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Radiosensitizing and synergistic targeted therapies for glioblastoma

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■ **Summary and Discussion**



CONCEPTUAL ADVANCES

Cancer is frequently associated with a dysfunction of one or a few specific genes in molecular pathways that regulate cell growth, proliferation, motility and survival (Hanahan and Weinberg, 2011). Overactivity of a single oncogene on which growth and survival of cancer cells depend is called 'oncogene addiction' of the cancer cell (Weinstein & Joe, 2008). Targeted inhibition of these oncogenes may interrupt oncogene addiction, resulting in tumor growth arrest and induction of apoptosis.

The PI3K/MAPK pathway is one of the key pathways that is frequently disturbed in glioblastoma (GBM). The pathway is controlled by various receptor tyrosine kinases (RTK) on the cell membrane which are activated upon ligand binding. The pathway is also pivotal in processing DNA-damage after irradiation. Targeted inhibition of the receptors and downstream molecules of the PI3K/MAPK pathway may therefore sensitize the effect of radiation in glioma cells.

The PI3K/MAPK pathway as a target to improve radiotherapy of GBM patients - The PI3K/MAPK pathway is under control of various receptor tyrosine kinases (RTK) on the membrane that are activated upon ligand binding and/or mutations. Activation of these RTKs drives growth, proliferation, motility and survival. Inhibition of these pathways can reduce cancer growth and induce apoptosis and thus provide oncogene addiction. However, inhibition of these targets failed or showed only weak therapeutic improvement over current standard of care for GBM in the clinic (Prados et al. 2009, Erlotinib, NCT0018746; Gilbert et al. 2014, Bevacizumab, NCT00884741; Hainsworth et al. 2010, Sorafenib, NCT00544817; Ma et al. 2014, Everolimus). We investigated the radiosensitizing potential of novel targeted agents in the PI3K/MAPK pathways in glioma in vitro and describe an oncogene-addiction independent role of the MAPK pathway. This role was until now overlooked and could be revealed only under specific growth conditions that better mimic the clinical situation, i.e. in 3D multicellular glioma spheroids maintained in culture for weeks after irradiation, allowing evaluation of long term effects. Importantly, since short pulses of the MAPK inhibitor MEK162 are given, drug resistance is avoided in contrast to oncogene addiction where a constant alleviation of the growth/survival cue may be necessary (Becker et al, 2011; Sun et al, 2014). The preclinical data presented in this thesis show for the first time that MEK inhibition combined with radiotherapy may be a novel therapeutic

strategy for glioblastoma patients. Our in vitro and in vivo findings provide a strong basis for clinical evaluation.

Parallel and multidrug synergies - By building a model based on retrospective analysis of reports of drug-drug synergy from the literature and by matching these data to public dose-response data, we found that many reported synergies target processes that affect independent pathways. From these analyses, we have developed a new concept that we have called 'parallel synergy' (see Figure 1). Given that inhibition of parallel survival pathways represents a new category of drug combination therapy, a major challenge remains to identify relevant inhibitors from the thousands of possible combinations. We showed a general strategy to identify these vulnerabilities. Importantly, since our method is based on monotherapy data, we can predict interactions that go beyond the scope of already tested combinations. After identification of parallel vulnerabilities, therapy predictive molecular markers may be identified to enable personalized therapies. In addition to this concept, there is an ongoing effort in linking high throughput synergy data to omics-based prediction models including the recent AstraZeneca synergy DREAM challenge (Menden et al., 2017 submitted).

