The influential factors and regulatory processes of mTOR activity in Hodgkin lymphoma models and tissue samples

PhD Theses

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Introduction

The signalling network plays an important role in cellular regulating mechanisms which are connected at several points. The activity of mTOR (mammalian target of rapamycin) kinase has a central role in monitoring the signalling of the growth and stress factors, nutrition and energy supplement respectively. The recent data about mutations and altered activity related to PI3K/AKT/mTOR signalling pathway highlight the role of its dysregulated function in tumorigenesis. In spite of the development of mTOR inhibitors, their application in clinical trials and their introduction started in therapeutic treatments, there are not enough relevant data about the factors and alterations of signalling which can contribute to mTOR hyperactivation in certain tumour types.

In my thesis the factors which influence the activity of mTOR signalling – as the role of Notch signal activity – and its regulatory function in metabolism and tumorigenesis were studied by mTOR inhibitors in human Hodgkin lymphomas (HL) *in vitro* and *in vivo*.

mTOR,(a serine threonine kinase) was named after its inhibitor rapamycin which was isolated from *Streptomyces hygroscopicus* as an antifungal agent in the 1970s on Easter Island (Rapa Nui), it was later found to use as an immunosuppressive agent.

The mTOR kinase can be found in two multiprotein complexes with several same components but its unique elements provide the differences in structure, function and inhibitor sensitivity. Raptor protein as an mTORC1 element and Rictor as an mTORC2 element are important scaffold proteins of mTOR complexes.

mTOR kinase participates in the regulation of several intracellular and extracellular processes, many of its regulatory disorders are well-known in numerous tumours. Both mTOR complexes belong to PI3K/AKT pathway. the activation of several receptors in the phosphorylation cascade, PI3K phosphorylates the inactive phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) that is negatively regulated by PTEN, a well-known tumour suppressor protein. The sequential phosphorylation is followed by the activations of phosphoinositide-

dependent protein kinase and AKT (a serine threonine kinase) activation that negatively regulates the inhibitory effect of tuberous sclerosis complex 1/2 (TSC 1/2). mTOR activity can be regulated by adenosine-monophosphate-activated protein kinase (AMPK) related to hypoxia and low energy supply, in parallel other hyperactivated signals – independently of the PI3K pathway – can control it as well.

Ribosomal S6 kinase (S6K) and 4E binding protein are important targets of mTORC1. These proteins can indicate oncogenic transcription via S6 phosphorylation and 4E regulation which control the 5' CAP -dependent translation such as c-MYC and cyclin D1 or VEGF. Moreover, some factors of metabolic processes and HIF1α related to hypoxia changes are regulated by mTORC1 as well. These processes can also influence the metabolic roles of C1 such as in nucleotide, lipid and protein synthesis, glucose utilization and autophagy. Increasing data are known about the regulatory roles of mTORC2 complex. Its hyperactivation induces AKT dependent processes (cellular survival, anti-apoptotic effects) and cytoskeletal remodelling to promote tumour progression and resistance.

The PI3K/AKT/mTOR axis can be connected to different pathways depending on cell type and in addition several factors can influence its activity, such as mutations of upstream hub proteins or other regulatory disturbances. Activating mutation of several oncogenes are well-known in this signal e.g.: PI3K catalytic subunit, IGFR, AKT; furthermore loss of function mutation of suppressor genes e.g.: PTEN, TSC 1/2 can contribute to elevated mTOR activity.

The physiological and pathophysiological importance of mTOR is known in other diseases (obesity, diabetes, cardiovascular or neurodegenerative diseases and aging). Neurodegenerative disorders are resulted by high mTOR activity related to the disturbance of translation and inhibition of autophagy causing protein accumulation. The mTORC1 plays important role in glucose homeostasis, its dysfunctional activity associates with II type diabetes and obesity. The inhibition of mTOR and reduced nutrient intake may lead to life-span increase and the deceleration of aging processes.

