Inhibition of BRAF mutant tumors: the role of PI3K pathway and combination therapies

Synopsis of PhD thesis

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1. Introduction

RAS/RAF and PI3K/Akt pathways have a key role in the development and maintenance of the majority of cancers. Cross-talk between these pathways and mutations in both pathways contribute to the failure of targeted therapy, especially in monotherapy. Therefore, the research of combinational inhibition and alternative targets is of utmost importance. In my PhD thesis we investigated the inhibition of BRAF mutant tumors, especially the role of PI3K pathway and certain combination therapies. Three studies are presented in my dissertation. First, sensitivity of BRAF and BRAF+PI3K/PTEN mutant cells was compared against horizontal combination inhibition of RAS/RAF and PI3K/Akt pathways. Second, sensitivity of a novel BRAF V600E mutant anaplastic thyroid cancer cell line was tested with monotherapy and combination inhibition of RAS/RAF pathway elements. Finally, effects of a lipophilic and a hydrophilic prenylation inhibitor were compared in melanoma cell lines harboring RAS/RAF/PI3K/PTEN mutations. All these results may contribute to the development of successful targeted therapy for patient with RAS/RAF and PI3K/Akt pathway mutant tumors.

2. Aims

In my work, I investigated the effectiveness of single agent and combination therapies in BRAF mutant cancer cell lines and the role of PI3K/Akt pathway in the mechanism of action with the following aims:

1. Comparison of BRAF and BRAF+PI3K/PTEN mutant cell lines to determine whether double mutant cell lines are more sensitive to the combination therapy against RAS/RAF and PI3K/Akt pathways.

2. Investigation the sensitivity of a novel BRAF mutant anaplastic thyroid cancer cell line against inhibitors of RAF and MEK alone or in combination.

3. Comparing inhibitory effect of two prenylation inhibitors, the hydrophilic zoledronic acid and its lipophilic analogue (BPH1222) in melanoma cell lines with different mutations (BRAF, BRAF+PTEN, NRAS or none of them), to determine whether the better physical-chemical properties of BPH1222 translate to higher antitumor effect.

3. Methods

3.1 Cell lines

Nine melanoma, two colorectal cancer, one lung adenocarcinoma and one anaplastic thyroid cancer cell lines with known RAS/BRAF/PI3K/PTEN mutation status were involved in our investigations.

3.2 Inhibitors

Two prenylation inhibitor bisphosphonates (zoledronic acid (ZA), BPH1222 (BPH)), one pan-RAF (sorafenib) and two BRAFV600E specific inhibitors (vemurafenib, dabrafenib), one MEK1/2 inhibitor (selumetinib) and one PI3K/mTOR dual inhibitor (BEZ235) were tested in our cell lines.

3.3 Viability assays, combinatory index (CI)

Short- and long-term viability assays were performed to analyze the toxicity of the inhibitors on 2D models. Sulforodamine B (SRB) staining for short-term (72h) viability assay, and crystal violet staining for long term (10 days) colony formation assay were applied. Bound dye was dissolved and optical density (OD) was measured. Cell viability via IC50 values and colony forming potential were determined. Combination index (CI) was calculated by CompuSyn software.

3.4 3-dimensional spheroid assay

Eight cell lines were studied in 3D spheroid assays. Spheroids were generated either spontaneously in 4-7 days or with additional spheroid formation supporting compounds (Matrigel, collagen). Images were taken during the treatment period and the volume of the spheroids were

calculated. Additionally, in certain experiments, CCK8 assay was applied at the end of the treatment to determine cell viability.

3.5 Cell migration analysis

The inhibitory effect of bisphosphonates on migration of the melanoma cells was investigated by a custom-designed video microscope. Pictures were taken in the first 24 hours after treatment in every 10 minutes and they were evaluated with a cell-tracking program. Displacement of the cells was measured prior to and after treatment.

