Studying mTOR signalling and altered cellular metabolism using *in vitro* tumour models

PhD theses

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1. Introduction

The latest examinations in tumour biology emphasise the importance of altered cellular metabolism of cancer cells for the development, progression and therapy sensitivity of tumours. The altered cellular metabolism can provide the bioenergetic background which is necessary for cell proliferation and tumour progression.

An increasing amount of data supports the view that tumour cells differ from normal cells in their metabolic characteristics (different metabolic profiles) as well as in their need for nutrients and their substrate utilisation abilities. High glycolytic activity even in the presence of oxygen (Warburg-effect), intensive glutaminolysis, fatty acid oxidation can be characteristic for cancer cells.

High metabolic adaptability of tumour cells also contributes to their long-term survival, therapy resistance and metastasis formation.

The aforementioned processes are influenced by the multi-faceted heterogeneity of tumour tissue, the variety of different cells, even genetic variety (various tumour cell clones), the co-operation and the symbiosis of different cells as well as their adaptation to the microenvironment. The metabolic differences and the adaptability of tumour cells and the metabolic tissue heterogeneity promote the formation of metabolic symbiosis within the tumour tissue.

The regulation of tumour cell metabolism is influenced by genetic and epigenetic factors. These factors also cause a change in the activity of signalling pathways, which plays a crucial role in tumour growth. Besides maintaining the activity of the growth signals, they also participate in the re-programming of cellular metabolism and their regulation.

The RTK-PI3K-mTOR axis participates in the regulation of numerous cell functions. One of its key elements is mTOR (mammalian target of rapamycin) kinase, which forms two complexes (C1 and C2) in cells. As important regulatory elements of the signalling network, the latter are sensors of the energy- and nutrient-supply of the cells. Relating to this, they regulate survival and proliferation, the biosynthetic or even degrading cellular processes of the elements which are necessary for the construction of new cells.

The oncometabolite production of tumour cells is a specific characteristic of metabolic alteration. Oncometabolites are defined as small molecules produced in high amount during metabolic processes, which support the malignant phenotype at various points.
The well-known oncometabolites are succinate, fumarate or D-2-hydroxyglutarate (2-HG); they accumulate as a consequence of certain metabolic enzyme (succinate dehydrogenase, fumarate hydratase, isocitrate dehydrogenase - IDH) mutation. In the most recent summaries, lactate produced in large quantities by (Warburg phenotype) tumour cells as a result of glycolytic activity is also regarded as an oncometabolite.
We have presented methods which are suitable for characterising the metabolic processes and profiles of tumour cells. I have studied the increased but individually varying glycolytic activity of tumour cell lines, the effects of 2-HG oncometabolite production (accumulating as a consequence of IDH), the differences in the substrate utilisating capacity of tumour cells and their correlation to increased mTOR activity.

2. Aims

An important characteristic of malignant tumours is their ability to alter their metabolism in order to survive and proliferate. In my work, the metabolic alterations (metabolic profiles) and substrate utilisation of different tumour cells were studied and in addition, the altered mTOR activity. The main objectives were the following:

A) Setting up analytical and other methods which are suitable for metabolic characterisation and bioenergetic profile determination using *in vitro* models (cell lines) with varying substrate utilisation characteristics.

B) Examining 2-HG oncometabolite production of isocitrate-dehydrogenase (IDH1)-mutant HT-1080 fibrosarcoma cells

- Elaboration and development of liquid chromatography - mass spectrometric methods
- Examination of the correlation between IDH mutation and mTOR activity change

C) Examining the effects of mTOR inhibitor treatments on metabolism and oncometabolite production in *in vitro* and *in vivo* experiments

D) Performing comparative and multi-aspect examinations on an IDH1 wild-type and -mutant enzyme overexpressing glioma cell line pair and other glioma cell lines

- Studying tumour growth and metabolic characteristics (metabolic profile and substrate utilisation, source of oncometabolite production) *in vitro* and *in situ* in human tissue samples.

