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)[37] are known to be redox regulated. Interestingly, most transcription factors underlying redox regulation are closely associated with cancer. Major targets for redox signaling are cysteines as they present a unique target for the reversible oxidation/reduction of their residues[38]. Following oxidation, cysteines form intra- or intermolecular disulfide bonds, which may alter protein structure and thereby regulate co-factor binding or dimerization. Additionally, they can be glutathionylated resulting in the regulation of enzymatic activity[39,40].

However, due to their high chemical reactivity, excessive ROS levels can damage cellular proteins, lipids as well as nuclear and mitochondrial DNA leading to cellular senescence, apoptosis and carcinogenesis[41,42].

1.2.2 Redox balance in cancer

Both an altered redox homeostasis and deregulated redox signaling present generally accepted hallmarks of cancer cells. Following the initiation of tumorigenesis by exposure to radiation or carcinogens, the acquisition of genetic, metabolic and microenvironment-associated alterations lead to persistently upregulated ROS levels[43]. In order to survive, cancer cells compensate these high intrinsic oxidative stress levels by increasing their anti-oxidant capacity[44–46], thereby shifting the overall redox balancing point upwards **(Figure 4a)**[43]. Paradoxically, this adaptation further promotes tumor growth by generating additional DNA damage and genomic instability[47]. However, simply increasing ROS levels to induce cancer cell death remains insufficient, which is likely due to the high plasticity of the oncogenic redox system[48] and its efficient mechanisms of ROS detoxification, promoting

growth under oxidizing conditions. Therefore, disabling the ROS defense system by targeting anti-oxidant or other non-oncogenic addiction enzymes has become a promising area of research **(Figure 4b)** [46,49].

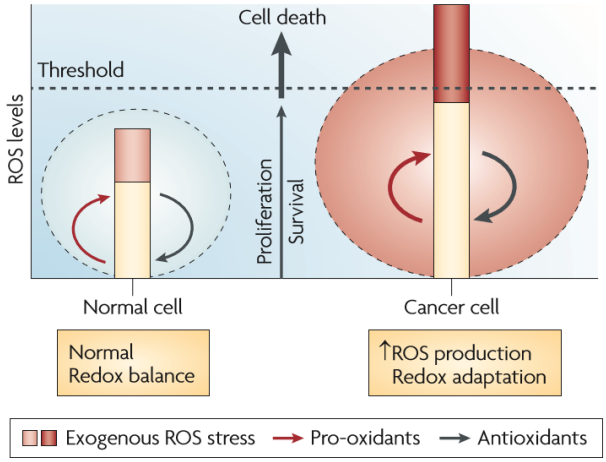
1.2.3 DNA integrity and oxidative damage in cancer

The genetic information of all living organisms, including humans, is enclosed in the DNA, or deoxyribonucleic acid, where it is stored as a code consisting of four chemical bases, the purine bases adenine (A) and guanine (G) as well as the pyrimidine bases cytosine (C) and thymine (T). Similar to letters in the alphabet, the order of these bases determines protein composition and hence, all information for building an organism and maintaining its multicellular functionality. Each DNA base can be supplemented with a sugar molecule to form a nucleoside, or with a sugar and phosphate molecules to form a nucleotide. For the incorporation into the DNA, purine bases always pair up with pyrimidine bases (A:T and G:C) to build up a complementary nucleotide double helix, the unique structure of our DNA[50].

As described by Nobelist Thomas Lindahl in 1993, the chemical stability of our DNA is constantly challenged by numerous endogenous and exogenous substances that cause DNA lesions via methylation, hydrolysis and oxidation[51]. Such lesions can be recognized by complex DNA protection and repair mechanisms to maintain genomic integrity and cellular fitness. However, misrepair of potentially lethal lesions may result in disturbed expression of oncogenes and tumor suppressor genes leading to malignant transformation and carcinogenesis.

Among other macromolecules in the cell, the DNA represents the major target of highly reactive ROS molecules. Aside from the nucleotide double helix, it is well established that the free deoxyribonucleoside triphosphate pool (dNTP) is especially prone to oxidative damage[41]. Among all nucleotides, deoxyguanosine triphosphate (dGTP) is most prevalently oxidized upon exposure to ROS due to its favorable chemical structure. Its product, 8-oxo-7,8-dihydroxy-2'-deoxyguanosine-5'-triphosphate (8-oxo-dGTP), can readily be incorporated into the DNA causing mutagenic G:C to T:A and *vice versa* transversions, subsequently leading to genomic instability and cell death[52,53].

a)



b)

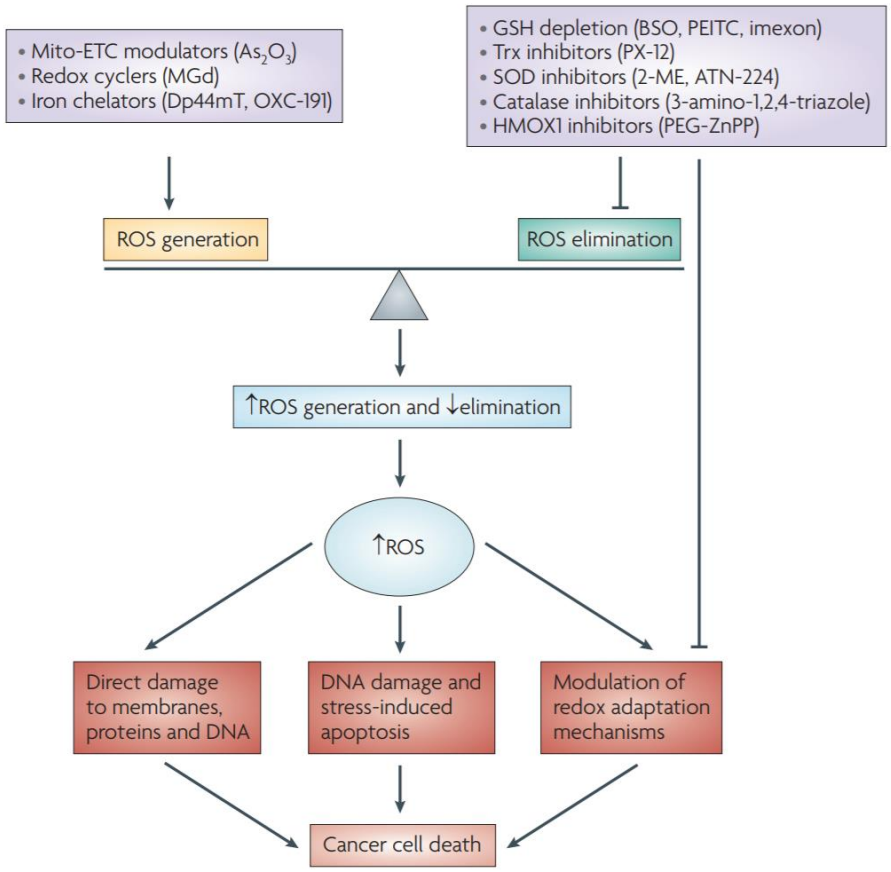


Figure 4: Targeting redox alterations in cancer

In order to survive and proliferate, cancer cells display a highly plastic redox environment and adapt to elevated ROS pressure by increasing their anti-oxidant ability, thereby shifting the overall redox balancing point upwards (a). As cancer cells depend on ROS elimination systems to ensure growth under oxidizing conditions while keeping ROS levels below the toxic threshold, they are more vulnerable to further oxidative insults. Hence, stimulating ROS generation and targeting ROS elimination system offers therapeutic selectivity to fight against cancer cells. Ideally, the simultaneous exposure to ROS-generating agents and compounds that interfere with cellular anti-oxidant systems may induce additive or synergistic effects to promote ROS-induced damage and subsequent cancer cell death (b). ROS, reactive oxygen species. Reprinted with permission from Springer Nature, from Trachootham *et al*., 2009.

1.2.4 MTH1, a sanitizing enzyme

As indicated before, for the maintenance of genomic integrity and cellular fitness, cells comprise complex and highly efficient DNA protection and repair mechanisms. Protection mechanisms include nucleotide sanitation enzymes that prevent the incorporation of deleterious bases into the DNA by promoting their immediate elimination[54]. However, if the amount of erroneous nucleotides outweighs the house-cleaning capacity of sanitation enzymes, first line protection mechanisms fail rendering the incorporation of damaged bases into DNA inevitable. For this case, cells have developed a variety of repair mechanisms including base excision repair (BER)[55] and the mismatch repair (MMR) machinery[56] to detect and excise deleterious bases.

Focusing on DNA protection mechanisms, the most common sanitation enzymes have been described as members of the nucleoside diphosphate linked to some other moiety X (NUDIX) hydrolase family[57]. Despite sequential and structural differences among the 22 family members described in humans, all members share a distinct amino acid NUDIX box domain, which determines their function: catalyzing the hydrolysis of nucleoside-like di- or triphosphate to respective monophosphates[58,59]. The variety of substrate preferences among NUDIX family members is linked to structural differences, respectively[59].

Human NUDIX hydrolase NUDT1 (NUDIX type 1), also known as human mutT homolog 1 (MTH1) has recently been identified as a sanitation enzyme in cancer cells[60,61]. Due to the altered redox homeostasis and increased oxidative pressure, cancer cells rely on efficient anti-oxidant systems and other non-oncogenic addiction enzymes, such as MTH1. It has been shown that the MTH1 enzyme sanitizes the oxidized dNTP pool by hydrolyzing 8-oxo-dGTP and 2-OH-dATP (2-hydroxy-2'-deoxyadenosine-5'-triphosphate) to their respective monophosphates[61], thereby preventing incorporation of these erroneous nucleotides into DNA and potentially lethal consequences[49] **(Figure 5)**.

Recently, we and others have extensively described MTH1 as a promising anti-cancer target[49,62–65]. Our group was able to show that depletion of MTH1 either by siRNA-mediated knockdown or exposure to in-house developed MTH1 small molecule inhibitors induces cancer-specific DNA damage and subsequent cancer cell death *in vitro* and *in vivo*[49,64] **(Figure 5a)**, while having minor effects on normal cells. In 2016, the role of MTH1 in cancer was challenged, since some small molecule MTH1 inhibitors were not cytotoxic despite inhibiting the MTH1 enzymatic activity *in vitro*[66–68]. Following extensive research to further understand the mechanism of action of our potent and cytotoxic MTH1 inhibitors, we now

know that they act via a dual mechanism to induce cell death by A) causing a mitotic arrest, which further induces production of ROS and 8-oxo-dGTP and B) inhibition of 8-oxo-dGTPase activity resulting in elevated incorporation of oxidized nucleotides into DNA **(Figure 5b)** (unpublished data).

Here, we determine the characteristics that render cancer cells sensitive to MTH1 inhibition. Based on the close relation to an elevated redox environment present in cancer cells, it is moreover inevitable to study the effect of MTH1 inhibition in highly aggressive and treatment-resistant glioblastomas, which suffer tremendous oxidative pressure.

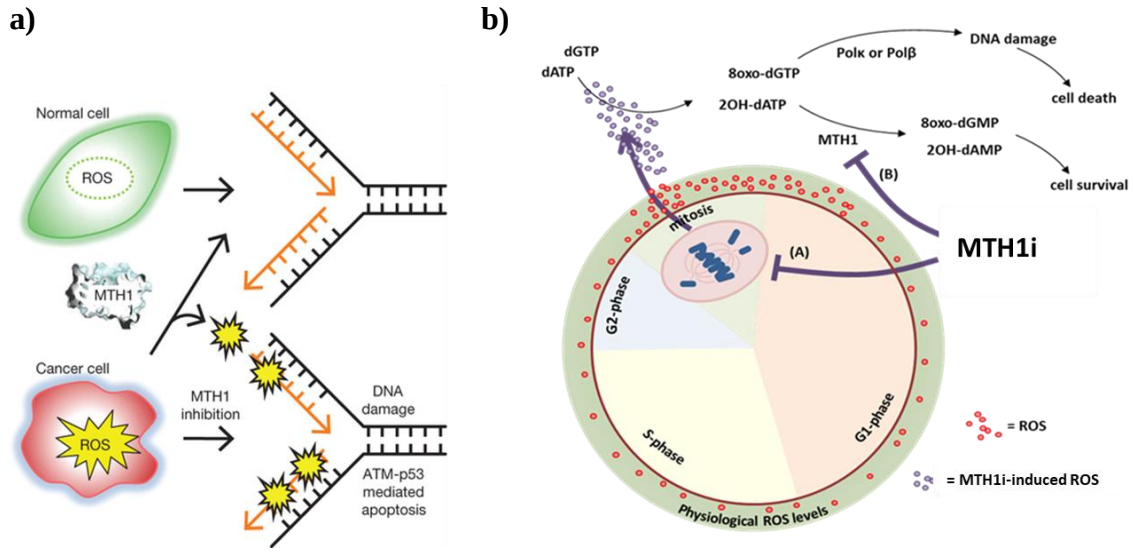


Figure 5: MTH1 inhibition as a promising anti-cancer therapy
As originally proposed, inhibition of sanitizing enzyme MTH1 leads to the incorporation of oxidized nucleotides into DNA and hence, induction of DNA damage with subsequent cancer cell death (a). According to the updated mechanism of action, cytotoxic MTH1 inhibitors interfere with tubulin dynamics, which arrest cells in mitosis and cause an increase in ROS, specifically in cancer cells. High levels of ROS oxidize the nucleotide pool to form oxidized deoxynucleoside triphosphates, such as 8-oxo-dGTP and 2OH-dATP. Additionally, cytotoxic MTH1 inhibitors prevent MTH1-mediated clearance of 8-oxo-dGTP and 2-OH-dATP, which results in Polκ- or Polβ-mediated incorporation of 8-oxo-dGTP and 2-OH-dATP into DNA, thereby causing DNA damage and cell death (b). MTH1, MutT homolog 1; MTH1i, MTH1 inhibitors; DNA, deoxyribonucleic acid; ROS, reactive oxygen species; 8-oxo-dGTP, 8-oxo-7,8-dihydroxy-2'-deoxyguanosine-5'-triphosphate; 2-OH-dGTP, 2-hydroxy-2'-deoxyadenosine-5'-triphosphate; Polκ, polymerase κ; Polβ, polymerase β. Reprinted with permission from Springer Nature, from Gad *et al.*, 2014.

1.3 GLIOMA AND GLIOBLASTOMA

Gliomas represent the most common tumors of the central nervous system (CNS) and are classified into clinical grades of ascending malignancy based on histopathological criteria by the WHO[69]. Grade IV gliomas, namely glioblastoma multiforme (GBM), are the most malignant and aggressive primary brain tumors displaying the worst prognosis with only 10 % of diagnosed patients surviving 5 years[70].