3.6 Immunoblot analysis

Baseline activation and/or expression of PTEN, MET, EGFR, Akt, S6 and Erk proteins was studied via immunoblot assay. Additionally, changes in the protein activation and/or expression of Akt, S6, Erk, Rheb, PCNA and cleavage of PARP upon treatment were also determined.

3.7 Cell cycle analysis

After treatment with bisphosphonates, ratio of the cells in the cell cycle phases was examined by the DNA content of the cells using the NucleoCounter image cytometer system. Changes in cell number ratio in the G0/G1, S, G2/M and subG1 phases upon treatment were compared to control treatment.

3.8 *In vivo* experiment

Animal experiments were carried out in accordance with the Guidelines for Animal Experiments and were approved for the Department of Experimental Pharmacology in the National Institute of Oncology. SW1417 and M24met cell lines were injected subcutaneously into NOD-SCID and SCID mice. Treatment was applied intraperitoneally or per os during the 17-23 day-long experiments. Length and width of the tumors were measured by caliper and tumor volume was calculated. Tumor mass was also determined after the mice were sacrificed.

3.9 Statistics

All statistical calculations were carried out via GraphPad Prism 5 program.

4. Results

4.1 Combinational inhibition of MEK and PI3K/mTOR in BRAF and BRAF+PI3K/PTEN mutant cancer cell lines

We determined the sensitivity of the cell lines against the inhibitors via short- (72h) and long-term (10 days) viability assays. Based on our results, the MEK inhibitor selumetinib was more effective in BRAF only mutant cell lines than in BRAF+PI3K/PTEN mutant cells in short- and long-term as well. Interestingly, the short-term effect of BEZ235 was similar in the cells independently from their mutational status, however, in long-term

experiments, double mutant cells were more sensitive than the BRAF only mutant lines. Combination of selumetinib and BEZ235 was tested in 30 different concentrations in long-term experiments. In most of the cases, combination index suggested additive interaction (CI≈1). However, in two double mutant lines (WM239, SW1417) synergistic interaction was confirmed (CI<1). Furthermore, baseline expression and activation of EGFR, MET, PTEN, Akt, Erk and S6 proteins were tested in the five double mutant cell lines (SKMEL28, A2058, WM239, HT29, SW1417). Expression of EGFR and MET was higher in BRAF+PI3K mutant colorectal carcinoma than in melanoma cell lines. In contrast, BRAF+PTEN mutant cells had enhanced p-Akt and p-Erk level compared to only BRAF mutant cells. Interestingly, p-S6 level was similar among the cells, except for HT29 line. In addition, Erk, Akt and S6 protein activation was tested upon treatment with selumetinib, BEZ235 or both of them in A375, WM239, HT29 and SW1417 cell lines. S6 and Erk activation decreased after treatment with BEZ235 and combination, and selumetinib and combination, respectively. Interestingly, p-Akt level decreased only in SW1417 upon treatment with selumetinib+BEZ235. In addition, enhanced c-PARP and reduced PCNA level were found in A375 and WM239 after treatment with selumetinib and selumetinib+BEZ235. Based on our results from spheroid experiments, selumetinib and selumetinib+BEZ235 had significant inhibitory effect on spheroid growth and viability in all cells. Interestingly, combination treatment was only in

case of SW1417 spheroids significantly more effective than single treatment. Finally, SW1417 cell line was injected subcutaneously into NOD-SCID female mice. While both selumetinib and BEZ235 single treatments had significant inhibitory effect on the tumor growth the combination of them was the most effective.

4.2 Investigation of a novel BRAF mutant anaplastic thyroid cancer cell line (PF49)

PF49 cell line was established from a 68-year old male patient who was diagnosed and treated with papillary thyroid cancer. However, 8 months later anaplastic dedifferentiation was observed (loss of TTF1 and thyroglobulin expression; retained CK18 and PAX8 expression). Despite adjuvant chemo-radiotherapy the tumor progressed rapidly with bone, pleural and lung metastases. Malignant cells were detected in the accumulated pleural effusion which was the origin of the newly established PF49 cell line. The patient finally deceased eleven months after the first diagnosis.

