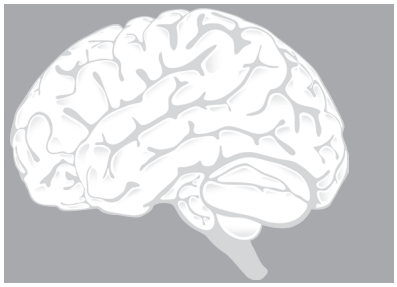
CHAPTER 7



General discussion and future perspectives

GENERAL DISCUSSION

New treatment strategies for high-grade brain tumors, including glioblastoma (GBM), medulloblastoma, and diffuse intrinsic pontine glioma (DIPG), are urgently needed since outcome remains poor for patients with these diseases. In addition, current treatment regimens are accompanied by short- and long-term sequelae, which have a severe impact on the quality of life of brain tumor patients. The aim of this thesis was to identify mechanisms underlying treatment resistance in high-grade brain tumors, to interfere with these mechanisms, and, thus, to improve the efficacy of currently used treatment modalities in GBM, medulloblastoma, and DIPG. We identified potential therapeutic targets and agents that could - at least partly - increase the response of high-grade brain tumors to temozolomide (TMZ; chapters 2 to 4) or radiotherapy (chapters 5 and 6).

Combination therapies

The novel treatment strategies described in this thesis may be potential therapeutic venues for the sensitization of high-grade brain tumor cells to the standard treatment modalities, i.e. radio- and chemotherapy. However, the putative sensitizers used in this thesis only partially increased the radio- and TMZ sensitivity of brain tumor cells and in the *in vivo* experiments almost all tumors eventually recurred. This partial response could be explained by redundancy of intracellular signaling pathways that promotes tumor progression and therapy resistance, in our case radio- and TMZ resistance. Targeting one specific protein or pathway is probably not sufficient to completely inhibit tumor growth, reverse therapy resistance and eradicate the tumor[1]. For example, the identified TMZ resistance factor EFEMP1 (chapter 2) is able to activate Notch signaling in GBM, however, it has also been suggested to activate other signaling pathways, including EGFR signaling, which could also convey resistance to GBM cells. Therefore, it was to be expected that inhibition of Notch signaling using the gamma secretase inhibitor RO4929097 could only partially restore sensitivity to TMZ. Thus, it may be necessary to combine this with inhibitors of EGFR or inhibitors of downstream effector molecules of EGFR signaling (e.g. PI3K, AKT, mTOR1/2, and/or MEK inhibitors[2,3]). Since redundancy of intracellular signaling pathways presents a significant obstacle for treatment of high-grade brain tumors, combinatorial strategies for the management of these diseases are needed to respond to this molecular redundancy within the tumor. This might be achieved by targeting tumor cells with agents that have multiple targets (including dual inhibitors such as NVP-BEZ235[4] targeting PI3K and mTOR and multi-targeting agents such as sorafenib[5,6] targeting PDGFR-α,β, VEGFR-2,3, BRAF, c-Kit) or with several single target agents combined in a cocktail or administered sequentially, which inhibit multiple signaling pathways or different constituents in the same pathway. Clearly, the affinity of inhibitors for therapeutic targets needs to be taken into account, and combinatorial use of inhibitors may also lead to enhanced toxicity to normal

cells and tissues. Future studies on drug combinations are warranted, in particular in combination with TMZ and radiation if considered in first-line therapeutic regimens.

Tumor heterogeneity

Another feature of high-grade brain tumors that contributes to treatment resistance and emphasizes the need for a multi-targeted approach in treating brain tumors is tumor heterogeneity, both between and within tumors[7–9]. The existence of molecular subtypes has been described for all three high-grade brain tumor types studied in this thesis[10–12]. The diversity of molecular aberrations detected in these subtypes explains the lack of optimal efficacy of a standard treatment regimen for all patients with a specific brain tumor type, which usually consists of a combination of radio- and chemotherapy. In addition, intratumoral heterogeneity further complicates the efficacy of a standard treatment regimen for individual patients as subpopulations of cells within the tumor portray distinct molecular aberrations. Recently, medulloblastoma metastases from an individual patient were shown to be genetically divergent from the matched primary tumor, caused by clonal selection of a restricted subclone of the primary tumor, and different molecular GBM subtypes within single GBM tumors have also been identified[13,14]. Therefore, the existence of tumor heterogeneity suggests that a personalized, tailored therapeutic approach consisting of multimodal treatment regimens is needed to combat these tumors. However, intratumoral heterogeneity can make it difficult to select therapeutic agents for individual patients since the selection of these agents will be based on the molecular aberrations found in a small piece of tumor tissue at a certain time point, which can result in sampling bias. The effect of sampling bias over space and time needs to be taken into account in the selection of targeted therapies for individual patients[15]. Therefore, for optimal therapy selection, it will probably be necessary to examine the molecular profile of several parts of the tumor. Furthermore, to be able to adapt the selection of therapeutic agents over time, changes in the molecular phenotype of the tumor should be determined over time, which requires a non-invasive detection method such as blood-based assays.