Over the past decade, scientists have discovered various genetic mutations and abnormalities in different pathways promoting the disease progression of GBM. Occurring in 60 - 90 % of all GBM cases, loss of heterozygosity (LOH) on chromosome arm 10q represents the most frequent gene alteration in GBM[71]. Common genetic abnormalities include *i.e.* epidermal growth factor receptor (EGFR) amplification[72] as well as mutations in tumor suppressor genes p53 and PTEN (phosphatase and tensin homolog)[73,74]. As the disease progresses, additional genetic alterations can be found. Some of the genetic alterations present in GBM are currently targeted for therapeutic use. However, identifying an optimal target is hampered by the fact that GBMs are composed of a highly heterogeneous mixture of tumor cells[75]. On top of that, high cellular plasticity promotes the dedifferentiation of tumor cells into a more stem cell-like state[76]. Until today, multiple studies support the theory of a prominin-1 $(CD133)^+$ stem cell population in GBM, which is responsible for both maintenance of a tumor and tumor recurrence after therapy[16]. Strongest evidence for the existence of CSCs is provided by generating a phenocopy of the original patient's tumor *in vivo* upon transplantation of $CD133^+$ but not $CD133^-$ cells into immunodeficient mice. Most recently, researchers have found heterogeneous CSC sub-clones exhibiting dissimilar phenotypes regarding morphology, self-renewal, proliferative capacities and therapeutic sensitivities[76], thereby further hampering the identification of a common target.

1.3.1 Standard therapy of care

Currently, the standard treatment of brain tumors consists of surgical resection, radiotherapy and adjuvant chemotherapy. Depending on the individual tumor size and shape, its location in the brain and surrounding blood vessels, surgeons choose between gross total resection (GTR) and subtotal resection (STR). Tumor-specific fluorescent staining such as 5-aminolevulinic acid (ALA) enables precise distinction between tumor and non-tumor cells in order to facilitate maximal resection of the malignant tumor mass[77]. Remaining tumor cells are targeted by subsequent radiotherapy, which induces DNA damage, most prevalently double-strand breaks (DSB), leading to apoptosis. Resistance mechanisms are usually

conferred by upregulated DSB repair machinery rendering tumor cells insensitive to radiotherapy[78]. The standard adjuvant chemotherapy against GBM is Temozolomide (TMZ), a prodrug that is able to pass the blood-brain barrier (BBB). Once converted to the active form, the alkylating agent MTIC (5-(3-methyltriazen-1-yl)imidazole-4-carboxamide), it methylates purines in the DNA[79]. If left unrepaired, these lesions induce tumor cell death. However, it has been shown that the sensitivity to TMZ correlates with the methylation state of the O^6-methylguanine-DNA-methyltransferase (MGMT) promoter in tumor cells. Epigenetic silencing of the MGMT gene results in low intracellular concentrations of MGMT, thereby increasing the sensitivity to TMZ and promoting tumor cell death[80].

To sum up, the therapeutic inefficacy of TMZ in GBM cells with high cellular concentrations of MGMT combined with the risk of possible side-effects such as TMZ-induced DNA damage in healthy cell highlight the urge to improve GBM therapy regarding efficacy and specificity.

1.3.2 Alternative treatment options

All together the combination of surgical resection, radiotherapy and adjuvant chemotherapy with TMZ has improved the average patient survival of 12.1 months to 14.6 months[81]. Other Food and Drug Administration (FDA)-approved drugs against GBM show similar modest effects such as anti-angiogenic drug Avastin (Bevacizumab)[82,83] and Lomustine, either alone or in combination[84].

Today's treatment options remain insufficient as patients suffer tumor recurrence due to inherent or acquired resistance mechanisms within 6 month after resection[70]. Additionally, GBMs are composed of a highly heterogeneous mixture of tumor cells with unique mutational profiles[75]. Therefore, there is an urgent need to fully understand the complex tumor biology of gliomas and GBM in specific in order to circumvent resistance mechanisms and identify new targets for effective clinical treatments.

Currently, various clinical studies investigate the safety and efficacy of novel therapeutic approaches including monoclonal antibodies[85], oncolytic viruses[86] and small molecules to inhibit cancer cell specific signaling pathways[87] or to reprogram the innate immune system[88]. Some of these clinical candidates are described as follows.

AG-120

Amongst other pro-oncogenic events, it has been shown that isocitrate dehydrogenase (IDH)-1 mutations initiate and drive cancer growth, thereby promoting the transition from low-grade gliomas to secondary GBM[89]. Point mutations in the active site of IDH-1 reduce the efficacy to convert isocitrate to alpha-ketoglutarate and simultaneously promote the ability to convert alpha-ketoglutarate to 2-hydroxyglutarate (2-HG), an onco-metabolite, which is believed to cause epigenetic changes that block normal differentiation of cells[90]. This block could be reversed by inhibiting mutated IDH-1, which results in low levels of 2-HG[91]. Thus, mutated IDH-1 represents a new therapeutic target to fight against GBM. Supported by promising results in preclinical *in vivo*[91] and clinical phase I studies[92] further clinical evaluations for AG-120, the first-in-class mutant IDH-1 inhibitor are warranted.

Dovitinib

As tyrosine kinases represent fundamental mediators of various signaling cascades involved in growth, differentiation, metabolism and apoptosis, it is generally accepted that mutation-based tyrosine kinase malfunctions may lead to oncogenic activation and thus, cancer initiation and progression. Recently, selective tyrosine kinase inhibitors have shown promising therapeutic effects, such as Imatinib as a treatment against Philadelphia chromosome-positive chronic myeloid leukemia (CML). Dovitinib, however, is a multi-tyrosine kinase inhibitor that targets several tumor-relevant tyrosine kinases including FGFR (fibroblast growth factor receptor), VEGFR (vascular endothelial growth factor receptor), PDGFRß (platelet derived growth factor receptor ß) and c-kit (receptor tyrosine kinase kit). As an increased activity of those tyrosine kinases is closely associated with GBM oncogenesis, multi-tyrosine kinase inhibition could be an effective treatment approach[93]. Dovitinib, which is able to cross the BBB, has recently been tested in phase I trials in patients with recurrent GBM[93].

Palbociclib

Pfizer has developed PD0332991 (Palbociclib), a cyclin-dependent kinase (CDK) 4/6 inhibitor, to treat patients with advanced estrogen receptor-positive and human epidermal growth factor receptor (HER) 2-negative breast cancer. The inhibition of CDK 4/6 leads to the blockage of retinoblastoma 1 (RB1) phosphorylation and consequently cell cycle arrest. As GBM exhibits similar alterations in the cyclin D1-CDK 4/6-RB1 pathways, Palbociclib represents a promising drug for the treatment of GBM[94]. First results indicate sensitivity of GBM cell lines to Palbociclib *in vitro*. However, the drug only seems to inhibit cell

proliferation in Rb1 proficient GBM cells, while RB1 deficient cells are resistant[95]. Further studies confirm the anti-proliferative effect of Palbociclib *in vivo* along with a survival benefit[96]. The clinical potential of Palbociclib against GBM has recently been tested in phase II trials in patients with Rb1 positive, recurrent GBMs. In this trial, Palbociclib has not been effective against recurrent GBM of a heavily pretreated patient population, and further exploration targeting the CDK 4/6 pathway is required[97].

Valganciclovir

Gene products of the human cytomegalovirus (HCMV) are known to dysregulate multiple signaling pathways involved in the oncogenesis of several cancer types[98]. Among other dysregulations, HCMV proteins are believed to control cell cycle progression by interacting with p53, Rb1 and cyclins, inducing chromosomal and DNA damage as well as inhibiting DNA repair and apoptosis[99]. As a high percentage of malignant glioma is infected by HCMV, therefore expressing multiple HCMV gene products, it is suggested that HCMV plays an important role in glioma pathogenesis[98]. Indeed, both HCMV replication and tumor growth are inhibited *in vitro* and in animal xenografts of tumors that are HCMV-positive *in vivo* using Valganciclovir[100], an anti-viral agent and prodrug of Ganciclovir. In a clinical trial, the combination of Valganciclovir to the standard therapy showed an increased survival benefit[101]. As only tumor cells are HCMV-positive, while the healthy surrounding tissue remains HCMV-negative, high treatment specificity is rendered, hence decreasing the risk of side-effects[99]. Supported by preliminary clinical data[102], further studies are recommended to investigate the effect of Valganciclovir in GBM patients.

1.3.3 Current animal models for glioblastoma

In order to enhance our knowledge on gliomas and GBM in specific, and to fully understand the complexity of molecular mechanisms underlying brain tumor initiation and progression, the use of animals as preclinical models is inevitable. Currently available *in vivo* models for primary brain tumors can be summarized in three categories including a variety of chemically induced models[103,104], xenograft models[105,106] and genetically engineered mouse (GEM) models[107], all extensively reviewed in numerous publications[108–110] **(Figure 6)**. Despite the fact that none of these *in vivo* models fully recapitulates the development and progression of human brain tumors, all of them have made significant contributions to the understanding of brain tumor biology.

Both chemically induced and GEM models benefit from an intact immune system while mimicking cancer initiation and progression from early stages on, hence providing a useful tool to investigate the molecular mechanisms of carcinogenesis within the CNS and to develop targeted therapeutics[111]. Xenograft models, which are based on the orthotopic transplantation of either patient-derived glioma cells or fresh brain tumor biopsy spheroids into immunodeficient mice, closely resemble the genetic and phenotypic heterogeneity of the original patient tumor[112]. Due to their high clinical relevance, xenograft models remain the gold standard in terms of performing safety and efficacy studies of novel drugs before registration by the FDA.

However, while recapitulating human brain tumors sufficiently well, all of these *in vivo* models come along with high cost and time-consumption as well as technical limitations and ethical burden, rendering them inapplicable for large drug screen projects to identify novel therapeutic targets against GBM.

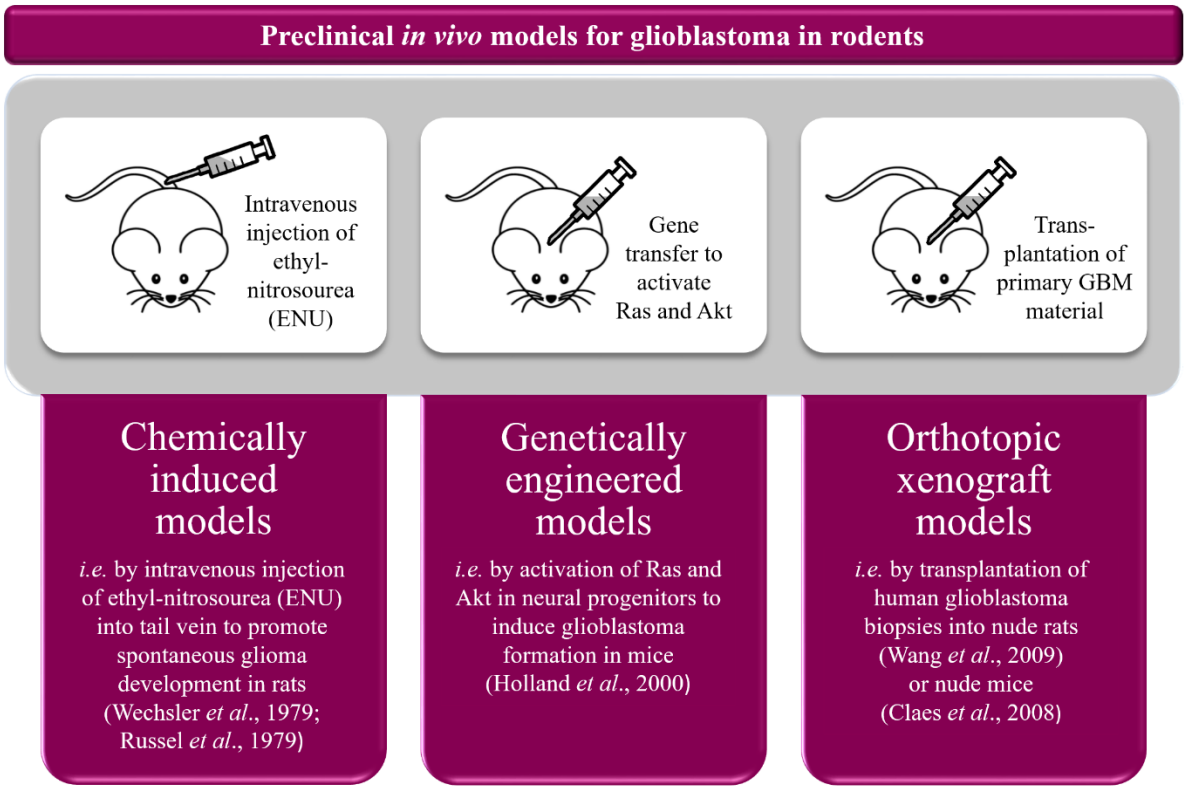


Figure 6: Overview on preclinical *in vivo* models for brain tumors in rodents
Currently available preclinical *in vivo* models for brain tumors are generated by three approaches including 1) chemically induced models, 2) genetically engineered models and 3) orthotopic models. Each category comprises a large variety of different models and while having contributed significantly to a better understanding of brain tumors initiation and progression, none of these models is applicable for large scale drug screens.

1.4 THE ZEBRAFISH AS A MODEL ORGANISM

1.4.1 Application in cancer research

During the last decades, the zebrafish (*Danio rerio*) has become a powerful animal model to study vertebrate development[113], integrative physiology[114] and toxicology[115]. More recently, the zebrafish emerged as a preclinical model for various cancer types, including liver[116] melanoma[117] and leukemia[118]. Compared to rodent models, advantages such as cost efficient husbandry, breeding of large embryo numbers, rapid development and small size/transparency of embryos render this model suitable for high-throughput drug screen projects[119,120]. In addition to that, the absence of a functional adaptive immune system up to 4 - 6 weeks post fertilization facilitates the microinjection of human primary tumor material into zebrafish embryos, thereby increasing the clinical relevance of the model by recapitulating heterogeneity of the original patient tumor.

1.4.2 Advantages and limitations of the zebrafish model

Due to numerous technical and biological advantages **(Table 1)**, the zebrafish has emerged as a powerful model organism for preclinical studies involving numerous human diseases including neurological disorders[121–123] as well as cancer[116–118].

Technical advantages include cost efficient husbandry, large number of offspring, rapid *ex utero* development as well as small size and transparency of embryos (< 48 hours post fertilization, hpf) which render the zebrafish a suitable model for high-throughput drug discovery screens[120,124]. Additionally, the zebrafish genome has been fully sequenced and numerous, well-characterized zebrafish strains are readily available. These include transgenic zebrafish strains like the *casper* mutant lacking complete pigmentation[125] or fluorescent reporter strains demarking distinct cell types such as epithelial cells in blood vessels[126] and numerous hematopoietic cells like T cells and macrophages[127,128]. Taken together, the availability of such transgenic fluorescent reporter stains allows for minimally invasive imaging techniques such as real-time imaging via light-sheet microscopy to study cell-cell interactions on a singular cell level.

