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ORIGINAL ARTICLE

Growth inhibitory effect of rapamycin in Hodgkin-lymphoma cell lines characterized by constitutive NOTCH1 activation

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Abstract Growing evidence suggests that deregulation of signalling elements of Notch and mammalian target of rapamycin (mTOR) pathways contribute to tumorigenesis. These signals play important roles in cellular functions and malignancies. Their tumorigenic role in T-cell acute lymphoblastic leukaemia (T-ALL) is well known; however, their potential interactions and functions are poorly characterized in Hodgkin lymphoma (HL). The aim of our study was to characterize mTOR and Notch signalling elements in HL cell lines (DEV, L1236, KMH2) and human biopsies and to investigate their cross-talk in the tumorous process. High mTOR activity and constitutive NOTCH1 activation was confirmed in HL cell lines, without any known oncogenic mutations in key elements, including those common to both pathways. The anti-tumour effect of Notch inhibitors are well known from several preclinical models but resistance and side effects occur in many cases. Here, we tested mTOR and Notch inhibitors and their combinations in gamma-secretase inhibitor (GSI) resistant HL cells in vitro and in vivo. mTOR inhibitor alone or in combination was able to reduce tumour growth; furthermore, it was more effective in xenograft models in vivo. Based on these results, we suggest that constitutively activated NOTCH1 may be a potential target in HL therapy; furthermore, mTOR inhibitors may be effective for decreasing tumour growth if resistance to Notch inhibitors develop.

Keywords Mammalian target of rapamycin (mTOR) · Notch · Gamma secretase inhibitor (GSI) resistance · Hodgkin lymphoma (HL) · Tumour growth

Introduction

Notch signalling influences cell fate and controls a number of cellular processes in haematopoietic progenitors toward lymphoid or myeloid lineages, and its excessive activation causes aberrant cellular proliferation, apoptosis and development [1]. The role of deregulated Notch signalling is well known in different malignancies, such as leukaemias, lymphomas, and breast cancer [2, 3]. The t(7;9) translocation was the first known oncogenic alteration in the *NOTCH1* gene in T-cell acute lymphoblastic leukaemia (T-ALL), resulting in a constitutively activated truncated protein. Mutations in the heterodimer domain (HD) and PEST (proline, glutamine, serine and threonine) domain leading to ligand-independent cleavage of the Notch receptor and reduced degradation of NICD1 (Notch intracellular domain 1) have also been described [4]. Aberrant Notch signalling has been shown to occur in more than 50 % of paediatric or adolescent T-ALL patients, and high NOTCH1 expression was detected in several other lymphoid malignancies, such as anaplastic large-cell lymphomas, diffuse large B-cell lymphomas, and HL [5–8]. In contrast, the tumour suppressor role of Notch signalling was also reported in myeloid malignancies [9].

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Activation of the four Notch transmembrane receptors (NOTCH1-4) can be triggered by their ligands (Jagged1, 2 or Delta like 1, 3 and 4), which is followed by consecutive cleavages by the ADAM metalloprotease and gamma-secretase, and the translocation of NICD into the nucleus. NICD binds to the CSL (CBF1/Su(H)/Lag-1) transcription factor complex and induces expression of target genes such as *c-MYC* and *HES1*, modulating cell growth, survival and differentiation [10, 11]. Small molecule gamma-secretase inhibitors (GSIs) target Notch signalling at the receptor activation level. DAPT (N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-Butyl ester) is the most frequently used GSI, but several new inhibitors have also been developed and tested recently [12]. SAHM1 (stapled α-helical peptides derived from MAML1) is a small peptide molecule, which is a highly selective inhibitor of NOTCH1 transcriptional activity in the nucleus, with a high therapeutic potential in haematopoietic malignancies or solid tumours, and—hopefully—less side effects [13].