3. Methods

Cell cultures – *in vitro* studies

The following human cancer cell lines were used in our studies: HT-1080, fibrosarcoma; Oscort, osteosarcoma; MDA-MB231, BT-474, ZR-75.1 breast cancers; HepG2, hepatocellular carcinoma; U937 acute myeloid leukaemia; A-2058, melanoma; U251 MG, U87 MG, U373 MG glioma cell lines. To examine the IDH1 mutation, IDH1 R132H overexpressing (genetically engineered) U251 MG glioma cell line was used.
mTOR inhibitors and drugs, which influence cellular bioenergetic pathways such as glutaminolysis, IDH-mutant enzyme, were applied. mTOR inhibitors: rapamycin (mTORC1 inhibitor); PP242 (mTORC1 and C2 inhibitor); NVP-BEZ235 mTOR complexes and PI3K inhibitor). To inhibit glutaminolysis, BPTES and Zaprinast were applied. Besides, several chemotherapeutics (doxorubicin, temozolomide) were used in proliferation studies *in vitro*. In addition, we studied the effects of 2-HG and/or GABA (gamma-aminobutyric acid) metabolites on cellular proliferation.
Alamar blue assay was used for proliferation studies and flow cytometer was applied for apoptosis measurements.

Xenograft studies with *in vivo* HT-1080 model

In vivo xenograft model was established by injecting subcutaneously HT-1080 cells in the sole region of SCID mice and the further engrafted palpable tumours were treated with Rapamune (per os 3 mg/kg 3 times/week). The tumour sizes were followed during the treatments and the tumour weights were registered at the end of 3-week treatment period.

Tissue microarray studies in clinical samples *in situ*

47 glioma patients (type: astrocytoma $n = 14$, oligodendroglioma $n = 14$, glioblastoma $n = 19$; sex: female $n = 23$, male = 24; WHO grade: II $n = 9$, III $n = 19$, IV $n = 19$; IDH1 R132H

mutation: positive $n = 32$, negative $n = 15$) were included in a tissue microarray (TMA) study. Three normal brain and renal tissues were included as controls.

Protein expression and mutation studies

Western blotting was used for quantitative analysis of p-mTOR, p-S6, Raptor, Rictor, lactate dehydrogenase A (LDH-A), glutaminase, glucose transporter 1 (GLUT1), β-F1-ATPase, GAPDH, pan-Akt, p-(ser473)-Akt 1, p-fructokinase-P, hexokinase-2, glutamine transporter (ASCT2), succinic semialdehyde dehydrogenase (SSADH) and GABA transporter 1 proteins with Vectastain Elite Universal ABC Kit and enhanced chemiluminescence.
p-S6, p-mTOR, LDH-A and glutaminase protein expressions were studied in HT-1080 xenografts. SSADH expression was analysed in human glioma TMA slides using immunohistochemistry (IHC) stainings.
The IDH1 mutation was also confirmed by anti-IDH1 R132H antibody.

Metabolic rewiring studies

Substrate oxidation by ^{14}C-labelled nutrients: Cells were labelled with 1-^{14}C-glucose or 1-^{14}C-acetate for 1 h. The $^{14}CO_2$ released by cells was trapped on solid alkaline adsorbent, attached to the air-flow chamber. Geiger–Müller counter was used to measure radioactivity.

Quantitative measurements of metabolites: Intracellular metabolites (lactate, citrate, malate, succinate, glutamate, 2-HG) were extracted from the cells. To determine the quantity of different metabolites liquid chromatography-mass spectrometry methods were used which were established with testing different derivations and graphite columns – modified C18 column.

Substrate utilisation studies after using stable isotope labelled substrate addition: Cells were incubated in DMEM D5030 medium with U-^{13}C-glucose or U-^{13}C-glutamine or 2-^{13}C-acetate addition for 1 h in labelling experiments before the extraction.

Seahorse measurements: Real-time measurements of oxygen consumption rate (OCR), reflecting mitochondrial oxidation and extracellular acidification rate (ECAR), indicated as a parameter of glycolytic activity, were performed on a Seahorse XF96 Analyzer using glioma cell lines. Substrate utilisation was measured by using different substrates in parallel wells. In addition, the oxidation of the following substrates was analysed: glucose, glutamine, citrate, GABA, lactate, malate, acetate and glutamate.