Further biological advantages include high genetic similarity to humans enhancing the model's value for preclinical studies. Zebrafish are vertebrates and share a high degree of sequential and functional homology with mammals, and in particular humans, conferring to the conservation of countless cell biological and developmental processes[129,130]. Despite the phylogenetic distance between teleost fish and mammals, the zebrafish holds 82 % of human

disease-causing genes, thereby providing useful insights into various disease progressions[120]. Compared to simple cell culture systems, the zebrafish offers a broader range of disease-related phenotypes that can be simply monitored in phenotypic screens including vital parameters, pain, vascular tone, and tumor metastasis and gut motility[120]. In addition to that, recent advances in genome editing techniques including transcription activator-like effector nucleases (TALEN) or clustered regularly interspaced short palindrome repeats (CRISPR) and CRISPR-associated systems (Cas) allow for easy introduction of genetic modification that mimic oncogenic mutation profiles and drive tumor progression[131,132], thereby creating an efficient strategy for target-based drug discovery.

Table 1: Summary of advantages and limitations using zebrafish as a model for drug screens

Technical and biological advantages	Limitations
• Vertebrate • Cost efficient husbandry • High fecundity • Large number of offspring • Rapid *ex utero* development • Small size • Optical transparent eggs	• Ectothermic • Genome duplication
• Fully sequenced genome • Availability of multiple (transgenic) zebrafish strains and disease models • High degree of genetic conservation incl. disease-causing genes • Accessibility for genetic engineering • Amenable for molecular and genetic analysis	• Lack of several human organs (breast, lungs, prostate) • Lack of adaptive immune system until 4 - 6 weeks post fertiliation • Activation of embryonic signaling
• Conservation of multiple biological and developmental processes • Unique possibilities for real-time imaging incl. minimally invasive whole organism imaging (*i.e.* by light-sheet microscopy)	• Easily influenced by environment • Drug metabolism not fully characterized
• Applicability for phenotypic drug screens	

By contrast, the immature state of the immune system represents a rather double-edged feature in developing zebrafish embryos. While the innate immune system is functional by 3 days post fertilization (dpf), the adaptive immune system develops around 4 - 6 weeks post fertilization[133,134]. On the one hand, the immature immune system averts the use of immune suppressing agents prior to transplantation of cancer specimen[135], a prerequisite for xenotransplantation of patient material. On the other hand, it limits studies focusing on the interplay between transplanted cancer cells and the immune system and how treatment efficacy is affected, as it may not reproduce the behavior of cancer in a fully immunocompetent host. Due to phylogenetic distance between teleost fish and mammals, including humans, the zebrafish offers a different microenvironment to transplanted human cancer cells. This becomes a problem when orthotopic transplantation is impossible due to the lack of corresponding organs in zebrafish[136]. Moreover, cancer predominantly arises during adulthood and cancer cells may display altered phenotypes upon transplantation into the embryonic environment of developing zebrafish[124]. However, it has been shown that numerous signaling pathways underlying embryonic development are reactivated during malignant transformation[137–139]. With that in mind, further studies are needed to fully understand the potential influence the embryonic zebrafish environment may have on transplanted cancer cells.

1.4.3 Zebrafish models for glioblastoma

Glioblastoma is the most aggressive form of primary brain tumors and despite combinatory treatments, tumor recurrence and patient death are inevitable[81]. The development of novel strategies to fight against this deadly disease is challenged by the lack of orthotopic animal models for GBM that are suitable for high-throughput drug screens. Due to its beneficial features listed before, the zebrafish represents a powerful platform for preclinical and drug discovery research[120,140].

Until today, several research groups have used the zebrafish to create either orthotopic or automatable xenograft models of GBM. For the latter one, researchers have injected primary GBM cells into the protruding yolk sac serving as a nutritional cache for developing zebrafish embryos to study tumor growth and invasion[141,142]. More recently, chemically induced rat GBM cells were injected into the yolk sac to study the effect of nitric oxide on tumor development[143]. Despite the fact that automatable injection comprises a major requirement for large drug screen projects, the disease-recapitulating quality of the zebrafish yolk sac as a non-orthotopic injection site remains questionable **(Figure 7)**.

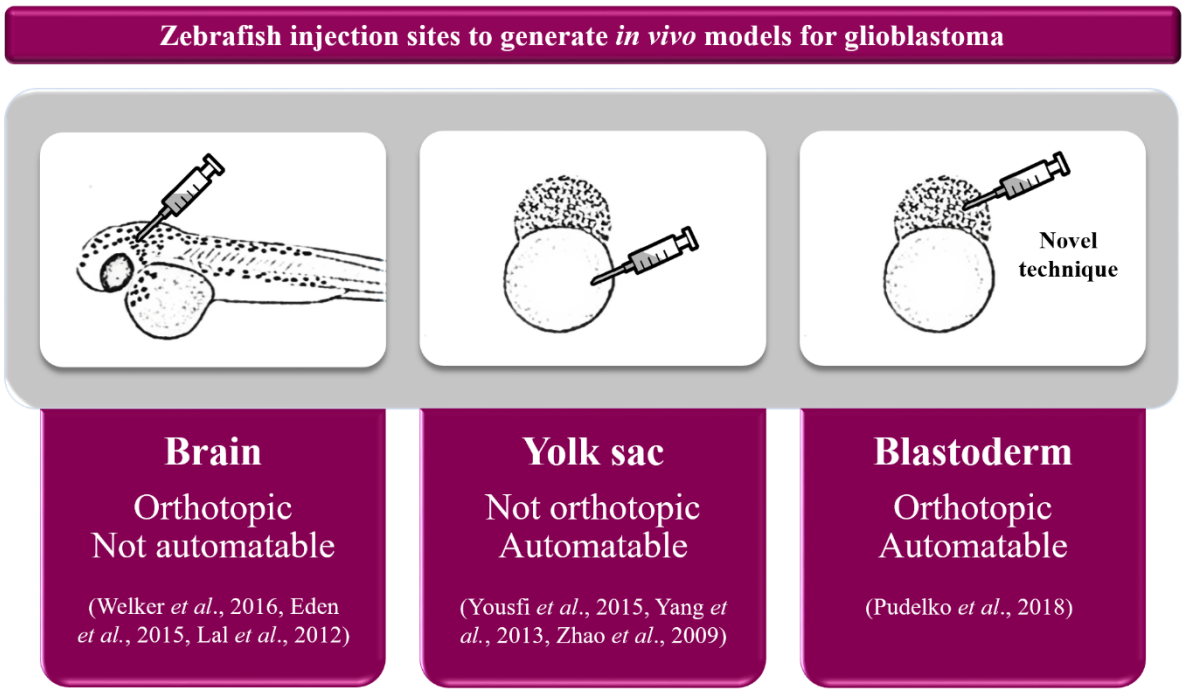


Figure 7: Comparison of zebrafish injection sites
Currently available zebrafish models for glioblastoma are generated by using two different injections sites, the brain and the yolk sac. While transplantation of GBM cultures directly into the brain is clinically relevant, though laborious, the disease-recapitulating capacity of the yolk sac remains questionable. Here, we introduce a novel transplantation technique to generate an orthotopic, and thus, clinically relevant zebrafish model for GBM, which is applicable for drug discovery screens.

In contrast to automatable xenograft models, other research groups have generated orthotopic zebrafish models for GBM to investigate angiogenesis, invasion and the role of signaling pathways in GBM cell differentiation[144]. More recently, one group orthotopically transplanted isolated pediatric mouse brain tumors and GBM cells, which retained histological characteristics of the tumor of origin, thereby creating an intermediary platform between high-throughput drug screens and mouse models[145]. Another group orthotopically transplanted patient-derived GBM cell cultures and neurospheres leading to progressive and heterogeneous brain tumor growth and increased lethality. Treatment of these tumors with TMZ resulted in a tumor size reduction *in vivo* and an increase of survival[146] **(Figure 7)**. These studies highlight the potential of the zebrafish model to serve as a clinically relevant screening platform to facilitate brain cancer drug discovery.

However, since the disease-recapitulating capacities of automatable, though non-orthotopic zebrafish models for GBM remain questionable and the generation of orthotopic zebrafish models for GBM is rather labor-intensive and not automatable as transplantation procedures

require sedation and precise positioning of the zebrafish as well as highly skilled personnel, a novel model circumventing these limitations is of high interest.

The optimal orthotopic zebrafish model for GBM should be suitable for fully automated high-throughput drug screens, including automatable tumor transplantation, embryo handling, drug exposure and read-out processes.

1.4.4 Clinical relevance

Despite numerous technical and biological advantages that render the zebrafish a highly attractive model system for phenotypic high-throughput drug discovery screens, questions about its clinical relevance to humans remain of central importance.

Although being an aquatic, ectothermic organism with anatomical differences compared to humans, genome sequencing has revealed that the zebrafish contains orthologues of 71 % of all human proteins including 82 % of disease-causing proteins[129]. In relation to that, it has been shown that protein targets of the ten most-prescribed drugs have zebrafish orthologues with sequence identity ranging from 54 % (glucocorticoid receptor) to 91 % (thyroid receptor)[120] **(Figure 8)**. As the sequence similarity is greater in the active side of enzymes, the pharmacological effect is still highly conserved in the zebrafish. Moreover, despite adaptations to the aquatic life, zebrafish physiology is well-conserved and exhibits many similarities to humans such as the hematopoietic system[147], glucose metabolism[148] and the cardiovascular system[149]. Intriguingly, the human cardiac electrophysiology shows greater similarity to zebrafish than to rodents **(Figure 8)**. To date, numerous cardiac disease models could be recapitulated in zebrafish including drug treatments showing the same pharmacological effects as in humans[149–152].

In regard to cancer, transgenic zebrafish models are of high clinical relevance as they allow investigation of cancer initiation and progression, induction of angiogenesis and metastasis as well as interactions with host cells such as the immune system. For example, the first study ever to investigate the effect of activated oncogene BRAF in melanoma development in an animal model was performed using zebrafish[153]. In this landmark study, transgenic zebrafish expressing the most common BRAF mutant form V600E under the control of melanocyte *mitfa* promoter showed increased patches of ectopic melanocytes. In addition to that, the induction of melanocyte lesions in p53-deficient zebrafish led to the development of highly invasive melanomas[153]. Following this study, further transgenic zebrafish models for multiple cancer types such as neuroblastoma[154], brain cancer[155] and leukemia[156] were established allowing to investigate cancer driving mechanisms and thereby, significantly contributing to

our knowledge of cancer biology. In addition to that, numerous zebrafish cancer studies involving xenotransplantation of patient-derived cancer cells such as breast cancer[157] and glioblastoma[146] were established and further confirm the clinical relevance of the zebrafish as transplanted tumor cells faithfully recapitulate the human disease *in vivo*. The generation of zebrafish patient-derived xenografts (PDX) allows to assess the aggressiveness of the original patient tumor and helps to predict disease progression by investigating the capacity to invade and metastasize.

Taken together, genetic and physiological similarities as well as supporting data generated by various studies involving transgenic zebrafish cancer models and zebrafish PDX models highlight the clinical relevance of the zebrafish as a powerful model to study cancer initiation and disease progression in order to develop novel anti-cancer therapies.

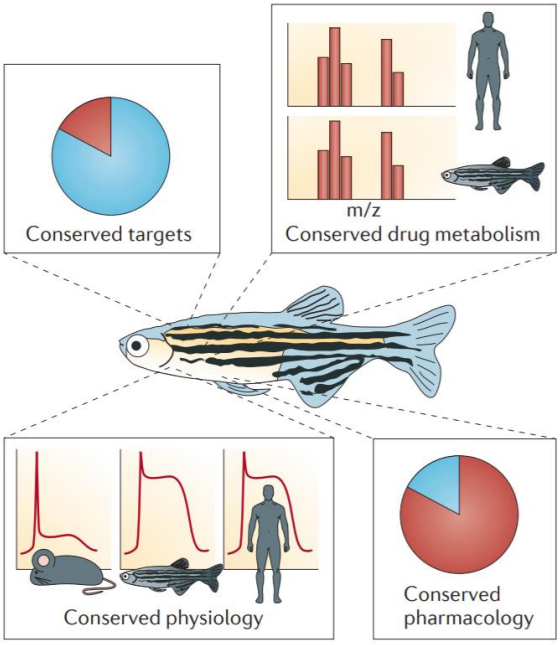


Figure 8: Clinical relevance of zebrafish for human drug discovery
Recent studies support the clinical relevance of the zebrafish model for human drug discovery by elucidating the degree of genetic similarity as well as conservation of target-proteins and drug metabolism pathways. Additionally, zebrafish physiology is well conserved as indicated by the example of cardiac electrophysiology which shows higher similarity to humans than rodents. According to that, several compounds discovered in zebrafish screens exhibit similar effects in rodent models and humans. Reprinted with permission from Springer Nature, from MacRae and Peterson, 2015.

2 DOCTORAL THESIS

2.1 THESIS OBJECTIVES

Cancer cells are characterized by an elevated and highly plastic redox environment. In order to compensate for intrinsic oxidative stress, cancer cells upregulate protective enzymes, such as MTH1, to eliminate erroneous bases and thereby promote both survival and growth under oxidizing conditions. Recently, we have developed potent in-house inhibitors that efficiently target MTH1 and induce cancer-specific cell death *in vitro* and *in vivo*[49,64].

As the reasons for cancer cells' sensitivity to our in-house MTH1 inhibitors need to be further characterized, in **Paper I**, we aimed to determine factors that render cancer cells sensitive to the inhibitors.

Based on the obtained results and in combination with the fact that the brain, due to its high energy consumption and metabolic rate, is more susceptible to oxidative stress than any other organ, in **Paper II**, we aimed to validate MTH1 as a target in GBM, specifically.

GBM represents the most aggressive form of brain cancer and as today's standard treatment fails to sufficiently eradicate the tumor, the prognosis remains dismal. Therefore, there is an urgent need to screen, identify and optimize novel anti-cancer drugs that could be used as future GBM treatments. In **Paper III**, we aimed to address this need by developing an orthotopic animal model for GBM that can be applied in automated drug discovery screens in order to facilitate the identification and optimization of novel promising anti-cancer drugs by simultaneously investigating drug toxicity and anti-tumor efficacy.