Based on the important role of mTOR activity changes in different diseases, beside the immunosuppressive effect of rapamycin, the new mTOR inhibitors have been developed. The classical mTOR inhibitors can be divided into two groups: rapamycin and derivates of rapamycin (rapalogs). Rapamycin was the first isolated allosteric inhibitor of mTOR, which inhibits the assembly and activity of mTORC1 complex. Due to the structural differences of the complexes, direct inhibition of mTORC2 cannot be achieved. However, the long-term and higher dose of rapamycin treatment can decrease the mTORC2 activity as well. Rapamycin was approved in 1999 in renal transplantations by FDA, and in 2002 t its anti-tumoural effect was described and approved for the treating of several malignancies. Rapalogs can be applied in mantle cell lymphomas (MCL) and in several tumour types with poor prognosis, since they have similar structure as rapamycin with better solubility and stability. The three clinically applied rapalogs are temsirolimus, everolimus and ridaforolimus. Everolimus is effective in non-Hodgkin and Hodgkin lymphomas (NHL, HL) with poor prognosis thus there is possibility for mTORC1 inhibitor administration by personal clinical judgement. Furthermore, it can be used in second- and third-line treatments in endometrial cancer, acute myeloid leukaemias (AML), glioblastomas and sarcomas.

The next generation of mTOR inhibitors due to their ATP competitive effect can suppress both the mTORC1 and mTORC2 complex activities or in parallel they can inhibit other proteins in the mTOR signal such as AKT or PI3K (dual inhibitors). The first mTORC1/2 inhibitor was PP242, its derivatives have been tested in trials of solid and haematological tumours. The dual inhibitors can suppress the negative feedback loop from p70S6K (e.g.: NVP-BEZ 235 – the most effective inhibitor of PI3K/mTOR complexes). The third generation mTOR inhibitor (e.g.: RapaLinks) tests have been started recently.

The PI3K/AKT/mTOR activity is associated with B- and T-cell receptors and many growth factors and cytokines also during the activation and differentiation of haematopoietic cells. Aberrant activity can cause myeloid and lymphoid development disorders. These disorders can be potential therapeutic targets in haematological

malignancies. Increased activity of PI3K/AKT pathway is characteristic for AML (>60%), ALL cells and other lymphomas (e.g.: MCL, Burkitt, Hodgkin and diffuse large B-cell lymphomas). We detected high mTOR activity in more than 90% of HL cases and found prognostic correlation between the activity status of the two mTOR complexes in DLBCL.

In my work three HL cell lines were used as models. The treatment of HL patients is effective with good therapeutic response, more than 80% of patients have at least five-year survival and in significant number of the cases complete remission is expected. However, the new targeted therapy treatments are important to be introduced in the therapy of patients with poor prognosis.

The mTOR activity and the two complexes connect to other cellular monitoring signalling pathways. The interaction of mTOR and Notch signals and the effects of their inhibitors were studied in my PhD work thus the introduction of Notch signal and its tumour biological aspects are summarised here below.

Beyond the regulation of differentiation and the function of lymphoid cells, the Notch signal has also an important role in the development of a whole organism. This signal is prominent in cellular communication but its most characterised roles are the developmental and functional regulations of immune cells. NOTCH1 expression is necessary for T-cell development, while the absence of its activation can lead to B-cells formation at the same time the constitutively activated Notch signal can inhibit the B-cell development.

Activation of the Notch receptors (NOTCH1-4) can be triggered by their ligands (Jagged1, 2 or Delta like 1, 3 and 4) which can induce sequential cleavages by ADAM metalloprotease and gamma-secretase. The intracellular domain of Notch receptor (NICD) translocates to the nucleus and induces the expressions of target genes such as c-MYC and HES1 by binding to the CSL (CBF1/Su(H)/Lag-1) transcription factor and other cofactors. The cellular effect of Notch signal is influenced by the attachment strength of Notch receptor and its ligand, the signal activity and the microenvironmental characteristics. The role of deregulated Notch signalling was described

at first in the emergence of haematological malignancies (T-ALL). High NOTCH1 activity was detected in Hodgkin and Reed-Sternberg cells which associated with cellular proliferation, survival and loss of B-cell identity as well.