Statistical analysis

The data are presented as mean with standard deviation and calculated from three independent experiments with minimum three or more parallels depending on the method used. Results were statistically evaluated through one-way ANOVA. Mann-Whitney U test and Kruskal-Wallis test were used to compare SSADH expressions and clinicopathologic parameters in human gliomas. $p < 0.05$ was considered statistically significant.

4. Results

Bioenergetic pathway studies in cell lines *in vitro*

Comparing ^{14}C-glucose and ^{14}C-acetate oxidation in human cancer cell lines

The majority of the assessed cell lines oxidised glucose to a more significant degree than acetate. The studied tumour cell lines showed minor individual variances in their glucose oxidation ability, while we experienced significant variances in acetate. Based on this, we can assume that the differences showing in the glucose/acetate ratio and characterising the cell lines may be attributed to varying acetate utilisation and the related variance in mitochondrial functions.
The most significant variation in the oxidisation ratio of the two substrates was measured in HT-1080 fibrosarcoma and ZR-75.1 breast cancer cells. Therefore, we conducted a detailed comparative bioenergetic analysis of these two cell lines.

Expression differences of enzymes and transporters related to bioenergetics pathways

In the course of further studies of glucose/acetate oxidation ratios, we examined the expression of the enzymes participating in the energy-metabolism (GAPDH – glycolytic enzyme; β-F1-ATPase – mitochondrial respiration chain protein) on the HT-1080 and ZR-75.1 cell lines. The ratio of the two proteins may indicate the ratio of glucose and oxidative phosphorylation. ZR-75.1 cells showed a higher β-F1-ATPase expression than HT-1080 cells.

Glucose utilisation studies – after using ^{13}C-glucose labelling and LC-MS measurements

In HT-1080 fibrosarcoma cells, the atoms originating from U^{13}C-glucose appeared primarily in lactate and glucose-6-phosphate and less in TCA intermediaries, as e.g. in ZR-75.1 breast cancer cells. We observed the incorporation of 2-3 ^{13}C atoms from glucose to malate in HT-1080 cells, while in ZR-75.1 cells we also observed the incorporation of 4 ^{13}C. Based on these, HT-1080 cells utilise glucose less in the TCA cycle than ZR-75.1 cells.
To demonstrate this difference, the characterisation of the ratio of glycolysis and oxidative phosphorylation the intracellular ^{13}C-lactate/^{13}C-malate ratio of the cells was given. In HT-1080 cells this ratio was 13.74, while in ZR-75.1 it was 1.17, which indicates the significant glycolytic shift of HT-1080 cells.

Testing the function of TCA cycle by ^{13}C-acetate labelling

Entering the TCA cycle, the second carbon atom of acetyl-CoA can incorporate into the citrate in various cycles. Thus, using 2-^{13}C-acetate labelling, it is possible to monitor the fate of the ^{13}C labelled atoms (derived from acetate), which can incorporate into the TCA cycle metabolites.
Using this method, the quantity of labelled incorporations, accumulations into citrate or further TCA metabolites and the increase

of the labelled metabolite ratio can indicate the intensity of TCA cycle processes within the given time frame.
After the cells were labelled with 2-^{13}C acetate, the amount of unlabelled (containing only ^{12}C atoms) and ^{13}C-labelled (containing ^{12}C and ^{13}C atoms) intracellular citrate was determined.
In HT-1080 fibrosarcoma cells, 1-2 incorporations were observed, while in ZR-75.1 breast cancer cells we also detected 6 ^{13}C labelled citrate. These results confirm the intensive mitochondrial function of ZR-75.1 cells, as well as lacking or damaged mitochondrial function in HT-1080 cells.