The specific objectives of constituent thesis papers are recapitulated as follows and further addressed by answering the research questions, respectively:

Paper I

To describe factors that render cancer cells sensitive to MTH1 inhibitors

- Identify factors that determine sensitivity to MTH1 inhibitors
    - *Does the redox environment influence sensitivity?*

- Introduce zebrafish as a tool to study MTH1 biology
    - *Do human MTH1 and zfMTH1 share high similarity?*
    - *Can the biology of oxidized nucleotides be studies in zebrafish?*

- Investigate the link between hypoxia, VHL and MTH1 *in vitro* and *in vivo*
    - *Is the hypoxia signaling axis involved in sensitizing to MTH1 inhibition?*
    - *Which role does VHL play in this szenario?*
    - *Do zebrafish experience increased oxidative stress upon activation of the hypoxia signaling axis?*

Paper II

To validate MTH1 as a target in GBM and GBM stem cells

- Study effects of MTH1 inhibitors on GBM and GBM stem cells *in vitro*
    - *Do MTH1 inhibitors efficiently target GBM?*
    - *How potent are MTH1 inhibitors compared to GBM standard therapy?*
    - *Which phenotypes do GBM cells lacking MTH1 display?*
    - *Do MTH1 inhibitors target GBM stem cells?*

- Apply GBM zebrafish model to investigate effect of MTH1 inhibitors *in vivo*
    - *Do MTH1 inhibitors target GBM* in vivo*?*

Paper III

To develop an orthotopic *in vivo* model for GBM to facilitate screening, identification and optimization of novel anti-cancer drugs

- Follow migration of GBM upon transplantation into zebrafish embryos
    - *Where do GBM migrate to upon transplantation into the blastoderm of zebrafish embryos? Is the migration phenomenon specific for GBM?*

- Characterize brain tumors *in vivo*
    - *Do transplanted GBM recapitulate clinical characteristics of the human disease?*
    - *Do transplanted GBM interact with host cells?*

- Apply GBM zebrafish model for a small drug screen
    - *Is it feasible to subject transplanted, tumor-bearing embryos to small drug screens?*

2.2 RESEARCH APPROACH

In order to address the specific objectives of the constituent thesis papers, we followed a multidisciplinary approach including the zebrafish as a universal key tool in all publications.

In a more general approach, we first began to identify factors that determine sensitivity of cancer cells to MTH1 inhibitors by combining biochemical and structural analysis with cellular assays and zebrafish *in vivo* studies. To get more specific, we next validated MTH1 as a target in GBM and GBM cells by first analyzing datasets for a possible connection between MTH1 expression and brain cancer followed by numerous molecular biology and cellular assays combined with zebrafish *in vivo* studies. Finally, for the development of an orthotopic and fully automatable animal model for GBM, we again focused on the zebrafish as our *in vivo* model of choice as it is the only vertebrate animal model suitable for large-scale drug discovery screens. Amongst other techniques, we combined xenotransplantation of GBM cultures with fluorescent imaging using confocal and light-sheet microscopy to characterize tumor growth *in vivo*.

A detailed outline of the research approach is listed as follows:

Paper I

Identification of factors for cancer cells to be sensitive to MTH1 inhibitors

1. Validation of redox environment as determinant of sensitivity to MTH1 inhibitors by altering redox levels with reducing and oxidizing agents to monitor survival and incorporation of oxidized nucleotides upon exposure to MTH1 inhibitors
2. Comparative biochemical analysis of human MTH1 and zfMTH1 ± MTH1 inhibitors to display structural and functional similarity
3. Microinjection of oxidized nucleotides into zebrafish eggs to investigate survival of zebrafish embryos upon exposure to MTH1 inhibitors
4. Investigate link between hypoxia, VHL and MTH1 *in vivo* by exposing zebrafish with activated HIF1α signaling axis (transgenic $VHL^{-/-}$ zebrafish or chemical induction) to MTH1 inhibitors to assesss zebrafish survival
5. Establish connection to redox environment by monitoring glutathione pool in transgenic zebrafish with activated HIF1α signaling axis ± MTH1 inhibitors

Paper II

Validation of MTH1 as a target in GBM and GBM stem cells

1. Dataset analysis to determine connection between MTH1 expression and GBM
2. RNAi-mediated knockdown of MTH1 *versus* in-house MTH1 inhibitors to investigate effects on cell proliferation, cell cycle and survival
3. Comparision of cell survival upon exposure to MTH1 inhibitors and GBM standard therapy
4. Analysis of DNA damage marker expression to relate effects of MTH1 inhibitors on GBM cells to oxidized nucleotide sanitation
5. Investigation of MTH1 inhibitor-induced effect on GBM stem cells (GSCs) by analysis of cell proliferation, cell cycle, survival and incorporation of oxidized nucleotides
6. Exploration of clinical relevance by investigating the effect of MTH1 inhibitors on GBM *in vivo* using transplanted zebrafish embryos

Paper III

Development of an orthotopic and fully automatable *in vivo* model for GBM

1. Identification of predominant migration site within zebrafish embryo by monitoring migration of transplanted GBM cells *in vivo*
2. Assessment for GBM specificity of migration site by transplanting and monitoring migration of different cancer types
3. Determination of clinical relevance of GBM zebrafish model by investigating clinical characteristics such as growth and invasion capacity of transplanted tumors as well as interactions with host cells such as the vascular and immune system by using transgenic zebrafish lines
4. Validation of applicability of GBM zebrafish model in drug discovery screens by performing a luciferase-based test screen (see chapter “Key Methodology”)

2.3 KEY METHODOLOGY

During the last decades, the zebrafish has become the flagship model organism to study vertebrate development[113] integrative physiology[114], toxicology[115] and more recently, cancer[116–118]. The application of zebrafish as a preclinical model for numerous cancer types has contributed significantly to a better understanding of cancer biology while simultaneously emerging as a promising, clinically relevant "tool" for high-throughput drug discovery screens potentially creating the leap towards precision medicine, the future of cancer treatments.

Thanks to its highly beneficial features including both biological and technical advantages (see chapter "The Zebrafish as a Model Organism"), the zebrafish is the only vertebrate animal suitable for high-throughput drug screens. This fact is strongly supported by a landmark screen performed in 2000, which highlights the general applicability of zebrafish in high-throughput drug screens by investigating toxicological effects of small molecules on organ development in whole zebrafish larvae using a 96-well format[158]. Until today, hundreds of such screens using zebrafish model organisms have been performed covering a diversity of research questions and compound library sizes[159] while complementing developmental phenotypes with the use of behavioral, cardiac, metabolic, proliferative and regenerative read-outs. Generally, phenotype-based screens are known for a higher success rate compared to target-based screens as they may identify effective drugs with a beneficial outcome in the absence of a validated target. As described by Swinney and Anthony in 2011, more than half of all approved first in-class drugs between 1999 and 2008 were discovered in phenotype-based screens[160], thereby significantly improving the life of many patients.

Here, we combine both biological and technical advantages of the zebrafish, most importantly its high clinical relevance indicated by the strong disease-capitulating quality of zebrafish xenograft models with the promising success rate of phenotype-based screens. In **Paper III** of this thesis, we present an orthotopic and fully automatable zebrafish model for GBM which opens up a new avenue for *in vivo* high-throughput drug screens offering great preclinical potential to 1) identify and optimize novel effective anti-GBM drugs, 2) accelerate repurposing of already existing drugs and 3) promote screening for tailor-made drugs, as a step towards personalized medicine.

2.3.1 Application of orthotopic GBM zebrafish model in drug screen

The following chapter focusses on describing the key methodology of this thesis, the generation of an orthotopic zebrafish model for GBM and its application in a small drug screen. It comprises detailed information on 1) the cultivation and preparation of human cell cultures and their transplantation into blastula stage zebrafish embryos, 2) the exposure of tumor-bearing zebrafish embryos to compounds and 3) the measurement of bioluminescence to determine tumor size *in vivo* **(Figure 9)**. Following a brief discussion about issues and complications that were overcome during the development of the underlying transplantation technique, the chapter closes with highlighting the advantages of our novel orthotopic zebrafish model for GBM, while balancing against limitations for its application.

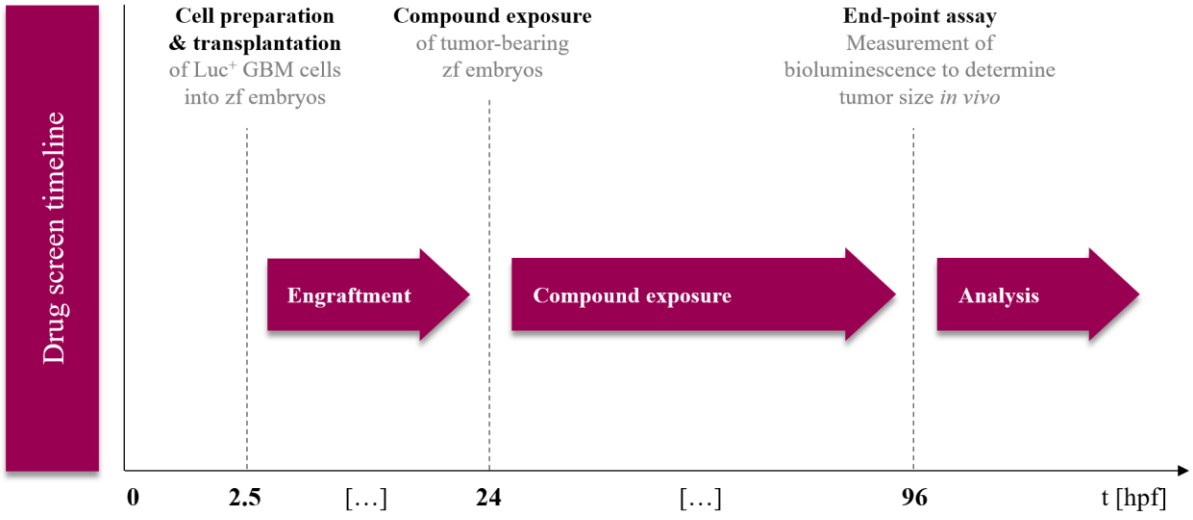


Figure 9: Schematic timeline of drug screen process
At 2.5 - 3 hpf, GBM cells stably expressing luciferase are harvested and transplanted into wildtype TL zebrafish embryos. Transplanted GBM cells migrate to the CNS to form a solid tumor. At 24 hpf, tumor-bearing zebrafish embryos are sorted, dechorionized and divided into groups to begin compound exposure. At 96 hpf, tumor size of transplanted and exposed zebrafish is determined by measuring bioluminescence individually in a luciferase end-point assay and quantitatively analyzed. Luc, luciferase; GBM, glioblastoma; hpf, hours post fertilization; zf, zebrafish.

Cell preparation and transplantation

For the drug screen presented in **Paper III**, we have used two GBM cultures, patient-derived GBM culture #18[161] and U343-MGa[162]. Both GBM cultures have been propagated in Minimum Essential Medium (Gibco) containing 2 mM glutamine (Gibco) at 37°C in a humidified atmosphere with 95 % humidity and 5 % CO_2. For further details on generation and characterization of GBM cultures, please view respective references.

In all experiments, GBM tumor size was determined by measuring bioluminescence using an *in vivo* luciferase reporter assay. For this, GBM culture #18 and U343-MGa were stably transfected with luciferase using standard lentivirus protocols.

After successful transfection and recovery, GBM cultures were transferred to complete neurobasal stem cell medium at least 5 days prior to transplantation to enhance tumor engraftment. GBM cultures were grown until subconfluency and harvested one hour before transplantation. For the generation of a single cell suspension, cells were passed through a pre-washed 20 µm cell strainer. Next, cells were washed, spun down and after removal of the supernatant, cells were resuspended in medium containing 2 % polyvinylpyrrolidone (PVP) to prevent clogging of the microinjection capillary (World Precision Instruments). Harvested cells were kept on ice until transplantation.

Immediately before transplantation, cells were spun down again and the supernatant was almost entirely removed, generating a highly concentrated cell suspension, which was loaded manually into a microinjection capillary.

Zebrafish embryos were collected at 3 hpf and immobilized in 2 % agarose injection plates. Approximately 100 cells were transplanted into the blastoderm of the zebrafish embryos. Following transplantation, zebrafish embryos were transferred to E3 fish medium into a 10 cm petri dish and incubated at 33°C for 24 hours before screening and drug exposure.

Compound exposure of tumor-bearing zebrafish embryos

The next day, transplanted zebrafish embryos were screened for viability and brain tumor establishment. To ensure homogenous drug exposure, zebrafish embryos were dechorionized using 2 mg/mL Pronase® (from *Streptomyces griseus*) and distributed to 6-well plates (20 - 25 embryos/well) in a total volume of 3 mL E3 fish medium containing 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) as buffer. Compounds of interest dissolved in DMSO (dimethyl sulfoxide) to 10 mM were added directly to the medium to reach a desired end concentration. DMSO was used as vehicle control. During drug exposure, zebrafish embryos were incubated at 33°C for 72 hours (unless stated differently).

End-point assay: Measurement of bioluminescence to determine tumor size *in vivo*

As described before, all transplanted GBM cultures were stably expressing a firefly luciferase enzyme, which converts substrate D-luciferin to its corresponding product oxyluciferin in the presence of cofactors ATP, magnesium and oxygen, while simultaneously emitting light as a by-product **(Figure 10)**. The amount of light produced is proportional to the amount of luciferase enzyme. In the context of our small drug screen, the amount of light produced reflects the number of GBM tumor cells, and hence tumor size *in vivo*.

Tumor size of transplanted and exposed zebrafish embryos was determined by measuring bioluminescence individually. For this, single zebrafish embryos were transferred to opaque 96-well plates (Perkin Elmer) and incubated for 30 min in lysis buffer (10 % glycerol, 1 % Triton-X 100, 1 mM DTT (1,4-dithiothreitol), pH 7.8). An equal amount of substrate solution (1 mM DTT, 1 mM ATP, 0.3 mg/ml D-luciferin, pH 7.8) was added for 5 min before measurement of bioluminescence (Hidex Sense).

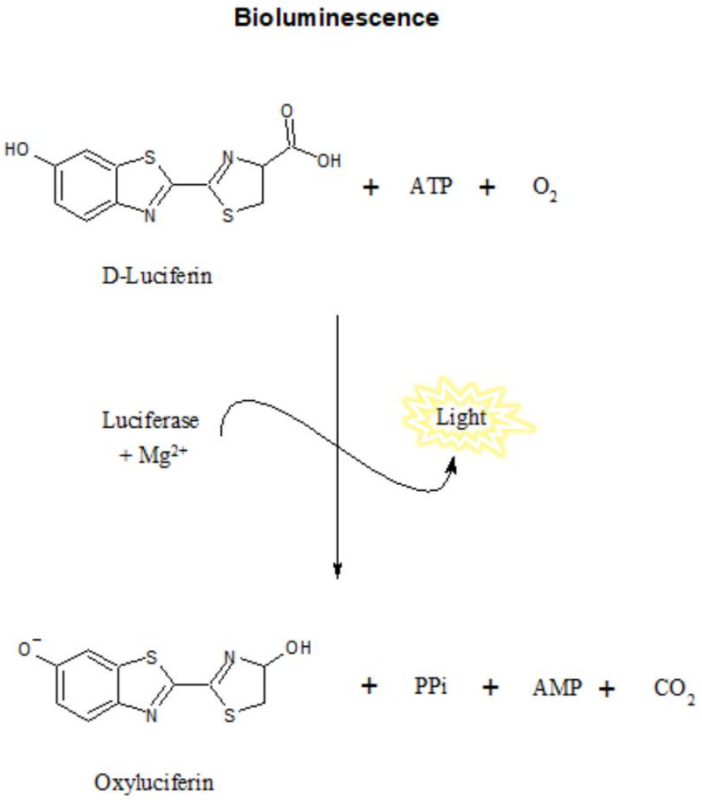


Figure 10: Simplified bioluminescence reaction mechanisms
Luciferase converts substrate D-luciferin to its corresponding product oxyluciferin in the presence of cofactors ATP, magnesium and oxygen, while bioluminescence is emitting as a by-product. Note: cofactor requirements are dependent on the luciferase used (here: firefly luciferase). ATP, adenosine triphosphate; Mg, magnesium; O_2, oxygen; PPi, diphosphate; AMP, adenosine monophosphate, CO_2, carbon dioxide.