The Notch receptor is a potential target in cancer treatment Gamma-secretase inhibitors (GSI) are the most frequently used small molecules for Notch inhibition such as DAPT (N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-Butyl ester); but several new derivates are also available. Promising results are shown that SAHM1 (stapled α-helical peptides derived from MAML1), is appeared to be a highly selective inhibitor of NOTCH1 transcriptional activity in preclinical trials. Notch and mTOR signalling can crosstalk via certain points e.g., changes in the activity and amount of AKT, PTEN, FBXW7 a ubiquitin ligase, and c-MYC.

Energy and new metabolites are necessary for cell division and growing; in parallel the catabolic processes are needed to be inhibited (e.g.: autophagy). According to the recent data, mTORC1 and mTORC2 play central role to maintain the balance in anabolic and catabolic processes. Lactate is produced from pyruvate under hypoxic condition by glycolysis. mTORC1 increases the translation of hypoxia-inducible factor 1-alpha (HIF1α) or c-MYC and can promote glycolysis and lactate production. Oxidative phosphorylation can also be suppressed and in addition the cells can utilise other substrates such as glutamine beside glucose by mTORC1 mediated glutaminolysis.

Metabolic activity changes like the immune cell activation can be observed in lymphoid tumour cells as well – for example the glycolytic reprograming of metabolism. Glucose is the main energy source of the activated lymphocytes that is converted to lactate under oxygen supply, thus the cells are characterised with aerobe glycolysis (Warburg effect) like solid tumours. The active lymphoid cells can utilise glutamine and fatty acid as alternative carbon and energy sources similar to tumour cells. The growing evidences suggest that these characteristics are well controlled processes. However, the metabolic processes which are characteristic for B-cell populations are less understood, there are many data about the mTOR activity changes in B-cell development and metabolic alterations of lymphoma and

leukaemia cells. Based on *in vitro* study, several lymphoma cells are characterised with intensive glycolytic activity such as HL. However, the activity of oxidative phosphorylation is intensive in HL cells *in vitro*, more than half of HL patients shown increased activity of glucose transporter 1 (Glut1). In addition elevated expression of lactate dehydrogenase enzyme was detected which suggests that oxidative phosphorylation could be significant in HL as well, according to *in vitro* studies.

Aims

1. Based on our previous results and literature data, the Hodgkin lymphoma cells are characterised with high mTOR activity in tissue environment; however, its molecular background has not cleared.

   Dysregulation of Notch signalling pathway in several haematological malignancies are known, therefore our aim was to investigate the co-operation between the activity of mTOR and Notch signals in HL cells.

   a. To characterise the Notch signal elements and activity in human HL cells and tissues.
   b. To determine the activity of mTOR complexes (the rates of both complexes) in HL cell lines *in vitro*.
   c. To investigate the mTOR inhibitor sensitivity and the effects of different inhibitors (tumour growth and protein expression level) *in vitro* and *in vivo*.
2. Beside the genetic alterations and protein expression changes which influence the development and activity of lymphoid cells, the metabolic changes are characteristic for lymphoid cells as well. Nowadays the metabolic regulatory role of mTOR has increasing interests, but little information is known about its metabolic alterations in lymphoma cells such as Hodgkin lymphomas. In my work the characterisation of bioenergetic changes related to the activity of mTOR complexes and the effects of mTOR inhibitors were studied in *in vitro* and *in vivo* HL models.

Methods

***In vitro* experiments**: Three classical HL cell lines (DEV, KMH2, L1236); T-ALL cell lines (Jurkat, MOLT4) were cultured and were treated with mTOR inhibitors (rapamycin, PP242, NVP-BEZ 235); Notch signal inhibitor (DAPT, GSI-XII, SAHM1); Jagged1 ligand.
Detection of cell proliferation – AlamarBlue – and apoptosis – flow cytometry – were measured

***In vivo* experiments HL xenografts** were established by injecting subcutaneously into the back region of SCID mice. Treatments: mTORC1 inhibitor – Rapamune, Torisel; mTORC1/2 inhibitor – PP242; dual inhibitor – NVP BEZ 235; gamma-secretase inhibitor – GSI-XII.

HL patient samples: The formalin fixed paraffin embedded HL tissue samples were provided by archive of 1.st Department of Pathology and Experimental Cancer Research.