Blood-brain barrier

When considering brain tumor treatment, efficient drug delivery to the tumor site is a major hurdle to take[16]. The blood-brain-barrier (BBB) impairs the uptake of chemicals from the circulation and, therefore, hinders delivery of drugs to tumors growing in the brain. In addition, drug efflux pumps on the endothelial surface and on tumor cell membranes play an essential role in drug uptake and form a second barrier for efficient drug delivery. Drugs that are targets of these specific drug efflux transporters do not penetrate into the brain and into tumor cells efficiently. Therefore, novel therapeutic agents that show preclinical potential must be able to pass the BBB in order to be of value in the clinic. The agents identified and tested in this thesis for their potential

to function as TMZ- or radiosensitizers have all been reported to pass the BBB and to penetrate into the brain tissue. Although hydroxyurea (HU) has been shown to be transported across the BBB in both guinea pigs and rats, and to increase blood brain tumor permeability, potentially improving the uptake of other chemicals into the brain[17–19], a pre-clinical analysis showed that it had no effect on permeability of Imatinib Mesylate (Gleevec) across the blood-brain barrier[20]. Whether HU increases the penetration of TMZ to the brain and/or to GBM tumors, thereby contributing to an enhanced therapeutic effect of TMZ, warrants further evaluation. Both the natural compound quercetin (identified in chapter 5) and the WEE1 kinase inhibitor MK-1775 have been shown to pass the BBB, making these compounds promising for brain tumor treatment, although quercetin has been reported to be a substrate of the ABC transporter BCRP1, possibly limiting its bioavailability to the brain[21–27]. The ability of the gamma secretase inhibitor RO4929097 to pass the BBB has been evaluated in a phase I clinical trial[28]. In this proof-of-concept trial, they observed detectable RO4929097 tissue levels both in areas with a disrupted BBB or an intact BBB, although the RO4929097 levels were significantly higher in the disrupted BBB areas.

Approaches for novel target/therapeutic agent identification

Two different approaches were employed to explore for novel targets and therapeutic agents that could improve the response of high-grade brain tumors to chemo- or radiotherapy. In chapters 2, 3, and 6, we used a functional genomics approach to identify novel therapeutic targets that could function as treatment sensitizers in GBM (Ch. 2 and 3) and DIPG (Ch. 6), whereas drug screens were employed in chapter 4 and 5 for the identification of potentially effective TMZ- or radiosensitizers in GBM (Ch. 4) and medulloblastoma (Ch. 5), respectively. Functional genomics, including transcriptomics, is a field of molecular biology that explores gene and protein functions and interactions on a global scale. This approach provides a powerful tool for the molecular dissection of cancer and we used it to explore TMZ resistance mechanisms in GBM by determining the differences in mRNA and miRNA expression between TMZ-sensitive and TMZ-resistant GBM cells. In chapter 6, WEE1 emerged as a potential drug target in DIPG by *in silico* transcriptome analysis. High-throughput experiments for determining transcript expression yield enormous amounts of complex data, which requires sophisticated computational tools to analyze these data. When analyzing these data sets to answer biological questions it is necessary to consider the effects of background noise, data normalization, and complex statistics on the obtained results, and to apply bioinformatics techniques, such as clustering, to extract relevant information. Moreover, the increasing diversity of experimental techniques and subsequent high dimensionality of the resulting data makes it difficult to compare results across different experiments. Integration of information from a multitude of functional genomics data sets such as applied in chapter 3 (mRNA and miRNA expression) adds to the complexity of the analysis of -omics data. Therefore, for biologists to be able to analyze and interpret the

results of these high-throughput experiments, they need to acquire specific knowledge and skills, and must collaborate with experts such as biostatisticians. To our benefit, more and more user-friendly tools have been developed that incorporate algorithms for data analysis and allow for the analysis of generated -omics data and for comparing results to data sets already deposited in publicly available repositories[29,30]. This will facilitate extraction of biological insights from these data.