Effect of glucose and acetate on adenylate energy charge

In our experiments, we examined the adenylate energy charge (AEC) in the presence and absence of glucose and acetate in HT-1080 and ZR-75.1 cell lines. For these tests the cells were cultured in DMEM D5030 medium (glucose-, glutamine- and serum-free condition). Comparing the results of the cells held in a nutrient-rich medium (AEC=0.75-0.85), a decreased value was measured in D5030 DMEM medium, which was higher in HT-1080 cells (0.38) than in ZR-75.1 cells (0.54). Under starving condition, it was the added glucose in HT-1080 cells or acetate in case of ZR-75.1 cells that increased the measured AEC value.

Putative regulators of the examined bioenergetic pathways

In our further studies, we focused on mTOR as it is an important central signalling integrator in the regulation of protein synthesis and bioenergetic mechanism. In this respect, it is noteworthy that different metabolic profiles of the two investigated cells could be associated to distinct expression patterns of mTOR complexes. HT-1080 cells expressed preferentially mTORC1 activity (represented by high p-mTOR and p-S6 with relative no/low Rictor expression). At the same time ZR-75.1 cells showed high p-mTOR level with lower p-S6 and higher Rictor level as signs of potential high mTORC2 expression and activity. Related to mTOR complexes the amount of Akt protein detected by pan–Akt antibody was similar in both cell lines, but higher

level of phosphorylated Ser473 form correlates to higher mTORC2 complex activity and functioning TCA cycle in ZR-75.1 cells.

2-hydroxyglutarate oncometabolite production and mTOR activity

2-HG production of HT-1080 fibrosarcoma cells

HT-1080 fibrosarcoma cells can be characterised by increased glycolysis, impaired TCA cycle and high mTORC1 activity. 2-HG production – high level of this oncometabolite was detected by LC-MS – and the decreased number and irregular arrangement of the cristae in mitochondria (detected by electron microscopy) represent other remarkable properties of HT-1080 cells. To elucidate the involvement of *IDH1* in the enhanced production of 2-HG the exons of *IDH1* gene were sequenced. Heterozygous *IDH1* gene mutation (R132C) was confirmed by Sanger sequencing in the studied HT-1080 cells. High level of 2-HG (~18 nmol/10^6 cells) regarded as oncometabolite was a characteristic feature of HT-1080. Glutamine was proved to be a remarkable source (~15% of 2-HG pool) of 2-HG using ^{13}C labelled substrates.

Rapamycin reduced 2-HG and lactate production in HT-1080 fibrosarcoma cells

Based on time-dependent proliferation assay and cell counting, rapamycin inhibited the proliferation of HT-1080 cells.
Significantly decreased intracellular levels of lactate and 2-HG were detected using LC-MS measurements after *in vitro* rapamycin treatment. The metabolic effect was verified by using one-hour ^{13}C-substrate labelling after rapamycin treatment then measuring-evaluating unlabelled and labelled metabolite levels by LC-MS. rapamycin treatment reduced the level of total lactate (both ^{12}C- and ^{13}C-lactate) by more than 50% and blocked the incorporation of ^{13}C from U-^{13}C-glucose into lactate using one-hour labelling period at the end of the treatments. 2-HG production was also reduced effectively after rapamycin treatment, U-^{13}C-glucose and U-^{13}C-

glutamine labelling studies confirmed that rapamycin inhibited 2-HG production. The total level of 2-HG was reduced by more than 75% and the amount of ^{13}C-labelled 2-HG was also reduced significantly after using either glucose or glutamine labelling in one-hour time period in the treated cells compared to controls. It was also concluded that U-^{13}C-glutamin did not label lactate in our experimental conditions.

The regulatory role of mTORC1 in lactate and 2-HG production in vitro and in vivo

Decrease of LDHA and glutaminase expressions were confirmed after rapamycin treatment in HT-1080 cells. To exclude non-target effect of rapamycin, as a chemical compound, we used an other mTOR inhibitor (PP242) and compared their effects on 2-HG and lactate production; and on LDH-A, glutaminase protein expression and on proliferation, as well. Beside its anti-proliferative effect, PP242 also decreased the expressions of LDH-A and glutaminase. Additionally, it reduced both the lactate and the 2-HG levels, respectively.
The *in vivo* inhibitory effect of mTORC1 inhibitor on tumour growth was also confirmed in HT-1080 xenografts. The inhibitory effect of the applied treatment on mTORC1 activity, on the oncometabolite levels – lactate and 2-HG – and on the expression of glutaminase and LDH-A were confirmed by immunostainings and LC-MS.