Statistical Analysis

All experiments were performed at minimum in triplicates. The results are presented in mean ± SD. Statistical significance was determined using the One sample *t*-test (online GraphPad Software, 2018). The following *P*-values were considered significant: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

2.3.2 Issues and complications

In the course of establishing the protocol for our novel orthotopic zebrafish model, we came across several issues that needed further optimization, or complications that have to be considered when performing the transplantation procedure, prospectively.

First of all, aside the fact that around 70 % of zebrafish embryos transplanted with GBM cultures developed congregated brain tumors at 24 hpf, we occasionally observed some of the transplanted candidates with severe developmental defects or malformations including the development of a second body axis, or reduction of the tail. Due to the fact that zebrafish embryos are highly susceptible to disruptions in their early development, we concluded that these phenomena have to be time-dependent issues, as the incidence of developmental defects could be reduced when performing transplantations from 2.5 - 3 hpf on.

In addition to developmental defects, at times we observed edema formation in transplanted zebrafish embryos around 48 hpf. Following a more detailed analysis, we found that zebrafish embryos harboring CNS tumors in close proximity to the heart region were more likely to develop edemas. Intriguingly, peritumoral edema formation is also commonly found in GBM patients as suggested by recent clinical data[163–165]. It is believed that glioma-related edema (GRE) formation promotes tumor cell invasion and significantly influences the prognosis[164], thus serving as a diagnostic marker for clinical outcome of GBM patients.

Another complication that we were facing while developing the protocol for our novel orthotopic zebrafish model was the large variation of tumor sizes *in vivo*. The reason for this variation is a technical issue: due to their large size as well as their morphological features, GBM cells tend to block the microinjection capillaries, which can be further aggravated by residual dye and dust particles. In case of a blocked microinjection capillary, several options exist on how to eliminate the blockage such as 1) increasing the pressure of the micro-injector, 2) increasing the diameter of the microinjection capillary to remove the particle from the capillary, or 3) preparing a new microinjection capillary. In any case, the number of transplanted cells will ultimately change. Following intense optimization of the cell

preparation protocol prior to transplantation by adding multiple washing steps or supplementing medium with PVP to increase viscosity, we could reduce the variation of tumor sizes *in vivo*. However, as the technical issue still persists in some cases, extreme tumor sizes revealed by luciferase measurement should be excluded from the analysis as outliers.

Currently, we determine individual tumor sizes by measuring bioluminescence in an endpoint assay. As mentioned before, large variations in tumor sizes minimize any biologically significant effect on tumor reduction. One way to further optimize the read-out would be to follow individual tumor sizes in real-time. Recently, a group has engineered a luciferin analog "AkaBLI", which produces light emissions *in vivo* that are 100 to 1000 brighter compared to conventional systems and therefore allow for noninvasive visualization of single cells in deep tissue[166]. The generation of GBM cultures stably expressing "AkaBLI" would significantly improve our zebrafish model as we could measure bioluminescence and hence, tumor volume in real-time at any desired time-point without lysing the zebrafish.

2.3.3 Advantages and limitations

Xenograft models remain the gold standard for understanding GBM tumor biology *in vivo*. Although recapitulating the human disease sufficiently well, orthotopic rodent models for GBM are both technically and ethically challenging as well as time-consuming, and therefore not suitable for drug screens. The zebrafish offers a clinically relevant alternative to rodent models; however, currently existing orthotopic zebrafish models for GBM are generated by laborious intracranial transplantation of single zebrafish embryos, which limits their use in drug screens. Our newly developed zebrafish model for GBM closes the gap by offering a clinically relevant yet fully automatable orthotopic model for GBM that is suitable for high-throughput drug screens. Some of its numerous advantages are listed as follows:

- **Tumor formation in the CNS within 24 hours**

  Patient-derived tumor material and established GBM cultures specifically migrate to the CNS upon transplantation in the blastoderm of zebrafish embryos at 2.5 - 3 hpf to form a congregated tumor within 24 hours.

- **Fast and fully automatable transplantation procedure**

  Tumor establishment in the CNS of zebrafish embryos is independent of injection site within the blastoderm; thus, transplantation of GBM cultures could be performed by existing robotic injection systems.

- **Implementation in fully automatable drug discovery pipeline**

  Zebrafish embryos can be raised, treated and screened in 96-well plate format. Depending on the instruments included in the drug screen pipeline, it is possible to simultaneously monitor tumor size as well as vital parameters to evaluate the overall being of exposed zebrafish embryos.

- **High clinical relevance**

  Within few days, transplanted GBM cells faithfully recapitulate the human disease in the zebrafish, which is characterized by the formation of brain microtubules and invasion of the surrounding brain tissue, vessel formation and interactions with the innate immune system such as macrophages. In addition to that, the formation of the BBB at 3 dpf limits potential drug screen hits to drugs that display chemical properties to actually pass the BBB to induce an anti-tumor effect.

- **Time-efficient pipeline**

  Robust and predictive drug screen within 5 days

- **No ethical restrictions**

  As no requirement of an ethical permit is needed below 5 days, the model complies with the 3R guidelines (replace, reduce, refine).

Despite numerous highly relevant advantages, our orthotopic zebrafish model for GBM comprises few limitations. As mentioned before, the adaptive immune system is not functional until 4 - 6 weeks post fertilization[133,134] and limits studies focusing on the interplay between transplanted cancer cells, the immune system and how treatment efficacy is affected as it may not fully reproduce the behavior of cancer in a fully immunocompetent host. Additionally, cancer cells might display altered phenotypes upon transplantation into the embryonic environment of a developing zebrafish embryo[124] which has to be considered when interpreting study results. Another limitation is presented by the duration of the experimental pipeline, which is as short as 5 days. Prolonging the experimental pipeline to follow treatment effects for an extended period of time is generally possible but requires further optimization regarding the tumor burden of transplanted zebrafish embryos. Additionally, zebrafish studies that exceed 5 days require ethical permits.

2.4 SUMMARY OF RESEARCH PAPERS

2.4.1 Paper I: Hypoxic Signaling and the Cellular Redox Tumor Environment Determine Sensitivity to MTH1 Inhibition

As explicitly addressed in the introduction, cancer cells are characterized by an elevated and highly plastic redox environment. To compensate for intrinsic oxidative stress, cancer cells upregulate sanitizing enzymes, such as MTH1, which eliminate oxidized nucleotides and thereby promote both survival and growth under oxidizing conditions. After the development of potent in-house inhibitors that efficiently target MTH1 and induce cancer-specific cell death[49], in **Paper I**, we aimed to determine factors that render cancer cells sensitive to MTH1 inhibitors, predominantly focusing on compound TH588.

Recent publications postulated that the loss of functional MHT1 correlates with an increased incorporation of 8-oxo-dGTP into DNA[49,62]. Based on the observation that elevated levels of total glutathione in cancer cells also correlate with increased sensitivity to MTH1 inhibition, we first investigated whether the redox environment itself determines sensitivity to MTH1 inhibitor TH588. Indeed, we could show that increasing oxidative stress in non-malignant cells by blocking their glutathione *de novo* synthesis induces their sensitization to MTH1 inhibition by TH588. In contrast to that, decreasing the oxidative pressure in cancer cells using general anti-oxidants such as N-acetyl-L-cysteine (NAC) protects against MTH1 inhibition by TH588 (Paper I, Figure 1a, 1b).

Next, we identified the zebrafish model as a useful tool to study MTH1 biology as human MTH1 and zfMTH1 share high similarity on amino acid level (70 % identity). Furthermore, we cloned, expressed and purified zfMTH1 and found that the human MTH1 inhibitor TH588 shows similar affinity to the active site of zfMTH1 as to human MTH1, a prerequisite to further study TH588 *in vivo* (Paper I, Figure 2).

In order to determine whether functional MTH1 is required to detoxify oxidized nucleotides *in vivo*, we microinjected 8-oxo-dGTP and 2-OH-dATP into zebrafish eggs. As the deoxy form of oxidized nucleotides can readily be incorporated into DNA, we discovered that the delivery of both 8-oxo-dGTP and 2-OH-dATP to zebrafish eggs is highly toxic in the absence of functional MTH1 (Paper I, Figure 3).

Based on the interplay between redox signaling networks and hypoxia sensing mechanisms, we next investigated whether an activated hypoxia signaling axis also sensitizes to MTH1 inhibition using TH588. For that, we mimicked activated hypoxia signaling either genetically

by developing homozygous VHL knockout zebrafish, or chemically by inactivating prolyl hydroxylases (PHDs) using DMOG (dimethyloxaloylglycine). In both cases, zebrafish embryos were sensitized to MTH1 inhibitor TH588 by showing significantly reduced viability (Paper I, Figure 4, Figure 5a, and b).

Lastly, using a transgenic zebrafish line to monitor the cellular redox state *in vivo*, we detected an increase in oxidative pressure upon activation of hypoxic signaling. As the pretreatment with anti-oxidant NAC protects embryos with activated hypoxia signaling against MTH1 inhibition, we concluded that an aberrant redox environment causes sensitization (Paper I, Figure 5c, and d).

Summarizing the results obtained in **Paper I**, we conclude that MTH1 inhibition may offer a general approach to treat cancers that are characterized by elevated oxidative stress levels and a deregulated hypoxia signaling axis by inducing cancer-specific cell death via incorporation of erroneous bases into DNA **(Figure 11)**.

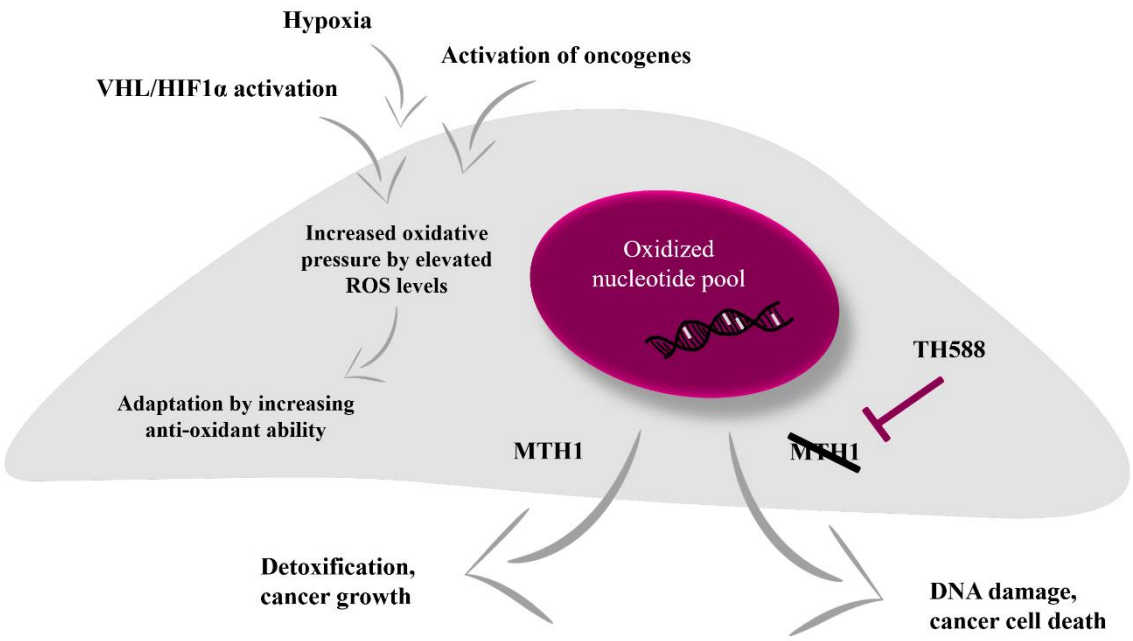


Figure 11: Overview on how elevated redox pressure sensitizes cancer cells to MTH1 inhibition
Due to the activation of oncogenes, hypoxic conditions as well as deregulated hypoxia signaling pathways, cancer cells suffer from elevated redox pressure. As the nucleotide pool is especially prone to oxidative damage, it requires the enzymatic activity of MTH1 for sanitation. If MTH1 is inhibited, oxidized nucleotides are incorporated into DNA leading to DNA damage and eventually, cancer cell death. MTH1, MutT homolog 1; DNA, deoxyribonucleic acid.

2.4.2 Paper II: Glioblastoma and glioblastoma stem cells are dependent on functional MTH1

Based on the results obtained in **Paper I**, we next planned to validate MTH1 as an anti-cancer target in a specific cancer type that is characterized by elevated oxidative pressure and increased anti-oxidant defense mechanisms. Knowing that the brain, due to its high energy consumption and metabolic rate, is more susceptible to oxidative stress than any other organs, in **Paper II**, we aimed to investigate the effect of our in-house MTH1 inhibitors in brain cancer.

First, we started by analyzing available cancer datasets for a potential connection between MTH1 and brain cancer, and found consistent evidence that MTH1 mRNA expression levels are upregulated in GBM compared to non-tumor brain tissue. Following this significant correlation, we investigated the effect of our in-house MTH1 inhibitor TH588 and its pharmacologically and pharmacokinetically improved version TH1579 on the survival of different GBM cell lines. Indeed, we found that both inhibitors decrease viability of all GBM cell lines following a 3- or 5-day treatment (Paper II, Figure 1b).

Intrigued by these initial findings, we continued to assess the requirement of functional MTH1 for GBM cell viability in a panel of patient-derived GBM cultures divided into type A and B depending on their tumorigenic activity and amount of GSCs. We found that both inhibitors TH588 and TH1579 significantly decrease viability of all GBM cultures independent of their intrinsic aggressiveness. To further confirm the requirement of functional MTH1, we chose a representative GBM culture of type A (GBM #18) and type B (GBM #7), and depleted MTH1 either by siRNA-mediated knockdown using different sequences or by small molecule inhibition using TH588 and TH1579. In both cases, we observed an efficient reduction of GBM survival as depicted by clonogenic survival assay as well as cell cycle analysis (Paper II, Figure 2).

Comparing our in-house MTH1 inhibitors TH588 and TH1579 to today's standard treatment and other clinical candidates targeting GBM, we found that TH1579 is more potent in eradicating GBM #18 cells (Paper II, Figure 3).

Next, we analyzed DNA damage markers to relate the effects of our MTH1 inhibitors on GBM cells to oxidized nucleotide sanitation. Indeed, we found prolonged tail moments by comet assay and increased numbers of γH2AX foci as revealed by immunofluorescent (IF) analysis, indicating that the effect of MTH1 loss on GBM is likely mediated through incorporation of oxidized nucleotides and subsequent DNA damage (Paper II, Figure 4).