DNA sequence analysis: Sanger and pyrosequencing were used for detecting the most frequently mutated exons of *FBXW7* (exons: 5, 9, 10, 11) and *PIK3CA* (exons: 9, 20). NGS was used to analyse the mutational hotspots of 50 oncogenes in HL cell lines by Oncompass Medicine – Molecular Diagnostic.

mRNA expression analysis: RNA was purified by PureLinkTM Micro-to-Midi kit. Notch receptor and ligand expressions were detected by reverse PCR. Real-time PCR of NOTCH1 target genes were performed with TaqMan Assay

Investigation of Protein expressions: p-S6, Rictor Raptor, cleaved-NOTCH1, cleaved-Caspase3, pHH3, Glucose transporter1, glutaminase primary antibodies were used with Novolink secondary antibody kit for **ICC and IHC** stainings.

Western blotting was used for quantitative analysis of pS6, Rictor, Raptor, cleaved-NOTCH1, NOTCH1 proteins with Vectastain Elite Universal ABC Kit and enhanced chemiluminescence.

Duolink staining was applied for *in situ* quantitative detection of protein modulations or complexes. Anti-p-S6 (rabbit), anti-S6 (mouse); anti-Rictor (mouse) and anti-mTOR (rabbit) antibody pairs were used. In case the antibodies positioned enough close to each other dot-like fluorescence sign was detected.

The metabolic concentration measurements: Unlabelled and ^{13}C labelled (with glucose, acetate or glutamine) cells were lysated and for derivatization. Norbert Szoboszlai and Zoltán Hujber helped in metabolic LC-MS measurements. The intracellular metabolic concentrations were given in releation to cell numbers and tissues weights.

Statistical analysis: Mean values and SD were calculated from three independent experiments. Past 3.05 and IBM, SPSS v.22 softwares were used with Student's t test and one-way analysis of variance as it was required. $p \leq 0.05$ was considered statistically significant.

Results

Activity of mTOR and Notch1 signalling pathway in HL cell lines

mRNA expression of NOTCH1 2 receptors JAGGED1, 2 and DLL1 ligands were characteristic for all of the three HL cell lines – KMH2, DEV, L1236. Activated NOTCH1 (cleaved-NOTCH1) was detected in all of the three examined cell lines but the intact full-length NOTCH1 receptor was undetectable in HL cell lines i*n vitro* by Western blot. The full-length receptor was only expressed in positive control cell lines, Jurkat and MOLT4. The activated NOTCH1 fragment was detected in HL patient tissue samples by IHC as well. The ligand independent high Notch1 signal activity was confirmed by 72 h *in vitro* GSI treatments.

The background of constitutively activated NOTCH1

Several genes (oncogenes or tumour suppressor genes) related to NOTCH1 and mTORC1 hyperactivation were tested. Mutations could not be detected (in case of *EGFR, FBXW7, NOTCH1, PIK3CA, TP53, PTEN, VHL*), every HL cell line was wild type.

The NOTCH1 and mTOR activity influencing treatments

The Jagged1 ligand, GSI and rapamycin (mTORC1 inhibitor) treatments could not influence the expression of NOTCH1 mRNA in HL cells. Beside, the cleaved-NOTCH1 expression was not altered as a result of mono- or combination treatment.

The time dependent effect (2, 24 and 72 h) of rapamycin was confirmed in HL cell lines. It significantly reduced p-S6 expression after two-hour treatment in all cell lines, however, this early effect was only stable in KMH2. In parallel, GSI treatment did not have a significant effect on mTOR activity.

Tumour progression effects of mTOR and NOTCH1 inhibitors

KMH2 was the most sensitive regarding to rapamycin treatment that showed inhibited proliferation and apoptosis induction. The monotherapy of GSI seemed to unaffected in all HL cell lines, however, SAHM1 could significantly inhibit the proliferation of these cells and induce apoptosis in L1236 and DEV cells. Surprisingly, the

combination of rapamycin and GSI significantly reduced the proliferation comparing to rapamycin monotreatment in the less sensitive HL cell line as well. GSI treatment reduced the tumour proliferation in L1236 xenografts, however, this effect was not significant. Beside, Torisel treatment decreased tumour progression significantly in the treated mice the GSI-Torisel combination proved to be the most effective treatment in it.