Bioenergetic study of human glioma cell lines and tissues – comparing IDH1 wild-type and IDH1-mutant tumour cell lines

Characteristics of cellular respiration and glycolytic activity in glioma cells

Basal respiration and extracellular acidification rates were studied using different glioma cell lines (U251 MG, U87 MG, U373 MG). Based on the results, U373 MG cells have the highest basal respiration. The cell lines have individual respiration, extracellular acidification and substrate oxidation capacity.

Respiration and extracellular acidification (glycolytic capacity) in IDH1 wild-type and IDH1-mutant glioma cell lines

U251 wild-type (wt) and its genetically engineered mutant IDH1 overexpressing counterpart - U251 (IDH1m) cells were compared in Seahorse oxygen consumption studies. Besides, we studied the short-term effects of 2-HG incubation in U251 wt cells to mimic the metabolic effect of mutation derived 2-HG exposition. Comparing the basal respiration of wt, 2-HG-treated wt and IDH1m U251 cells, significantly elevated OCR and decreased acidification were observed in case of IDH1m and 2-HG-treated wt cells. The altered characteristics related to IDH1 mutation and 2-HG treatment were supported by Western blot analyses of certain metabolic enzyme expressions in these models.

Source of 2-HG in IDH1-mutant U251 MG cells

To identify which substrates are the prominent sources for 2-HG production in IDH1-mutant U251 MG cells, the cells were fed with ^{13}C-labelled energy substrates (U-^{13}C-glucose, U-^{13}C-glutamine, 2-^{13}C-acetate) in DMEM D5030 medium. Based on our 1-h-labelling results, both glutamine and glucose could be sources of 2-HG, but acetate could not. Performing 24-h-labelling experiments, glutamine was proved to be the main source of 2-HG – 88% of the total intracellular 2-HG pool containing glutamine-derived ^{13}C-labelling, as

compared to ^{13}C-incorporation percentages of 15.95 and 15.7% from glucose and acetate, respectively. Of note, ASCT2 (glutamine transporter) and glutaminase expressions were higher in IDH1m U251 cells than in their wild-type counterpart.

IDH1 mutation and 2-HG related different substrate oxidation in U251 MG models

Decreased oxidation of glutamine, glutamate and malate was measured in IDH1m cells. 2-HG-treated U251 MG wt cells mimicked IDH1m cells regarding to oxidation capacity. Intriguingly, significant GABA oxidation was detected in U251 MG wt cells (OCR was elevated with 20%). However, IDH1m cells did not oxidise GABA and this could be mimicked in 2-HG treated U251 MG wt cells. The expressions of GABA oxidation related proteins were also studied. U251 MG cells express SSADH and GAT1 proteins as shown by Western blot.

Short- and long-term GABA treatment and their pro- (anti-) proliferative effect on U251 MG model cell lines

The pro- or anti-proliferative effects of short- and long-term GABA, 2-HG and GABA+ 2-HG combination treatments were also tested in U251 MG wt and IDH1m cells. Long-term GABA treatment (for more 3.5 weeks) significantly enhanced the proliferation of U251 MG wt cells, whereas significantly lower increase in proliferation was found in IDH1m cells. Short-term (72 h) GABA, 2-HG treatments and their combination showed similar tendency in wild-type cells, however, no significant effect could be observed.

SSADH expression in human glioma tissues

High SSADH expression was found in 97% of human glioma biopsies, it was characteristic for all histological subtype. This SSADH expression showed no associations with clinicopathological parameters such as age, gender, tumour type, grade or IDH mutation status. The SSADH expression in peritumoural cerebral tissues

showed differences, high expression was observed in the cortical region, however, white matter astrocytes showed low to moderate expression.