One major factor that determines GBM aggressiveness and treatment resistance is the highly heterogeneous nature of the GBM tumor bulk, which comprises a large number of GSCs characterized by cell surface marker CD133. Therefore, we aimed to explore if our MTH1 inhibitors target both $CD133^+$ and $CD133^-$ GBM cells. Indeed, we found that inhibition of MTH1 with TH588 and TH1579 decreases both populations by impairing $CD133^+$ and $CD133^-$ GBM cell viability. Moreover, life cell imaging of GSCs characterized by transcription factor SOX2 (SRY (sex determining region Y)-box 2) revealed a significantly prolonged mitosis upon exposure to TH588 and TH1579, proving further evidence that MTH1 inhibition targets GSCs (Paper II, Figure 5).

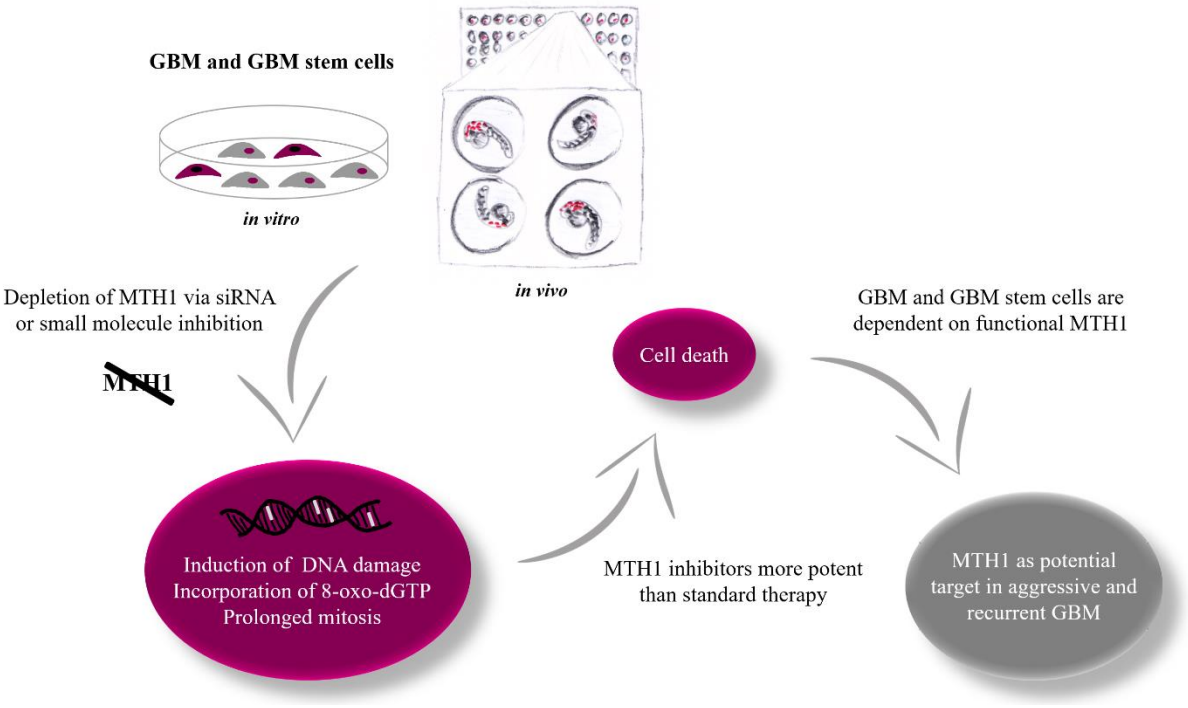


Figure 12: Dependency of GBM and GBM stem cells on functional MTH1
GBM cultures display elevated MTH1 expression levels, which correlate with GBM aggressiveness characterized by proliferation rate and amount of GBM stem cells, amongst others. Following depletion of MTH1 either by siRNA-mediated knockdown or small molecule inhibition using in-house synthesized MTH1 inhibitors TH588 and TH1579, viability of GBM cultures independent of intrinsic aggressiveness is significantly reduced *in vitro* and *in vivo*, using an orthotopic zebrafish model for GBM (shown in this figure: zebrafish embryos orthotopically transplanted with GBM and incubated in a 96-well plate). The effect of MTH1 loss on viability is most likely mediated by incorporation of oxidized nucleotides and subsequent DNA damage, rendering MTH1 as a promising target for GBM therapy. GBM, glioblastoma; MTH1, MutT homolog 1; DNA, deoxyribonucleic acid; 8-oxo-dGTP, 8-oxo-7,8-dihydroxy-2'-deoxyguanosine-5'-triphosphate.

Finally, to investigate the effect of our MTH1 inhibitors on GBM *in vivo*, we transplanted luciferase positive GBM cells enriched for CD133 into zebrafish embryos and exposed them to TH1579. Bioluminescence measurement of tumor size *in vivo* after 5 days of treatment revealed a significant reduction in tumor volume compared to the control. Additionally, *in vivo* real-time imaging of exposed tumors via light-sheet microscopy revealed numerous GBM cells undergoing cell death confirming the overall reduction in tumor volume by 25 % (Paper II, Figure 6).

To conclude, with these *in vitro* and *in vivo* results, we provide supporting data that the inhibition of MTH1 using our in-house inhibitors TH588 and its pharmacologically and pharmacokinetically improved version TH1579 might present an efficient strategy to target heterogeneous GBM tumors **(Figure 12)**.

2.4.3 Paper III: An orthotopic glioblastoma animal model suitable for high-throughput screenings

GBM is the most aggressive form of brain cancer and as today's standard therapy fails to sufficiently eradicate the tumor, there is an urgent need for novel GBM treatments. However, the development of novel GBM treatments is hampered by the lack of orthotopic animal models that can be implemented in high-throughput drug discovery screens. In **Paper III**, we aimed to address this issue by developing an orthotopic animal model for GBM that can be applied in automated drug discovery screens in order to facilitate the identification and optimization of novel promising anti-cancer drugs against GBM by simultaneously investigating drug toxicity and anti-tumor efficacy.

Based on fate-map analysis and previous data suggesting that early zebrafish embryos could provide lineage-specific trophic support to human cells, we hypothesized that human GBM cells transplanted into the zebrafish blastoderm might migrate into CNS structures of the developing zebrafish embryo. Indeed, 24 hours later around 70 % of transplanted zebrafish embryos develop an intracranial tumor (Paper III, Figure 1). By transplanting patient-derived GBM cultures, more established GBM cell lines as well as colorectal cell lines cells to compare the migration sites, respectively, we elucidated that the migration behavior into the CNS regions is GBM specific, but independent from the transplantation site within the blastoderm of the zebrafish embryo (Paper III, Figure 2).

After confirming the robustness of our model, we explicitly analyzed its clinical relevance. Real-time light-sheet microscopy revealed that transplanted GBM cells actively proliferate *in vivo* leading to an increased tumor volume within 24 hpf (Paper III, Figure 3a - d). In addition to that, transplanted GBM cells invade into the healthy surrounding brain tissue by developing extended tumor microtubules, which steadily grow (until the humane endpoint of the experiment at 6 dpf is reached, Paper III, Figure 3e - f). Moreover, transplanting GBM cells into transgenic zebrafish embryos (fli:eGFP) that harbor a fluorescent blood vessel system revealed ongoing tumor vascularization (Paper III, Figure 4a, and b). On top of that, upon the transplantation of GBM cells into transgenic zebrafish embryos expressing fluorescent proteins in macrophages (mpeg1:mCherry), we observed tight interactions between the innate immune system and the transplant (Paper III, Figure 4c).

In order to provide evidence that our orthotopic model for GBM is applicable for drug discovery screens, we performed a small drug screen testing the anti-tumor efficacy of several tyrosine kinase inhibitors (TKIs) including Erlotinib, R-Crizotinib, Gefitinib and

Afatinib on GBM *in vivo*. First, we engineered both the well-established GBM cell line U343-MGa as well as patient-derived GBM culture #18 to stably express luciferase. Next, we transplanted zebrafish embryos and exposed them to the TKIs. Following an exposure of 3 days, tumor volume of single embryos *in vivo* was assessed by bioluminescence measurement and revealed that Erlotinib reduces tumor burden of U343-MGa transplanted zebrafish embryos most significantly compared to the control. Albeit displaying similarly significant responses to the treatment, patient-derived GBM culture #18 are generally less sensitive to the TKIs (Paper III, Figure 4g).

Taken together, the thorough characterization of the orthotopically transplanted GBM tumors *in vivo* highlights numerous features including active proliferation, tumor growth, and formation of tumor microtubules, induction of angiogenesis as well as interaction with the innate immune system of the host, which are highly reminiscent of human brain tumors. Hence, our model faithfully recapitulates the human disease *in vivo*, while enabling the transplantation of thousand embryos per hour, thereby being applicable for fully automatable drug discovery screens **(Figure 13)**.

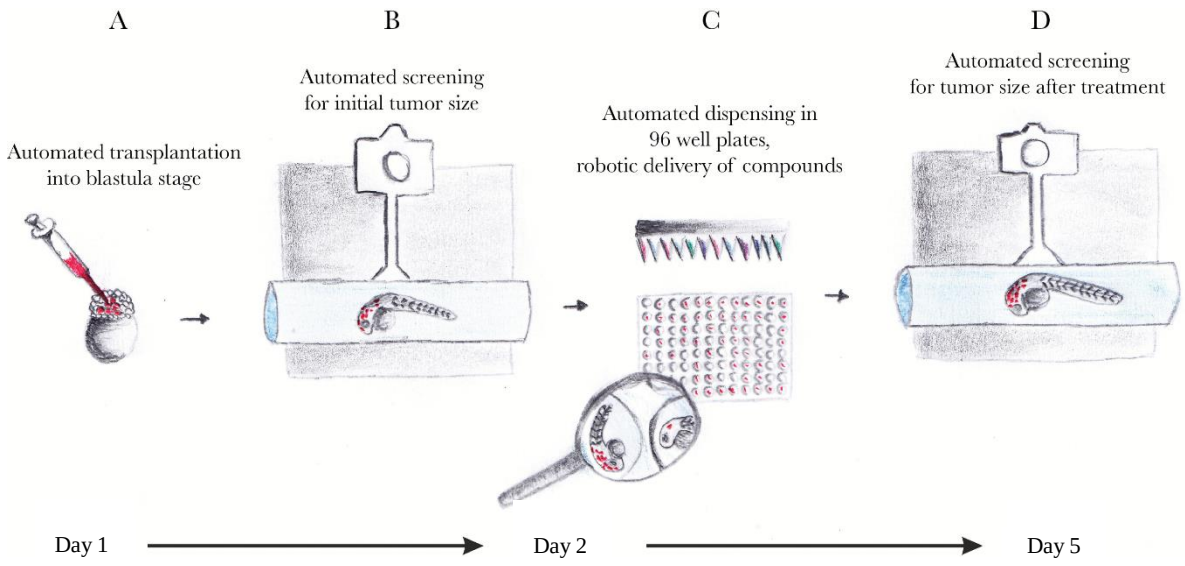


Figure 13: Application of orthotopic zebrafish model for GBM in automated large-scale drug screen
At 2.5 - 3 hpf, hundreds of zebrafish embryos can be lined up in agarose injection plates to be transplanted with GBM material by robotic injection systems (A). At 24 hpf, transplanted embryos are screened for tumor size by automated imaging systems (B) and automatically distributed into 96-well plates preloaded with candidate compounds (C). After 3 days incubation time, individual embryos are automatically sampled, imaged, and analyzed with commercially available instruments (D). GBM, glioblastoma; hpf, hours post fertilization. Reprinted with permission from Oxford University Press, from Pudelko *et al*., 2018.

2.5 DISCUSSION

As currently available treatment options for neurological cancers are limited and lethal tumor recurrence is inevitable, GBM remains one of the most intractable forms of cancer, creating an urgent need for the development of innovative therapeutic approaches. Advances in sequencing technology and the resulting discovery of GBM-associated oncogenic driver mutations and altered protein expressions opened the promising avenue of targeted therapy[72–74]. However, despite the discovery of various targets[167], the development of potent drugs leading to complete destruction of the tumor remains challenged by 1) the complex biology of GBM including its extensive tumor heterogeneity, cancer cell plasticity and the presence of cancer stem cells[16,75,76], 2) physical barriers that hamper drug delivery such as the BBB and blood-tumor barrier (BTB) and 3) the lack of predictive animal models suitable for high-throughput drug discovery screens.

In the underlying work of this PhD thesis, we first addressed the issue of GBM tumor heterogeneity as a major challenge for the development of efficient therapies by focusing on a more general/broad cancer-specific target: the elevated and highly plastic redox environment of cancer cells. To compensate the detrimental effect of increased ROS production, cancer cells and GBM in particular depend on effective anti-oxidant defense systems and non-oncogenic addiction enzymes such as MTH1, an enzyme that detoxifies oxidized bases to prevent DNA damage and subsequent cell death.

At the time this thesis work commenced, we have developed potent in-house inhibitors that efficiently target MTH1 and induce cancer-specific cell death *in vitro* and *in vivo* [49,64]. As the reasons for cancer cells' sensitivity to our in-house MTH1 inhibitors required further characterization, in **Paper I**, we first aimed to determine factors that render cancer cells sensitive to our MTH1 inhibitors. Following the hypothesis that the brain, due to its high energy consumption and metabolic rate, is more susceptible to oxidative stress than any other organ, in **Paper II**, we aimed to validate MTH1 as a target in GBM, specifically.

In the last part, we addressed the lack of predictive animal models for GBM that are suitable for high-throughput drug discovery screens. Following the introduction of the zebrafish as a clinically relevant model for human malignancies including cancer, in **Paper III**, we have developed an orthotopic and fully automatable animal model for GBM with the potential to 1) identify and optimize novel effective anti-GBM drugs, 2) accelerate repurposing of already existing drugs and 3) promote screening for tailor-made drugs, as a step towards personalized medicine.

2.5.1 Paper I

Activated hypoxia signaling frequently displayed in solid cancers and associated with poor clinical prognosis is a direct consequence of overexpressed HIF1α either induced by hypoxic tumor regions or by oncogenic signaling and growth factor stimulation[20,168,169]. Indirect activation of the hypoxia signaling axis can be induced by the absence of tumor suppressor protein VHL as a consequence of somatic mutations[170]. Increased HIF1 activity alters cellular metabolism leading to persistently upregulated ROS levels. Cancer cells ensure survival and growth under oxidizing conditions by increasing their anti-oxidant ability, which consequently shifts the overall redox balancing point upwards **(Figure 4a)** and fuels a vicious cycle of further adaption, continuous proliferation and acquisition of DNA damage and genomic instability. While simply increasing ROS levels to induce cancer cell death remains insufficient, disabling ROS defense systems by targeting anti-oxidant or other non-oncogenic addiction enzymes such as MTH1 represents an area of therapeutic opportunities[46,49].

Following the development of potent in-house inhibitors that efficiently target MTH1 and induce cancer-specific cell death *in vitro* and *in vivo*[49], we aimed to understand the underlying mechanism of cancer cells' sensitivity to the MTH1inhibitors.