The mutation status of the PF49 cell line was determined by next generation sequencing. BRAF V600E and TERT promoter mutations were detected in the cells which are common in older patients with anaplastic thyroid cancer. Of note, no RAS, TP53 or PI3K mutations were detected in the cells, despite the fact, that these mutations are also common in anaplastic thyroid cancers.

Short term (72h) viability assays were performed to determine sensitivity of PF49 cell line against RAF inhibitor sorafenib, BRAF inhibitor dabrafenib and vemurafenib, and MEK inhibitor selumetinib. A BRAF V600E mutant melanoma cell line (A375) was used as a control. We showed that the inhibitors were able to decrease viability of the cells in a concentration dependent manner, but PF49 was less sensitive than A375. The effect of the combination therapy (selumetinib+vemurafenib) on cell viability was tested in different concentrations. Based on the calculated combinatory indexes (CI), strong synergism (CI<1) was observed between the inhibitors in most of the concentrations. Furthermore, PF49 was found to have a strong migratory capacity, therefore, inhibition of migration was also tested. Both vemurafenib and selumetinib were able to decrease motility but the combination therapy was the most effective.

4.3 The antitumor effect of lipophilic bisphosphonate BPH in melanoma cell lines

Short-term (72h) SRB assay and long-term (10 days) clonogenic assay were used to compare the inhibitory effect of zoledronic acid (ZA) and BPH1222 (BPH) on the melanoma cell lines. Most of the cell lines were more sensitive to BPH than ZA both in short- and in long-term experiments. Of note, M24met line was more sensitive to ZA than BPH. Apoptosis induction of the compounds was tested via cell cycle analysis and immunoblot. The highest ratio of cells in the subG1 phase was

observed upon treatment with BPH in M24met, A375 and VM47 lines. In addition, ZA had stronger apoptotic effect in M24met than BPH. Cleaved-PARP was observed after BPH treatment mostly in BRAF and BRAF+PTEN mutant cell lines. Interestingly, *in vitro* ZA was more effective than BPH only in M24met cell line. Next, the effect of treatments on protein activation in RAS/RAF (Erk) and PI3K/Akt (Akt, S6, Rheb) pathways was also investigated. Inhibitors had no effect on Erk phosphorylation, in contrast, p-S6 and p-Akt levels reduced in A375, M24met and VM47 cells especially upon treatment with BPH. Interestingly, reduction of prenylated Rheb level was observed in A375, VM47 and WM35 cells after BPH treatment and we found lower total Rheb protein level in M24met cell line upon treatment with both ZA and BPH. Based on cell migration capacity (50 μm displacement in 18h was the cut-off value) cells were divided into fast and slow groups. BPH was able to inhibit migration of three of four fast cell lines (WM239, A2058, VM47). In contrast, ZA increased migration in three out of four fast cells (WM239, A375, VM47). In slow cells, migration did not change upon treatment. Furthermore, spheroid growth assays were performed with four lines (A375, A2058, M24met, VM47). A375 spheroids dissociated after 6-day-long treatment with BPH due to its strong growth inhibitory effect. In addition, BPH had significant inhibitory effect on spheroid growth in the other three lines. Of note, in the case of M24met cell line ZA showed still higher antitumor effect than BPH. Therefore, M24met xenografts

were established in female SCID mice and intraperitoneal treatment was applied for 23 days. Interestingly, based on tumor volume results, BPH was significantly more effective to inhibit tumor growth than ZA. Also tumor mass data presented a slightly stronger antitumor effect of BPH than ZA.