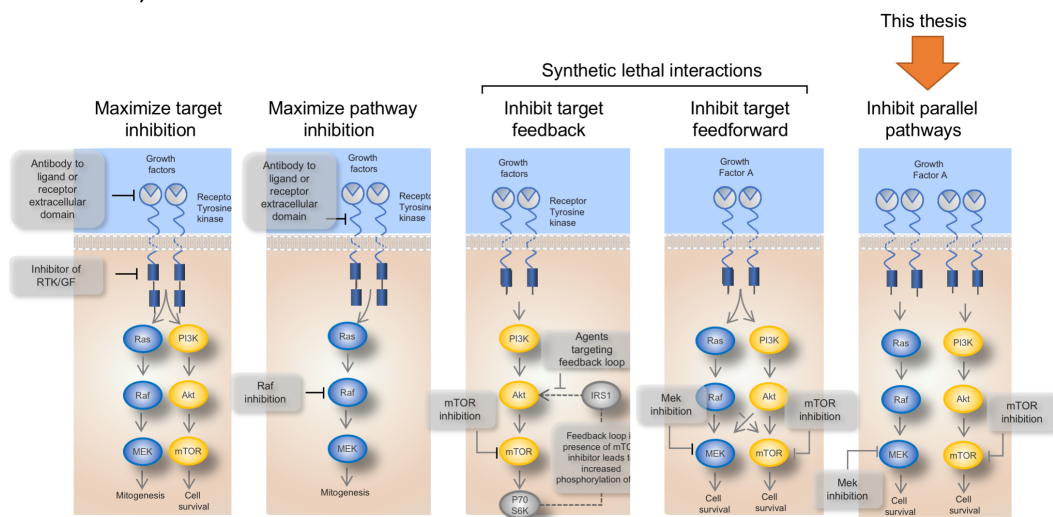


Figure 1. Strategies for synergistic combination therapies described in this thesis (see also chapter 1)

SPECIFIC CHALLENGES:

1 Selecting a biological rationale

The literature study presented in **chapter 2** demonstrated the importance of the AKT-pathway in the response of GBM to irradiation. Aberrations of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway have been frequently reported in GBM and are responsible for disturbed cell growth and increased cell survival leading to a competitive growth advantage, metastatic potential and therapy resistance. Both the importance of AKT pathway in GBM biology and in DNA damage response are described, highlighting the PI3K-AKT pathway as a rational therapeutic target in GBM treatment. Furthermore, an overview is given of specific inhibitors of AKT which have been tested in preclinical and in early phase clinical studies.

In **chapter 3**, we investigated the potential of the allosteric AKT inhibitor MK2206 to enhance current standard therapy, consisting of irradiation and temozolomide (TMZ) in GBM cell cultures either growing as monolayer or as multicellular spheroids. Effects were assessed on cell proliferation, clonogenic cell survival, cell invasion, cell migration and protein expression by Western blot. Low dose MK2206 reduced phosphorylation of AKT in both adherent cells and spheroids, delayed spheroid growth and sensitized spheroids to both irradiation and TMZ in a synergistic way. Effective inhibition of cell invasion and cell migration was observed only at higher doses of MK2206. The data presented show that MK2206 has potent synergistic efficacy to the current standard of care for GBM patients.

In **chapter 4**, glioma spheroid cultures were treated with a panel of small molecule targeted drugs, as single treatment and in combination with irradiation. Spheroid growth after exposure to MEK162 and radiation led to a synergistic growth inhibition of spheroids. MEK162 down-regulated and dephosphorylated the cell cycle checkpoint proteins while increasing DNA damage signals. Combined with radiation, this led to a prolonged DNA damage signal. In vivo data in tumor bearing animals demonstrated a significantly reduced growth rate, increased growth delay and prolonged survival time. In addition, RNA expression of responsive cell cultures correlated to mesenchymal stratification of patient expression data. In conclusion, the MAPK inhibitor MEK162 was identified as radiosensitizer in GBM spheroids in vitro and in orthotopic GBM xenografts in vivo. The data are supportive for implementation of this promising targeted agent in an early phase clinical study in GBM patients.

In **chapter 5**, we created a cytostatic and cytotoxic accumulation of autophagosomes by inhibiting both mTOR and the fusion between autophagosomes and lysosomes. U87 and U251 human glioma cells were exposed to the allosteric mTOR inhibitor RAD001 or the active site mTOR inhibitor MLN0128 in combination with the late stage autophagy inhibitor chloroquine. In addition, the radiosensitizing potential of this combination treatment was evaluated. Our preliminary data demonstrate that a combined inhibition of mTOR and autophagy has a more than additive inhibitory effect on GBM cell proliferation. However, the combination neither induced glioma cell death, nor sensitized glioma cells to radiation. Based on preliminary mechanistic data obtained after exposure to inhibitors in combination with irradiation, the timing parameters of the experiments may be improved to reach higher efficacies.