The investigation of mTOR complex activity

We found significant differences in the expressions of mTORC1 and mTORC2 in HL cell lines. The highest expression of p-S6 protein (marker of mTORC1 activity) in parallel the most intensive mTORC1 activity was detected in KMH2 compared to DEV and L1236 which were characterised with lower mTORC1 activity. The amount of Raptor (element of mTORC1) and Rictor (element of mTORC2) proteins were investigated by ICC and Duolink techniques. According to Duolink results, high expression of mTORC2 elements were detected in DEV cell line while KMH2 cells showed high mTORC1 activity. Additionally, both complexes were intensively expressed in L1236

The tumour growth inhibitory effect of mTOR inhibitors in HL cells

Rapamycin (mTORC1 inhibitor) was less effective in cell lines with high mTORC2 expression. *In vitro* results proved the differences of mTOR inhibitor sensitivity, PP242, NVP-BEZ 235 (new generation mTOR inhibitors) could be more effective in proliferation inhibition and apoptosis induction comparing with rapamycin treatment in HL cell lines with high mTORC2 activity *in vitro*. The proliferation was reduced significantly after 72 h NVP-BEZ 235 treatment in all HL cell lines while rapamycin was significant proliferation inhibitor only in KMH2 cells. Significant apoptosis induction was detected after 96 h by using a new generation mTOR inhibitor treatment in L1236 cells.

The inhibitory effects of the applied therapeutic agents on tumour growth were confirmed in *in vivo*. Rapamune and Torisel could significantly inhibit the tumour growth in all HL cell lines; and in line

with the *in vitro* results, the new generation mTOR inhibitors could decrease tumour progression in DEV and L1236.

Metabolism of HL cell lines in correlation with mTOR inhibitory sensitivity

High intracellular lactate concentration was measured in all HL cell lines similar to activated lymphoid cells. Lactate level was found the highest in L1236 cell line. The decreased glycolytic lactate level was characteristic for the KMH2 and DEV cells after a 48 h mTOR inhibitor treatment *in vitro*, while lactate level was increased after rapamycin and NVP-BEZ 235 (dual inhibitor) treatments in L1236 cell line. Similar metabolic alterations were detected in xenograft models *in vivo*.

The intensive glycolytic activity of all cell lines was confirmed after 1 h incubation with ^{13}C isotope labelling - 15-23% of lactate appeared to be labelled. The role of mTOR activity in glycolysis was confirmed. The glucose utilisation was reduced after 48 h rapamycin treatment *in vitro*, in parallel, the expressions of Glut1 and p-S6 were decreased as well. The cellular glutamine consumption could be followed by the integration of labelled glutamine into metabolites. We observed that the regulatory role of glutaminase expression was depend on mTORC1; glutaminase expression was decreased in lymphoma cells by rapamycin treatment.

Discussion

Our results highlight that the PI3/AKT/mTOR pathway is a central regulator and has an important role in tumour biology. My results are considered to extend the knowledge related to mTOR kinase activity help to improve the better understanding of these processes in human Hodgkin lymphomas and could promote the underlying of therapeutic development in the future.

In our previous studies we characterised the mTOR activity in HL patients which was associated to mTORC1 activity in HL tumour cells. We proved that certain HL cell lines (KMH2, DEV, L1236) have significant mTOR activity that can be caused by oncogene or tumour suppressor gene mutations related to mTOR signal. However, these mutations were not characteristic for HL cell lines, all HL cell lines were wild type. The effects of cytokine, chemokine (e.g.: IL-5, CCL5) and receptor-ligand interaction are well-known, for instance CD40-CD40L or NOTCH1 and Jagged1 provide an appropriate microenvironment for cellular survival and proliferation activity. My results of HL cells experiments evidenced the expressions of NOTCH1-3 and Jagged1, 2 which were contributed by Jundt et all. Here, the constitutive activity of cleaved-NOTCH1 independently of ligand activation was detected at first time in HL cell lines and human tissue samples. The background of GSI resistance was occurred mainly by mutations of NOTCH1, PTEN or FBXW7, ubiquitin ligase but these are not presented in HL cell lines.