5. Conclusion

1. The combination of the MEK and PI3K/mTOR inhibitors resulted in a synergistic inhibitory effect on two double mutant (BRAF+PI3K/PTEN) cell lines. The combination therapy decreased p-Akt level and spheroid growth significantly only in the case of the most sensitive SW1417 cell line. In addition, an *in vivo* experiment with SW1417 confirmed that the combination of selumetinib and BEZ235 is more effective than single treatments. However, according to our results, concomitant BRAF and PI3K/PTEN mutation could not be confirmed as predictive factor to the effectiveness of combination therapy.

2. Viability of the BRAF V600E mutant, newly established PF49 anaplastic thyroid cancer cell line was decreased with RAF, BRAF and MEK inhibitors, however with lower efficacy than in A375. Combination of the BRAF and MEK inhibitors had strong synergism in both cell lines. Furthermore, in PF49 cells combination treatment had the strongest migration inhibitory effect compared to single treatments.

3. Comparing the antitumor effect of hydrophilic zoledronic acid (ZA) and lipophilic BPH on viability of melanoma cell lines, BPH was found to be more effective in most of the cases. Additionally, in migration assays, fast cell lines were more sensitive to BPH than ZA. Interestingly, activation of the proteins in the PI3K/Akt pathway (Akt, S6, Rheb) decreased upon BPH treatment in the most sensitive cell lines. Also spheroid growth assays presented stronger growth inhibitory effect of BPH than ZA, except for M24met cell line. However, in *in vivo* experiment, BPH was more effective to inhibit tumor growth of M24met than ZA. Our results confirmed the hypothesis, that the better physicochemical properties and bioavailability of BPH lead to stronger antitumor effect in melanoma cell lines compared to ZA.

6. List of publications

6.1 Publications related to the thesis

Rittler D, Baranyi M, Molnar E, Garay T, Jalsovszky I, Varga IK, Hegedus L, Aigner C, Tovari J, Timar J, Hegedus B. (2019) The Antitumor Effect of Lipophilic Bisphosphonate BPH1222 in Melanoma Models: The Role of the PI3K/Akt Pathway and the Small G Protein Rheb. Int J Mol Sci, 20: 4917.

Hegedűs L, **Rittler D**, Garay T, Stockhammer P, Kovács I, Döme B, Theurer S, Hager T, Herold T, Kalbourtzis S, Bankfalvi A, Schmid KW, Führer D, Aigner C, Hegedűs B. (2020) HDAC inhibition induces PD-L1 expression in a novel anaplastic thyroid cancer cell line. Pathol Oncol Res, DOI: 10.1007/s12253-020-00834-y.

6.2 Publications not related to the thesis

Molnár E, **Rittler D**, Baranyi M, Grusch M, Berger W, Döme B, Tóvári J, Aigner C, Tímár J, Garay T, Hegedűs B. (2018) Pan-RAF and MEK vertical inhibition enhances therapeutic response in non-V600 BRAF mutant cells. BMC Cancer, 18: 542.

Molnár E, Garay T, Donia M, Baranyi M, **Rittler D**, Berger W, Tímár J, Grusch M, Hegedűs B. (2019) Long-Term Vemurafenib Exposure Induced Alterations of Cell Phenotypes in Melanoma: Increased Cell Migration and Its Association with EGFR Expression. Int J Mol Sci, 20: 4484.

Baranyi M, Molnár E, **Rittler D**, Hegedûs B, Tímár J. (2019) [Impact of prenylation inhibition on RAS mutant tumors in preclinical studies]. Magy Onkol, 63: 320-329.

Baranyi M, **Rittler D**, Molnár E, Shirasawa S, Jalsovszky I, Varga IK, Hegedűs L, Németh A, Dank M, Aigner C, Tóvári J, Tímár J, Hegedűs B, Garay T. (2020) Next Generation Lipophilic Bisphosphonate Shows Antitumor Effect in Colorectal Cancer In Vitro and In Vivo. Pathol Oncol Res, 26: 1957–1969.