Notch signalling is able to influence several other pathways, such as the PI3K (phosphatidylinositol-3-kinase)/AKT/mTOR pathway, which plays an important role in regulating cellular functions and tumour development as well [14, 15]. mTORC1 and C2 complexes (mTORC1 and mTORC2) contain characteristic proteins (such as Raptor and Rictor, respectively) and have different sensitivity to rapamycin. mTORC1 can be effectively inhibited by rapamycin, whereas mTORC2 is primarily resistant to it (however, it may show some sensitivity in the long term) [16]. mTORC1 regulates protein synthesis, promotes cell growth and proliferation through phosphorylating its target proteins S6K (ribosomal S6 kinase) and 4-EBP1 (Eukaryotic translation initiating factor 4E-binding protein1). mTORC2 phosphorylates AKT, SGK (Serum- and glucocorticoid-inducible kinase) and PKC (Protein kinase C), and it is able to promote cell survival, actin cytoskeleton reorganization and migration [17].

mTOR and Notch signalling share a number of common regulators (e.g. c-MYC, AKT, PTEN [Phosphatase and tensin homologue] and FBXW7 [F-Box And WD Repeat Domain Containing 7, E3 Ubiquitin Protein Ligase]), suggesting an existing crosstalk between the two pathways, which may contribute to therapeutic resistance and poor prognosis in different malignancies [18].

We previously characterized mTOR activity in a number of human lymphoma types including childhood ALLs and HLs, and tested the effect of rapamycin in cell lines representing low and high grade lymphomas in vitro. We showed that rapamycin was able to reverse resistance to the negative regulator TGFb in vitro in high grade lymphoma cells, and it was able to modify cellular composition and protein expression within the microenvironment in vivo [19–21]. We reported mTORC1 related high mTOR activity in the majority of human HL biopsies by immunohistochemistry as well [21, 22]. However, the molecular background of deregulated mTOR activity has not been extensively studied in HL cells [23, 24]. Beside intrinsic (e.g.: CCL5, IL-4, CXCL16) and other factors, Notch ligands have also been found in the background of altered regulation in the microenvironment [25]. Given the role of Notch signalling in the pathogenesis of multiple myeloma and HL, we hypothesized that Notch may be a potential activator of mTOR signalling in HLs [26, 27].

Here, we characterized the expression and the activity of Notch and mTOR signalling elements in three Hodgkin lymphoma cell lines and in human HL biopsy samples. The interaction of the two pathways was investigated using different Notch inhibitors, Jagged1—a NOTCH1 ligand—and mTOR inhibitors, and their effect on tumour growth was examined in vitro and in vivo.

Methods

Cell cultures and treatments

Hodgkin-lymphoma cell lines (L1236, KMH2—classical HL with mixed cellularity; DEV—nodular lymphocyte predominant HL) and T-ALL cell lines (Jurkat, Molt4) were cultured and treated in RPMI 1640 medium (HyClone, GE Healthcare Life Sciences) supplemented with 10 % fetal bovine serum (FBS; HyClone; for L1236, KMH2, Jurkat and Molt4 cultures) or 20 % FBS (for DEV cultures) and 0.4 % gentamycin (Sandoz) at 37 °C in a 5 % CO_2 atmosphere.

Experiments were performed at 1.5×10^5 or 5×10^5/ml cell density. Cells were treated with rapamycin (50 ng/ml, Sigma), DAPT (gamma-secretase inhibitor IX, 1 μM, Cayman Chem. Co.), GSI XII (gamma-secretase inhibitor XII, 4 μM, Calbiochem), SAHM1 (stapled α-helical peptides derived from MAML1, 5 μM, Calbiochem) and Jagged1 ligand (1 μg/ml, R&D Systems; plates were coated with the ligand at 37 °C for 2.5 h before plating the cells) for different time periods. 1 % DMSO (Sigma) was used as a vehicle for some of the compounds and was added to control cells as required. The applied concentrations were based on previous publications and our data.

Detection of cell proliferation and apoptosis

Apoptosis was measured by flow cytometry. The percentage of apoptotic cells (sub-G1 population) was determined according to Mihalik R. et al. using a FACScan flow cytometer (BD Biosciences, Erembodegem, Belgium) [28]. Data were analysed by WinList software (Verity Software House, Topsman, ME, USA). Alamar Blue assay (10 %, Thermo Fisher Scientific) was used to determine proliferation capacity. After 4 h incubation, the resulting fluorescence was measured at excitation wavelengths of 570 and 590 nm by Fluoroscan

Ascent FL fluorimeter software (Labsystems International). Percentage of proliferation was given relative to control samples.