Despite the highly plastic intracellular redox environment, we were able to show that the exposure of healthy cells to oxidants or the depletion of the cellular glutathione pool increased their sensitivity to MTH1 inhibition. This was in line with previous findings indicating that the exposure to hydrogen peroxide sensitized both fibroblasts and cancer cells to siRNA-mediated knockdown of MTH1[171]. In addition, we could show that decreasing the oxidative pressure in cancer cells using anti-oxidants such as NAC protected against MTH1 inhibition. Based on our data we concluded that the redox environment is a key factor in determining sensitivity to MTH1 inhibition.

Due to the close connection between the oncogenic redox environment and deregulated hypoxia signaling described earlier, we employed transgenic zebrafish embryos to study if MTH1 inhibition is connected to VHL activity. Indeed, we were able to show that the loss of endogenous VHL or the chemical activation of HIF1α signaling sensitized to MTH1 inhibition *in vivo*. According to the hypothesis that hypoxia leads to a more oxidized cellular and mitochondrial environment induced by ROS formation at complex III of the mitochondrial electron transport chain (ETC), using a transgenic zebrafish embryos genetically encoding a glutathione sensor, we could show that chemical activation of HIF1α signaling indeed increased oxidative pressure. As zebrafish embryos can be protected against

MTH1 induced death by exposure to anti-oxidants, we concluded that an elevated redox environment in combination with deregulated hypoxia signaling might be the underlying cause for sensitization to MTH1 inhibition.

Targeting MTH1 as a secondary ROS defense system

Cellular ROS defense systems contain endogenous enzymatic anti-oxidants and non-oncogenic addiction enzymes such as MTH1. MTH1 plays an important role in nucleotide metabolism by hydrolyzing 8-oxo-dGTP and 2-OH-dATP to their respective monophosphates[61], thereby preventing incorporation of these erroneous nucleotides into DNA and potentially lethal consequences[49] **(Figure 5)**. While being non-essential in non-transformed cells, as shown by MTH1 knockdown mice that live long and grow old[49,172], cancer cell depend on MTH1 as a nucleotide sanitizing enzyme. Attributed to this function, MTH1 is part of a *secondary* ROS defense system and represents a favorable and druggable target in cancer cells by performing oxidative damage control[173]. In contrast to MTH1, other endogenous anti-oxidant defense systems are involved in the *primary* regulation of redox homeostasis and potentially provide additional targets, such as the anti-oxidant master regulator Nrf-2. Playing a crucial role in tumor progression and correlating with poor clinical outcome, Nrf-2 regulates anti-oxidant response genes involved in glutathione (GSH) synthesis, metabolism and other cyto-protective mechanisms[174]. Moreover, the plant derived compound Brusatol was able to decrease Nrf-2 protein levels, thereby improving sensitivity to chemotherapeutics such as Cisplatin and Gemcitabine[175]. Other groups have developed small molecule inhibitors against Nrf-2[176] or used retinoic and ascorbic acid to suppress Nrf-2 activity[177]. However, it remains debatable if the therapeutic window between healthy and malignant cells is sufficient when systemically targeting Nrf-2, or if this could lead to adverse side-effects in healthy cells to the extent of promoting malignant transformation. The same will likely be true for systemic inhibition of cellular anti-oxidants such as GSH, thioredoxin (TXN) or superoxide dismutase (SOD). Although several studies support the theory that direct inhibition of these cellular anti-oxidants sensitized cancer cells to chemo- and radiotherapy *in vitro*[178–181], there is a realistic chance that such treatment, *i.e.* using natural compounds, can evoke adverse effects in patients, dependent on dosing and timing[182]. Thus, targeting more cancer-specific *secondary* ROS defense systems, *i.e.* by inhibiting MTH1, seems to be a more favorable approach than disrupting *primary* multifunctional ROS defense systems in order to eliminate heterogeneous cancer cells while sparing healthy cells.

Clinical application

With the results presented in **Paper I,** we have provided evidence that both, activated hypoxia signaling as well as the oncogenic redox environment, determine sensitivity to MTH1 inhibition, which originates from an increased reliance on damaged nucleotide sanitation. Indeed, overexpression of MTH1, highly associated with poor prognosis, can be found in several cancer types including GBM, liver, gastric and lung cancer[183–186]. Supported by preclinical studies, MTH1 inhibition could be used as monotherapy[187–189] or in combination with conventional chemotherapy as well as ROS inducing agents for enhanced sensitivity[187,190] to target different cancer indications, even those displaying tumor heterogeneity and poor prognosis. Given the fact that radiotherapy induces endogenous ROS production and promotes nucleotide damage-induced cell death, it is plausible to speculate that MTH1 inhibitors could be used as radio-sensitizers or in combination with radiotherapy[190].

Aside from cancer, other major health issues including neurodegenerative diseases such as Alzheimer[191] and Parkinson[192] as well as rheumatoid arthritis[193] are characterized by a specific, more oxidized redox environment, offering further clinical indications for MTH1 inhibition.

Supported by promising preclinical *in vitro* and *in vivo* data, clinical candidate Karonudib (TH1579) is currently evaluated for the treatment of cancer in a clinical phase I study (ClinicalTrials.gov Identifier: NCT03036228).

Challenges

Despite the development of several potent small molecule inhibitors against MTH1 as well as the support of preclinical *in vitro* and *in vivo* studies[49,187–189], the emerging role of MTH1 as a promising anti-cancer drug target has been challenged. Studies from independent groups report that some of the new and potent inhibitors for MTH1, albeit inhibiting the 8-oxo-dGTPas activity of MTH1 *in vitro,* fail to induce cancer-specific cell death[66–68]. Intriguingly, these compounds did neither increase 8-oxo-G levels nor oxidative stress levels, potentially explaining the lack of cellular toxicity. Other studies have reported that small molecule inhibitors TH287 and TH588 were able to induce cancer-specific cell death independent from MTH1 inhibition, indicating uncharacterized off-target effects[194]. To date, MTH1 is the only enzyme known to hydrolyze 8-oxo-dGTP to the corresponding monophosphate[61]. However, in addition to inducing DNA damage, it has been reported that 8-oxo-dGTP regulates telomere elongation by interacting with telomerase[195]. Depletion of MTH1 could therefore

increase telomere dysfunction leading to enhanced cancer-specific cell death. Regarding inhibitor specific off target effects, preliminary data indicates that MTH1 inhibitors TH588 and TH1579 affect microtubule structures in addition to oxidative stress to promote mitotic catastrophe and cancer-cell specific death (Gad *et al.*, submitted manuscript).

In summary, MTH1 biology seems to be more complex than initially thought and further characterization of the mechanistic link between MTH1 inhibitors, especially clinical candidate Karonudib, and induction of cancer-cell death is required.

Conclusion Paper I

In **Paper I** we obtained supporting data that increased oxidative pressure and deregulated hypoxia signaling determine sensitivity to MTH1 inhibition *in vitro* and *in vivo*. Resulting from malignant transformation, cancer cells commonly display an increased oxidative environment, while relying on functional ROS defense systems, including endogenous anti-oxidants and non-oncogenic addiction enzymes such as MTH1. As the disruption of the *primary* anti-oxidant defense systems for therapeutic purposes is debatable due to the high risk of adverse side effects, MTH1 inhibition may offer a promising approach to specifically target many different cancer indications either as mono- or combination therapy, by selectively killing heterogeneous cancer populations.

2.5.2 Paper II

In **Paper II** we focused on some of the major challenges that drug discovery against GBM is currently facing: extensive tumor heterogeneity, cancer cell plasticity and the presence of cancer stem cells[16,75,76]. However, all cancer cells within a glioblastoma can be tracked to a common denominator: they arise from neuronal brain cells. In order to fuel ATP intensive neuronal activity, the human brain consumes 20 % of the total basal oxygen and 25 % of circulating glucose[196,197]. Free oxygen radicals (*i.e.* superoxide; O_{2}-) and non-radicals (*i.e.* hydrogen peroxide; H_2O_2) generated as metabolic by-products play essential roles in tightly orchestrated redox signaling pathways[198,199], which are highly sensitive to disruptions. Due to the lack of extensive endogenous anti-oxidant defense systems[200,201], the brain is, more than any other tissue, susceptible to oxidative stress, which may lead to neurodegeneration and malignant transformation. In this regard, several studies have shown that brain cancers, independent of their mutational profile, are characterized by high oxidative pressure and a resulting reliance on functional DNA repair and sanitizing enzymes[202–204]. In regard to GBM, researchers have identified the amplification of chromosome 7 in combination with the loss of chromosome 10 as early genetic events in GBM ontogeny[205,206]. Intriguingly, it has been reported that the MTH1-gene is also localized on chromosome 7 (7p22) and by analyzing three different datasets, we found MTH1 transcripts to be significantly overexpressed in GBM. Furthermore, it has been suggested that the expression of MTH1 correlates with GBM aggressiveness and proliferative potential[184,202], thereby providing a broad GBM-specific target. Based on this, we hypothesized that MTH1 inhibition may present a promising strategy to eliminate heterogeneous GBM populations.

Initial investigations were performed using a panel of patient-derived GBM cultures[161,207]. We found that depletion of functional MTH1 either by siRNA-mediated knockdown or small molecule inhibition using MTH1 inhibitors TH588 and TH1579 resulted in decreased viability of all GBM cultures, irrespectively of their intrinsic aggressiveness. In line with our findings, two other groups provided additional supporting data showing that MTH1 is indispensable for GBM growth and survival[184,208]. Next, we aimed to relate the effects of our MTH1 inhibitors on GBM cells to oxidized nucleotide sanitation. Indeed, we found 8-oxo-G lesions in the DNA of analyzed GBM cultures using a modified comet assay and consequently, an increase of general DNA damage upon exposure to TH588 and TH1579. Thus, our results are in line with the working model presented by Gad *et al.*[49] **(Figure 5)**. In addition to its DNA damaging potential, it has been reported that modified guanine nucleotides may affect the polymerization of tubulin *in vitro* and *in vivo*[209,210]. Since our

MTH1 inhibitors induce G2/M arrest and mitotic catastrophe, further studies are currently ongoing to investigate potential effects of MTH1 inhibition on tubulin dynamics.

MTH1 inhibition to overcome GBM heterogeneity

Heterogeneous GBM populations comprise large numbers of GSCs, which are characterized by so called stemness traits including a quiescent state, protection against oxidative stress and overexpression of drug efflux pumps, all conferring to GBM aggressiveness and treatment resistance. Due to previous observations that our MTH1 inhibitors target GBM cultures potently and independent of aggressiveness, we exposed isolated GSCs to MTH1 inhibitors TH588 and TH1579 and investigated if they are also dependent on functional MTH1. Indeed, we found that depletion of MTH1 resulted in decreased viability and clonogenic potential of isolated GSCs. In addition to that, we observed increased incorporation of 8-oxo-dGTP into DNA as well as a dramatic prolongation of mitosis and mitotic catastrophe by following fluorescently labeled GSCs in real-time. Potential reasons for these MTH1-induced phenotypes in GSCs are debatable. Despite the malignant nature and in contrast to normal cancer cells, it is generally accepted that cancer stem cells, or in this context GSCs, reside in hypoxic niches and exhibit low ROS levels in order to maintain their stemness traits[23,211]. In respect to that, several studies have shown that increasing ROS levels in stem cells induces differentiation and/or exit of the quiescent state[36,212]. Therefore, it is easy to speculate that disrupting ROS defense systems in GSCs *i.e.* by MTH1 inhibition may lead to increased oxidative stress and subsequent exit of the quiescent state, which further promotes the accumulation of erroneous nucleotides in the DNA, prolonged mitosis and eventually, mitotic catastrophe. In contrast, a different study suggests that GSCs already suffer from higher oxidative pressure and reliance to anti-oxidant defense systems compared to non-GSCs[213], which could also explain their addiction to functional MTH1.

Comparison to standard therapy against GBM

In addition to treatment resistant GSCs which may promote tumor recurrence, additional resistance mechanisms are conferred by an upregulated DSB repair machinery rendering GBM insensitive to radiotherapy[78] and/or the standard chemotherapeutic agent TMZ[214]. Pursuing a monotherapy approach, we compared the anti-tumor efficacy of our MTH1 inhibitors to TMZ and found that TH588 and TH1579 were more potent in eradicating GBM. Most importantly, this effect was completely independent of the intrinsic MGMT expression levels of tested GBM cultures. Therefore, it is tempting to speculate that MTH1 inhibition could be used to target TMZ-resistant or even recurring GBM tumors. As mentioned earlier,

it is furthermore plausible that MTH1 inhibitors could be used as radio-sensitizers or in combination with radiotherapy[190]. In addition to that, a different study suggests that induction of oxidative stress by depletion of glutathione synthesis could sensitize GBM tumors to TMZ or Cisplatin[179]. It would be interesting to investigate if oxidative stress mediated by MTH1 inhibition could sensitize GBM tumors to TMZ therapy.

Penetration of the blood-brain barrier

Another major challenge that hampers GBM therapy is the presence of physical barriers such as the BBB and the BTB, which separate the circulating blood from the brain and thus, brain tumors. The BBB is a semipermeable border formed by endothelial cells, astrocyte end-feet and pericytes. Critical substances are retained passively by the selectivity of tight junctions or actively through efflux proteins (ATP binding cassette transporter and P-glycoprotein) within the BBB[215]. In contrast, the BTB is more complex than the BBB due to aberrant cellular compositions of newly developed microvessels[216]. Most drugs penetrate the barriers by transmembrane diffusion, a mechanism favored by hydrophobicity and molecular weight[217]. Despite the development of several *in vitro* BBB models[218], it remains inevitable to determine drug penetrance of the BBB *in vivo* in order to provide reliable predictions on the therapeutic outcome. Recently, it has been shown that the zebrafish develops its BBB between day 3 and 10 post fertilization, while sharing structural and functional similarities with that of mammals[219,220]. Supported by these findings, the zebrafish has been introduced as a suitable model to investigate BBB penetration, which further increases its clinical relevance.

In **Paper II** we applied our orthotopic zebrafish model for GBM to investigate if our MTH1 inhibitors efficiently target both GBM and GSCs *in vivo*. Following transplantation, tumor-bearing zebrafish were swimming freely in TH1579-containing fish water. After an exposure of 5 days to TH1579, we found a significant reduction in tumor volume compared to the control. Additional *in vivo* real-time imaging of exposed tumors via light-sheet microscopy revealed numerous GBM cells undergoing cell death confirming an overall tumor reduction of 25 %, thereby suggesting that MTH1 inhibitor TH1579 is able to reach GBM cells *in vivo*. However, further investigations in higher organisms are necessary to confirm these initial results.

Conclusion Paper II

In **Paper II** we evaluated MTH1 inhibition as a strategy to target heterogeneous GBM populations. Irrespectively of the GBM cultures' intrinsic aggressiveness, the amount of GSCs, or MGMT expression levels and thus, resistance mechanisms to the standard therapy TMZ, our MTH1 inhibitors TH588 and TH1579 efficiently induced cancer cell death in all tested GBM cultures *in vitro* and *in vivo*. Further studies to characterize the underlying mechanism of action of our clinical candidate Karonudib (TH1579) as well as its BBB-penetrating ability using higher organisms are currently ongoing. While the anti-tumor efficacy of Karonudib as combination therapy in preclinical studies also remains to be validated, based on the obtained results, we conclude that MTH1 inhibition indeed represents a promising targeted approach to selectively eradicate heterogeneous GBM populations.