In **chapter 6** we tested our hypothesis that tumors carrying the KIAA1549: BRAF fusion are probably particularly sensitive to mTOR and MEK inhibition since it was described that KIAA1549: BRAF induces neuroglial cell growth through an MEK-mediated, mTOR dependent manner (Kaul et al, 2012). To study this, mTOR was inhibited by MLN0128 (ATP-competitive mTORC1/2 inhibitor) and MEK was inhibited by AZD6244 (MEK1/2 inhibitor). The combination reduced the cell viability in a BRAFV600E and BRAF:KIAA1549 background which correlated with down-regulation of MAPK and AKT/ mTOR targets. Alternative RAS/MAPK activation by loss of NF1 cells resulted in moderate sensitivity to AZD6244 and showed increased apoptosis compared to NF1 wild type cells. Additionally, reactivation of MAPK and mTOR activity was seen when treated with MLN0128 and MLN0128+AZD6244. Proliferation of NF1 wild type cells could neither be inhibited with single agent AZD6244 nor in combination with MLN0128, this despite visible down-regulation of MAPK and mTOR targets. Together, combinatorial mTOR + MEK targeting shows synergistic interaction in vitro which might be beneficial for a subset of glioma patients diagnosed with BRAFV600E, BRAF:KIAA1549 alterations or NF1 loss. Therefore, we think that this combination is an example of maximal pathway inhibition that could have clinical implications.

In **chapter 7**, we show that identification of synergistic drug combinations by using an empirical setting (by actual testing all combinations) can be complemented by a novel approach that we call the drug atlas. The drug atlas enables to identify a limited number of molecular mechanisms for cell survival/proliferation and hits these by combinations of targeted

drugs. We validated this by performing a drug-combination screen against 9 glioblastoma cell lines using 30 drug combinations. Benchmarking of our prediction model showed that our model meets the predictive power of landmark datasets but only needs a fraction of the predictive features to train these models. From identified dual-therapies we were able to predict and validate a triple-drug synergy. This approach could lead to identification of unforeseen personalized multi-drug combination approaches and preferably drug-combinations that cross the blood brain barrier and that have long term cytostatic/cytotoxic effects should be selected for clinical translation.

2 The blood-brain barrier

We performed two in vivo studies wherein the transfer of drugs over the blood brain barrier may have affected the outcome of the experiments. In **chapter 4** we validated the in vivo efficacy of the combination of the MEK-inhibitor MEK162 with fractionated irradiation, but the combination was less effective than we had anticipated based on the in vitro data. The end point of the experiments was tumor volume measured by bioluminescence; the study was not limited by toxicity. MEK162 is known to be actively eliminated out of the brain by P-gp and BCRP efflux pumps located at the blood brain barrier (personal communication Dr. O. van Tellingen, Netherlands Cancer Institute). Since MEK162 may not pass the blood-brain barrier in sufficient amounts, other MEK inhibitors may be better candidates, such as trametinib which can reach 200 nM levels in the brain (preclinical data, Vaidhyanathan et al, 2014). Furthermore, fractionated irradiation could improve the delivery of small molecules by transient disruption of the blood brain barrier (Appelboom et al, 2016).

In **chapter 7** we used a triple-combination of targeted drugs which were selected based on their clinically relevance and on matching molecular targets, good blood brain barrier transfer and low toxicity. Thus, we selected Tagrisso (Osimertinib, AZD9291) which targets EGFR, AZD2014 (Vistusertib) which targets MTOR1/2, and docetaxel, a cytostatic drug which targets microtubules. Tagrisso has been shown to pass the blood brain barrier (Ballard et al, 2016). Although Tagrisso was developed to target EGFR-T790M, it has an EGFR inhibitory effect. AZD2014 passes the blood brain barrier and has shown target engagement in the brain. Although docetaxel poorly penetrates the blood brain barrier, the synergistic combination of drugs will allow target engagement (i.e. disruption of microtubules) by docetaxel at much lower doses than required when given as a single drug,

without causing intolerable systemic toxicity.