In line with other preclinical results and our experiments, SAHM1 – the new inhibitor, which can reduce the transcription activity of NOTCH1 – is more effective comparing with GSIs. Significant biological alteration (proliferation inhibitor and apoptosis inducer) was resulted in all HL cells, however the transcription changes of target genes (*c-MYC* és *HES1*) were not significant. The targets of NOTCH1 can regulate the regulator proteins of other signalling pathways. Our results draw attention to tumour cells with high NOTCH1 and mTORC1 activity as new targets in HL therapy. Rapamycin (a specific mTORC1 inhibitor) was a significant tumour growth inhibitor in *in vitro* and *in vivo*, beside GSI resistance and in mono-or combination therapies. The tumour growth inhibitory effect

of combination treatment is related to the mTOR inhibitory effect of rapamycin (neither GSI inhibited mTORC1 activity nor rapamycin altered the NOTCH1 activity) which should overwrite the effect of constitutive NOTCH1 receptor activity. Rapamycin sensitivity of the investigated HL cell lines was different in *in vitro* and the combination with GSI can enhance its effects *in vitro* moreover *in vivo* as well, that derives from the microenvironment modifying effect of GSI. According to our results, HL associated constitutively activated NOTCH1 can be a potential therapeutic target that could be influenced by other regulatory treatments of NOTCH1 activity such as mTOR inhibitors.

According to our *in vitro* experiments, the rapamycin sensitivity of the studied HL cell lines correlated with mTORC2 expression. In line with preclinical studies – in rapamycin resistant haematology or other tumours – only the new generation mTOR inhibitors had tumour growth inhibitory effect in cell lines with mTORC2 expression and activity. In spite of the *in vitro* detected differences, mTORC1 inhibitors (Rapamune/Torisel) showed similar tumour growth inhibitory effect in HL xenograft as *in vivo* combination treatment. These results could be explained by long-term treatment (more than one month) and rapalogs could be used in clinical treatments.

Recently the effects of mTOR in metabolic regulation have become better known. High lactate level and intensive glycolytic activity were found in the studied HL cell lines after the determination of intracellular metabolite concentrations. Our previous and recent results about the unique differences of cellular glycolytic activity and the literature data draw attention to the inhibitory effect of mTORC1 in context to metabolic changes. It can cause bioenergetic changes and rearrangement of the anabolic and catabolic balancing. We observed general intracellular metabolite concentration decreasing effect of mTOR inhibitors, related mainly to the new generation inhibitors (mTORC1/C2 and dual inhibitors) in different HL cell lines. Our results suggest that the metabolic regulator function correlates to mTOR activity beside the cellular survival and proliferation effects in HL cells. These results should be considered for introducing a potential targeted therapy with a well characterised mTOR activity and for applying new metabolic target determination.

The new statements about Notch1 and mTOR signal activity

- We first described the ligand and GSI independent constitutive NOTCH1 activation.
- The mutations of mTOR and Notch signal regulator genes (*EGFR*, *AKT*, *FBXW7*, *NOTCH1*, *PIK3CA*, *PTEN* and *TP53)* do not play any roles in the hyperactivation of either pathway.
- We confirmed the mTOR inhibitory effect of rapamycin in HL cells, in parallel, its tumour growth inhibitory and apoptosis inductor effects were increased by GSI treatment *in vitro* and *in vivo* as well.
- We determined that different activity levels of mTORC1/C2 correlate with mTORC1 sensitivity and the mTORC1/C2 or dual inhibitors can be effective tumour growth inhibitors in HL cell lines with mTORC2 complex activity.

Statements about mTOR activity changes associated metabolic effects

- We found that intensive glycolysis and active mitochondria were characteristic for the HL cell lines *in vitro*, however unique differences were detected. In parallel the mTOR inhibitor treatment dependent cellular sensitivity was detected by studying of intracellular metabolite concentrations.

Publications

Publications in context of the thesis

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