2.5.3 Paper III

In the last part of this doctoral thesis, we addressed another major challenge that hampers the discovery of potent drugs against GBM: the lack of predictive GBM animal models that are suitable for high-throughput drug discovery screens. In the last decade, the zebrafish has emerged as a clinically relevant model for all kinds of human malignancies including cancer, and orthotopic transplantation of GBM material was found to faithfully recapitulate the human disease. Although biological and technical advantages render the zebrafish suitable for drug discovery screens, none of the currently available transplantation procedures in zebrafish can be used in high-throughput screens[144–146]. Therefore, in **Paper III**, we aimed to refine existing orthotopic zebrafish models for GBM and to develop an approach which allows for fully automated high-throughput screens, including automatable tumor transplantation, embryo handling, drug exposure and read-out processes.

Migration potential of transplanted GBM cultures and other cancer types

As suggested by fate-map analysis and previous data indicating that early zebrafish could provide lineage-specific trophic support to human cells[221], we hypothesized that human GBM cells transplanted into the zebrafish blastoderm might migrate into CNS structures of the developing zebrafish embryo. Indeed, 24 hours after transplantation we found that the majority of transplanted zebrafish embryos developed intracranial tumors. By transplanting both patient-derived GBM cultures as well as established GBM cell lines in comparison to colorectal cancer lines, we further elucidated that migration behavior into CNS regions was GBM specific, but independent from the transplantation site within the blastoderm of the zebrafish embryo. This might be due to the fact that ectodermal precursor cells can be found at all latitudes in the blastula fate map[222].

To further investigate migration potential, we transplanted several other cancer types, including different human malignant melanoma lines, prostate and bladder cancer as well as non-malignant, immortalized retinal pigmented epithelial (RPE) cells and followed their migration behavior *in vivo*. Transplanted human prostate and bladder cancer lines formed tumors alongside intestinal regions of the developing zebrafish embryos, while RPE cells did not establish tumors (own unpublished data). In comparison to transplanted GBM cultures that migrated deeply into the brain and other CNS regions of developing zebrafish embryos, transplanted malignant melanoma lines predominantly formed superficial tumors in the skin of the zebrafish tail region (own unpublished data). These observations contradict previous study results showing that human metastatic melanoma cells transplanted into blastula stage

embryos failed to form tumors in host organs, while non-cancerous melanocytes migrated to the normal microenvironment of the skin[221]. One plausible explanation for the lacking tumor formation might be the absence of melanoma stem cells or melanoma cells that have undergone EMT to promote migration and invasion of the tumor microenvironment or in this case, the developing zebrafish embryo[223]. In our case, most of the GBM cultures that were transplanted into zebrafish embryos display high levels of invasive GSCs, which can be identified by cell surface marker CD133. Already in **Paper II**, we observed that GBM cultures depleted of CD133 positive cells failed to migrate into the CNS to establish a congregated tumor. In line with that, here we could show that GBM cells dissociated with trypsin, an enzyme degrading surface molecules and potentially depleting GBM cells of CD133 significantly reduced their homing capacity to the CNS when compared to dissociation with Accutase®, a more gentle dissociation reagent. Based on these results, we conclude that surface molecules of transplanted cells could be involved in the perception of homing cues and promote migration potential, similarly to cancer cells that have undergone EMT to promote invasion and metastasis[223–225].

Clinical relevance of zebrafish for drug development against GBM and beyond

Following the development of a robust orthotopic model for GBM using zebrafish embryos, we continued with a detailed characterization to support its clinical relevance. In line with the literature[144–146], we could show that transplanted GBM cells proliferated *in vivo* leading to increased tumor volume, and invaded healthy surrounding brain tissue by developing extended tumor microtubules. Moreover, we observed tumor vascularization and interaction between the innate immune system of the zebrafish and the transplant. Thus, our model faithfully recapitulates the human disease *in vivo* and opens numerous avenues for drug discovery by targeting GBM cells directly or interfering with tumor vascularization or the innate immune system to inhibit tumor growth.

As briefly mentioned in the previous section, the zebrafish has been introduced as a suitable model to investigate BBB penetration, which is clearly of outmost importance when it comes to GBM drug discovery. In this regard, several studies could show that the BBB in zebrafish displays structural and functional similarities to that of mammals[219,220,226] as it starts to develop around day 3 post fertilization. Therefore, evaluating the anti-tumor efficacy of selected drugs against GBM using our orthotopic model and the suggested experimental set-up **(Figure 12)**, ultimately provides information regarding the drug's ability to penetrate the BBB. In **Paper III** we applied our orthotopic GBM model to test the anti-tumor efficacy of

selected TKIs, which are currently in clinical studies, on TMZ-resistant GBM cultures. Only Erlotinib displayed superior anti-tumor effects compared to other tested TKIs, indicating sufficient BBB passage. Our findings are in line with other studies showing that Erlotinib has the best BBB penetrance and lowest brain efflux rate of our tested TKIs in humans[227–230], while inducing the highest response rate in patients. This indicates that our orthotopic model for GBM exhibits enormous predictive value by faithfully recapitulating the *in vivo* pharmacology of clinically tested drugs.

Besides high conservation of numerous drug targets and physiological processes, as thoroughly elaborated on in the introduction, it is postulated that drug metabolism and pharmacology also display a close correlation between zebrafish and humans[120,231]. Several human drugs have been screened for conserved effects in zebrafish, *i.e.* drugs that induce repolarization cardiotoxicity or modulate cardiac contractility and vasomotion[150,232]. Notably, the majority of effects in humans were recapitulated in zebrafish. Additionally, around 80 % of compounds discovered in diverse zebrafish screens evoked a similar response in rodents[120]. Due to the high probability of direct correlation between effects in zebrafish and humans, but also zebrafish and rodents, it is likely that drug distribution, metabolism and excretion are highly conserved among these species. Recently, a research group has reported that many human cytochrome P450 (CYP) enzymes possess direct orthologues in zebrafish[231], providing first indications that metabolism is indeed highly conserved between zebrafish and humans.

Although further studies are required to fully understand all *in vivo* pharmacology – essential ADME (administration, distribution, metabolism, and excretion) parameters in aquatic model systems, the number of compound screens[120] as well as the number of discovered compounds using zebrafish is steadily increasing[233,234]. In a landmark study published in 2007, Professor Zon and his research group performing zebrafish drug screens identified a stabilized derivate of prostaglandin E2 (PGE2), also known as Prohema, which improves the engraftment of transplanted umbilical cord blood cells by enhancing the homing effect to the bone marrow[235]. Notably, Prohema was the first drug identified in zebrafish, which was subsequently recommended for clinical evaluation. After passing clinical phase I in 2013, Prohema is currently tested in clinical phase II. Besides facilitating the discovery of novel drugs, drug discovery screens using zebrafish may furthermore accelerate studies that focus on new drug combinations and the repurposing of existing drugs[236] in order to improve the life of many patients.

Conclusion Paper III

In **Paper III**, we developed an improved orthotopic zebrafish model for GBM, which faithfully recapitulates the clinical characteristics of GBM tumors *in vivo*. In contrast to currently existing orthotopic zebrafish models for GBM, it does not require technically challenging intracranial transplantations, and can directly be implemented in fully automated high-throughput drug screens, including automatable tumor transplantation, embryo handling, drug exposure and read-out processes. Most importantly, as a robust and predictive drug screen using our model can be performed in zebrafish embryos younger than 5 days, no ethical permit is required in most countries. Moreover, the application of our model as a useful tool and complementary animal system could decrease the number of drugs that need to be tested in higher organisms. Due to these benefits, our orthotopic zebrafish model for GBM complies with the 3R guidelines for animal research, namely replace, reduce and refine, and offers great potential to 1) identify and optimize novel effective drugs against GBM, and potentially, other cancer indications 2) accelerate repurposing of already existing drugs and 3) facilitate screening for tailor-made drugs, as a step towards personalized medicine.

2.6 CONCLUSION AND FUTURE PERSPECTIVE

Cancer drug discovery is hampered by the complex and dynamic nature of cancer including constant alterations in molecular and cellular interactions, activation of cancer resistance mechanisms, as well as ineffective drug delivery systems and life-threatening side-effects. Drug discovery against GBM is particularly challenged by extensive tumor heterogeneity, the presence of physical barriers such as the BBB and the lack of orthotopic animal models, ideally suitable for high-throughput drug screens.

In the first part of this doctoral thesis, we have addressed these challenges by pursuing a targeted therapy approach focusing on deregulated redox systems, representing a cancer cell's Achilles' heel. For this, we have combined established biochemical and cellular assays with the broadly applicable zebrafish model **(Figure 14)**.

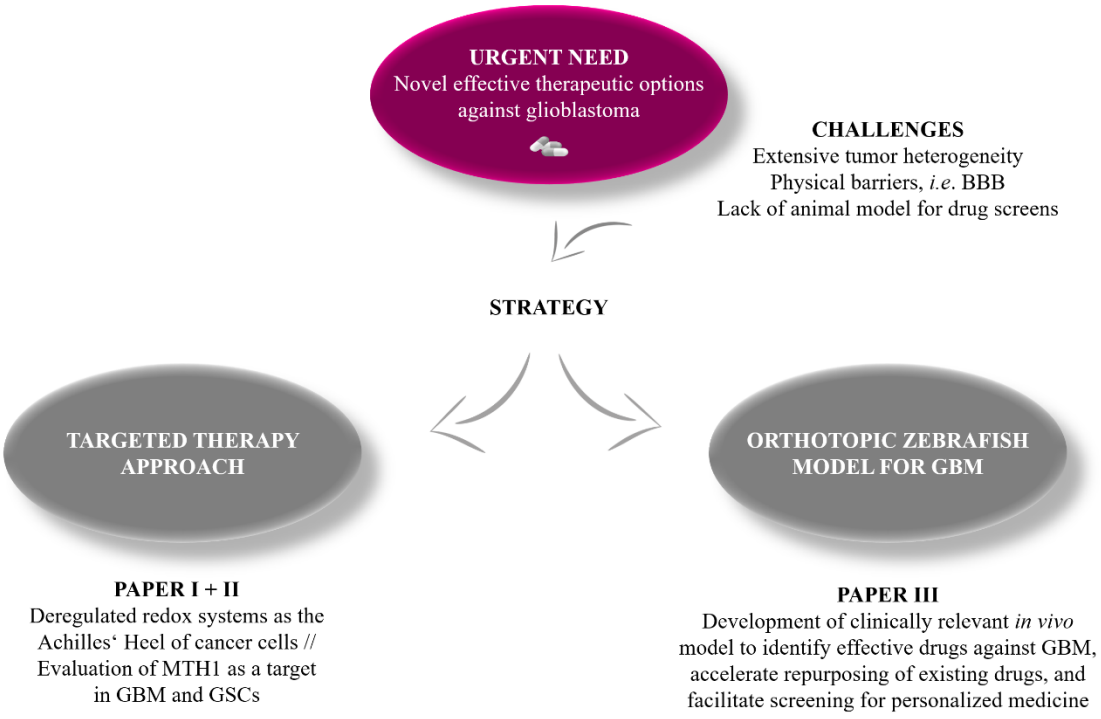


Figure 14: Illustration of strategic research approach
Although novel effective therapeutic options against GBM are urgently needed, drug discovery is severely challenged by extensive tumor heterogeneity, the presence of physical barriers such as the BBB and the lack of orthotopic animal models suitable for high-throughput drug screens. In this doctoral thesis, we have addressed these challenges by pursuing a targeted therapy approach focusing on deregulated redox systems, representing a cancer cell's Achilles' heel, and evaluating MTH1 as a potential target in heterogeneous GBM populations. Moreover, we developed a clinically relevant *in vivo* model using zebrafish to facilitate high-throughput drug screens.

Following the introduction of MTH1 as a promising anti-cancer target, in **Paper I,** we aimed to characterize factors rendering cancer cells to MTH1 inhibition. We found that increased oxidative pressure and deregulated hypoxia signaling determine sensitivity to MTH1 inhibition *in vitro* and *in vivo*. As cancer cells commonly display an increased oxidative environment, enhancing their reliance on functional ROS defense systems, MTH1 inhibition may offer a promising approach to specifically target many different cancer indications either as mono- or combination therapy, by selectively killing heterogeneous cancer populations.

In **Paper II** we tested this hypothesis by validating MTH1 inhibition as a strategy to target heterogeneous GBM populations. Indeed, we found that our MTH1 inhibitors TH588 and TH1579 efficiently induced cancer cell death in all tested GBM cultures, independent of intrinsic aggressiveness, heterogeneity or resistance mechanisms to the standard therapy TMZ, both *in vitro* and *in vivo*. With this, we have provided supporting evidence that MTH1 inhibition may indeed represent a promising targeted approach to selectively eradicate heterogeneous GBM populations.

However, future preclinical studies including further characterization of the mode of action of our MTH1 inhibitors, especially of the clinical candidate Karonudib (TH1579), and *in vivo* studies confirming its BBB-penetrating ability using higher organisms are needed. In addition to that, it is important to identify potential resistance mechanism to MTH1 inhibition as early as possible to successfully overcome them, possibly by enhancing anti-tumor efficacy of Karonudib as part of a combination therapy.

To address the urgent need of an animal model suitable for high-throughput drug screens, in **Paper III**, we developed an orthotopic zebrafish model for GBM, which faithfully recapitulates the clinical characteristics of GBM tumors *in vivo*. Performing a novel transplantation technique using blastula stage zebrafish embryos, our model can directly be implemented in fully automated high-throughput drug screens, including automatable tumor transplantation, embryo handling, drug exposure and read-out processes. Within 5 days, a robust and predictive drug screen can be performed to identify and optimize novel anti-GBM drugs, accelerate repurposing of already existing drugs, as well as to determine effective drug combinations with low systemic toxicity.

Moreover, due to its clinical relevance, our orthotopic zebrafish model allows for implementation in precision oncology platforms alongside other clinical applications to improve identification of tailor-made therapies. Freshly excised tumor specimen from patients could be transplanted into zebrafish using automated injection robotics, generating

individual zebrafish avatar libraries to screen for the most effective treatment for the corresponding cancer patient. The future is now: in September 2018, a co-clinical study using zebrafish embryos was launched by the University of Pisa (ClinicalTrials.gov Identifier: NCT03668418). This study combines an observational trial on patients operated on hepato-biliar-pancreatic or gastro-intestinal cancer undergoing chemotherapeutic treatment with an animal trial using zebrafish embryos transplanted with patient cancer cells to demonstrate the zebrafish model's ability to predict the most effective treatment option for each patient. This co-clinical study and our orthotopic zebrafish model for GBM presented in **Paper III** raise high hopes for personalized GBM medicine and beyond.

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