3 Drug resistance, inter- and intratumoral molecular heterogeneity

Based on inter-patient differences in treatment outcome to combined MEKi + radiotherapy in **chapter 4**, we have generated RNA signatures and classified patients accordingly in potential good or poor responding groups. Good responders to MEKi + radiotherapy were seen for the group of patients with poor survival outcome, indicating those patients may benefit from the combination treatment. In addition, patients with the mesenchymal GBM subtype, which are characterized with high tumor invasion and drug resistance, could potentially also benefit from the combination approach. Of note, since NF1 mutations are frequently observed in mesenchymal tumors, this patient group might profit from MEKi treatment strategies (**chapter 6**). These predictions based on RNA signatures should be interpreted with caution, since in vitro derived signatures may not be extrapolated to the clinical context. Moreover, these predictions may be prone to overfitting as a result of small sample numbers compared to the number of features, which may cause false-positive as well as false negative outcomes.

Corollary to the MEKi + radiotherapy response described in **chapter 6**, in **chapter 7**, we generated therapy response predictive models for triple drug combination treatments. In this chapter, the predictive model was based on experimental drug sensitivity data rather than on molecular features. As we also found in the previous **chapter 6**, there is a chance that false positive or negative results are obtained as a result of overfitting.

4 Pharmacodynamics/Pharmacokinetics and Toxicity

In **chapter 7** we used a triple-combination and for this study we used drugs that were predicted to give low toxicity (see also Challenge 2). As described above, we evaluated the potential acute and/or sub-acute toxicity of Tagrisso, AZD2014 and Docetaxel, in female Athymic Nude-Foxn1nu mice. The conditions of this drug combination toxicity (safety) screen were based on information obtained from in vivo drug studies reported in the literature. In this study, we found that drug combinations at dose levels inferred from extrapolated clinical doses, gave noticeable toxicity after 10-days of treatment. The present literature on the pharmacokinetics of the three drugs suggests that there only is a limited time window of six hours after drug administration wherein synergy can be expected on tumor growth delay. A major challenge remains to find the optimal drug doses,

sequence and administration timing to yield a maximal tumor effect with minimal toxicity.

OUTLOOK FOR MEKI + RADIOTHERAPY STUDY IN GBM PATIENTS

MEK as a radiosensitizer in the clinic - Given the long-term effect that we have seen of MEK inhibition in combination with radiotherapy in our preclinical models (**chapter 4**), we think that this therapy is the best candidate for clinical application. Currently, at the VU University medical center, a clinical study is in preparation to investigate a MEK-inhibitor in combination with radiotherapy in GBM patients after completion of an ongoing preclinical study to identify MEK inhibitors with a better blood brain barrier penetrating ability (see also de Gooijer et al, 2018). For the clinical study for which written informed consent is required, GBM patients will be pre-treated with the MEK inhibitor for two weeks before surgery (i.e. the time needed to reach a steady state concentration of the drug in the tumor). During surgery, tumor tissue will be obtained for analysis to determine the inhibitory effect of the drug in the tumor, by measuring the phosphorylation of key target proteins (e.g. pErk) and their effectors (i.e. DNA damage response through γ H2AX measurement). Drug levels in the tumor will be measured using HPLC. Next, based on the data from the former study, a clinical Phase I feasibility study will be performed in GBM patients, consisting of hypofractionated radiotherapy (40,05 Gy in 15 fractions of 2.67 Gy), temozolomide and MEK inhibitor. If necessary, addition of a P-glycoprotein and/or BCRP BBB pump inhibitors such as elacridar will be investigated in five additional patients. If this second step shows tolerability of the combination treatment, a next step is to determine the maximum tolerated dose of drug in combination with radiotherapy and concurrent and adjuvant temozolomide. In this phase I-II study, the dose of the MEK inhibitor will be increased in subsequent groups of five patients until the maximum tolerable dose is reached.

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