5. Conclusions

Characterisation of cellular energy metabolism, mTOR activity and its regulative role *in vitro*:

1) Liquid chromatography-mass spectrometry methods are suitable for determining the intracellular metabolite concentrations; using ^{13}C-labelled energy substrates (such as U^{13}C-glucose or 2-^{13}C-acetate), the dominant bioenergetic pathways of cancer cell lines can be determined.

    a) One of our model cell lines, ZR-75.1 breast cancer cell line could use acetate as an energy substrate; and it showed active TCA cycle

    b) whereas intensive glycolysis with impaired mitochondrial functions and structure were characteristic for HT-1080 fibrosarcoma cells

2) The main bioenergetic sources of 2-HG oncometabolite is glutamine in IDH1-mutant HT-1080 and glioma cells. However, in glioma cells, acetate can be an alternative source of 2-HG production supporting tumourigenesis based on the results of long term stable substrate labelling.

3) mTOR activity is associated with metabolic activity and oncometabolite production.

    a) The detected metabolic features of HT-1080 cells are related to mTORC1 dominancy, IDH1-mutant genotype and 2-HG oncometabolite production

    b) ZR-75.1 cells have balanced bioenergetics (can use glycolysis and mitochondrial oxidation well) and high mTORC2 activity

c) In HT-1080 cells the mTORC1 inhibitor rapamycin has anti-proliferative effect and additionally, it can diminish lactate and 2-HG oncometabolite production both *in vitro* and *in vivo*

4) 2-HG and lactate production are mTORC1 activity dependent processes and are in good correlation to glutaminase and LDHA expression and activity, respectively.

Bioenergetic characterisation and energy substrate oxidation of human gliomas; IDH1 mutation related metabolic feature studies:

1) The examined glioma cells showed individual substrate oxidation and extracellular acidification characteristics

   a) The studied glioma cell lines (which are not carrying IDH mutation) have different basal respiration and different glycolytic functions
   b) IDH1-mutant overexpressing cells have elevated basal respiration and lower extracellular acidification rates comparing to its IDH1 wild-type counterpart
   c) SSADH expressing wild-type glioma cell line is able to use GABA as a bioenergetic substrate, while its IDH1 mutation carrying cell line pair is not
   d) 2-HG and 2-HG production as a consequence of IDH1 mutation are associated with inhibited GABA oxidation and inhibition of GABA treatment related pro-proliferative effects

2) More than 90% of studied human glioma cases exhibited high SSADH expression independently from IDH mutational status, gender and age. These data indicate further studies about alternative regulatory role of the detected SSADH expression and potential GABA utilisation in cellular metabolism.

6. Publications

Publications in context of the thesis:

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2.) Jeney A*, **Hujber Z***, Szoboszlai N, Fullár Sz, Oláh J, Pap É, Márk Á, Kriston C, Kralovánszky J, Kovalszky I, Vékey K, Sebestyén A. (2016) Characterisation of bioenergetic pathways and related regulators by multiple assays in human tumour cells. Cancer Cell Int, 16:4. (*contributed equally) **IF: 2.740**

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5.) **Hujber Z**, Horváth G, Petővári G, Krencz I, Dankó T, Mészáros K, Rajnai H, Szoboszlai N, Leenders WPJ, Jeney A, Tretter L, Sebestyén A. (2018) GABA, glutamine, glutamate oxidation and succinic semialdehyde dehydrogenase expression in human gliomas. J Exp Clin Cancer Res, 37(1):271. **IF: 6.217**

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2.) Krencz I, Sebestyén A, Pápay J, Jeney A, **Hujber Z**, Burger CD, Keller CA, Khoor A. (2018) In Situ Analysis of mTORC1/2 and Cellular Metabolism-Related Proteins in Human Lymphangioleiomyomatosis. Hum Pathol, 79:199-207. **IF: 3.014**

3.) Sticz TB, Molnár A, Dankó T, **Hujber Z**, Petővári G, Nagy N, Végső G, Kopper L, Sebestyén A. (2018) The effects of different mTOR inhibitors in EGFR inhibitor resistant colon carcinoma cells. Pathol Oncol Res, doi: 10.1007/s12253-018-0434-4. [Epub ahead of print]. **IF: 1.935**