The other approach we employed in this thesis to identify potential therapeutic agents for high-grade brain tumor treatment was drug screening. The number of drug libraries available for screening is enormous and includes libraries consisting of natural compounds, FDA-approved drugs, kinase inhibitors, customized libraries, etc. This method allows for high-throughput drug screening to identify agents that show cytotoxicity in cancer cells or, in our case, to identify agents that could sensitize cancer cells to radiation or chemotherapy. However, there are some limitations concerning this approach. Usually, only a single fixed drug concentration is used for all compounds, which might not be optimal for all drugs, increasing the likelihood of discarding otherwise effective drugs from further analysis. Furthermore, we assessed the effect of the drugs in combination with TMZ or radiotherapy. Timing of the treatment modalities is pivotal for the identification of putative therapy sensitizers. TMZ and the potential TMZ sensitizers used in chapter 4 were added simultaneously to the medium, while in chapter 5, drugs were added to the cells 30 minutes before radiation was applied. The choices for these treatment schedules were usually based on logistics and on the limitations of *in vitro* cell culture techniques but exploiting one treatment schedule will increase the likelihood of discarding potentially effective treatment sensitizers. In addition, we applied only a single dose of radiation or TMZ to the cells in most *in vitro* assays performed in this thesis while in the clinic patients are treated with fractionated doses of radiation or multiple daily doses of TMZ for several weeks. This is a limitation of our approach and translation of our results to the clinic. In chapter 4 however, we did treat primary neurospheres for five consecutive days with TMZ and determined the effect of TMZ on sphere formation, growth and recovery. This assay setup more closely mimics the clinical situation and an effort should be put in mimicking the treatment schedules used in the clinic when searching for treatment sensitizers *in vitro*. Further validation in *in vivo* models will facilitate the use of more clinically relevant treatment schedules.

Preclinical models

Relevant and reliable preclinical models are essential for the identification of treatment resistance mechanisms, for interfering with these mechanisms, and for developing novel treatment strategies. In this thesis, we employed both *in vitro* and *in vivo* models to find novel targets and therapeutic agents for high-grade brain tumor treatment. As mentioned in the introduction of this thesis, there are some limitations concerning these models, and especially the relevance of established cell lines for target discovery

and drug testing is questionable. These cells have usually undergone selection and adaptation to the cell culture conditions and do not fully resemble the original tumor anymore[31]. It is likely that primary cells, obtained from biopsy or autopsy, recapitulate the characteristics of the original tumor more closely. We used primary cell cultures in chapters 2, 4, 5, and 6 to determine the efficacy of the identified agents in combination with conventional treatment. In our studies, drug testing on these primary cultures was shown to be difficult due to a slow proliferation rate and overall vulnerability of these cells in culture conditions. Therefore, improved culture methods are needed to retain viability of these cells in culture while maintaining the characteristics of the original tumor. *In vitro* models lack the microenvironment in which brain tumors grow in patients, implying that issues as drug delivery and infiltrating tumor cells cannot be addressed in these models. *In vivo* models seem more relevant in the clinical setting because of the presence of a microenvironment surrounding the tumor mass and the infiltrative tumor cells. In this thesis, we used both xenograft models generated from established human brain tumor cell lines (chapters 2, 4, and 5) and from primary brain tumor cell cultures (chapters 2, 4 and 6). The tumors generated after injection of established cell lines often do not recapitulate the infiltrative growth pattern of high-grade brain tumors observed in patients. Instead, primary cells injected orthotopically mimic this infiltrative growth pattern more closely, although these models show an altered tumor immunology and absence of the natural microenvironment[15,32]. Therefore, continued focus is needed on the development of *in vivo* brain tumor models obtained by injection of primary brain tumor cells in the mouse brain that could be used for future drug testing.

FUTURE PERSPECTIVES

The potential usefulness of the identified targets and therapeutic agents in this thesis warrants further research. Some of these studies are in a more advanced stage and translation of the results to the clinic is already launched. An effort is made to start a clinical trial in which the conventional treatment schedule for GBM is supplemented with HU. As mentioned above, the gamma secretase inhibitor RO4929097 is already being tested in a clinical trial (NCT01119599) for newly diagnosed malignant glioma patients. Similarly, the WEE1 kinase inhibitor MK-1775 is being tested in clinical trials for newly diagnosed/recurrent glioblastoma (NCT01849146) and newly diagnosed DIPG (NCT01922076). It will be of interest to see whether these agents are as promising in the clinic as they were preclinically. Due to redundancy of intracellular signaling pathways and tumor heterogeneity, it is likely that addition of only one agent to the conventional treatment regimen will not be sufficient to cure high-grade malignant brain tumors. Future studies on drug combinations are warranted, in particular in combination with TMZ and radiation if considered in first-line therapeutic regimens. In addition, continued focus is needed on the development of clinically relevant preclinical models

for drug testing, which will facilitate translating preclinical findings to the clinic. Finally, further research into the identification of molecular aberrations and critical pathways in high-grade brain tumors is necessary to obtain a more complete overview of the diverse molecular phenotypes of these tumors. This will eventually guide optimal treatment selection and subsequently could improve survival and quality of life of patients with high-grade brain tumors.

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