Xenograft models

Tumour xenografts were established by injecting 3×10^7 L1236 cells with Matrigel (Sigma Aldrich) subcutaneously (s.c.) into the back region of SCID mice in a total volume of 250 μl (125 μl cell suspension and 125 μl Matrigel). Palpable tumours were removed, cut into equal pieces and transplanted into new recipient mice randomized into four groups (control, Torisel, GSI, Torisel + GSI treated). Treatments (Torisel: 10 mg/kg intraperitoneally [i.p.], Pfizer [Temsirolimus CCI-779]; GSI XII: 4 mg/kg [i.p.], Calbiochem; control groups: DMSO or saline [i.p.]—based on previously published studies and our data [21]) were initiated when tumours were palpable, twice a week for 4 weeks. Tumour growth was measured twice a week by a calliper; tumour volume was calculated using the following formula: $\pi/6*((2*\text{width} + \text{length})/3)^3$ [29]. At the end of in vivo experiments, mice were euthanized, tumours were removed, fixed in 4 % paraformaldehyde and embedded in paraffin.

Immunocyto- and immunohistochemistry

Methanol fixed cytospin preparates and 4–5 μm tissue sections were used. Deparaffinized tissue sections underwent antigen retrieval (pH = 6 citrate buffer, in a pressure cooker). Slides were incubated with anti-phospho-S6 (Ser235/236, Cell Signalling 2211, 1:100), anti-Rictor (Bethyl Lab., Inc. A500-002A, 1:800), anti-Raptor (Abcam AB40758, 1:100), anti-Notch-1 (binds to amino acids 2300–2556, Novus NBP1-78292, 1:200), anti-cleaved-Caspase3 (Asp175, Cell Signalling 9664, 1:500) primary antibodies at 4 °C overnight, followed by Novolink Polymer Detection System (Novocastra, Wetzlar, Germany), DAB (Dako) staining and haematoxylin counterstaining.

Western blotting

At least 1.5×10^6 lymphoma cells were lysed (50 mM Tris, 10 % glycerol, 150 mM NaCl, 1 % Nonidet-P40, 10 mM NaF, 1 mM PMSF, 0.5 mM $NaVO_3$, pH 7.5). Equal amounts of proteins (quantitated by Quant-iT protein assay – Invitrogen) were separated in 8 % SDS-PAGE gels and transferred onto PVDF membranes using the semi-dry blot system (BioRad). Membranes were incubated with anti-phospho-S6 (Ser235/236, Cell Signalling 2211, 1:1000), anti-cleaved-Notch-1 (Gly1753 and Val1754, Cell Signalling 2421 1:1000), anti-Notch-1 (Novus NBP1-48289 1:1000), anti-Rictor (Cell Signalling 2140, 1:500) and anti-Raptor (BioLegend 623202, 1:500) antibodies overnight at 4 °C. Blots were incubated by biotinylated secondary antibodies followed by avidin-HRP complex (Vectastain Elite Universal ABC Kit, Vector Laboratories), developed by enhanced chemiluminescence (Advansta Inc.). Equal protein loading was confirmed using anti-β-Actin (Sigma Aldrich A2228, 1:5000). The expression of pS6 was quantitated by ImageJ 1.46r software.

Gene expression analysis

RNA was purified by PureLink™ Micro-to-Midi kit (Invitrogen) from at least 1.5×10^6 homogenized cells (QIAshredder, Qiagen). Isolated RNA was quantified by NanoDrop (ND-1000 v.3.3). 1 μg RNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen) and random hexamers (Invitrogen). Notch receptor and ligand expression was detected from 25 ng cDNA by specific primer pairs and RedTaq polymerase (Sigma) or FailSafe TM PCR reaction (Epicentre) and documented on a Kodak Image Station 4000 MM (Eastman Kodak, Rochester, NY, USA) (Online Resource 1a).

Quantitative real-time PCR of *HES1* (Hs00172878_m1), *c-MYC* (Hs00153408_m1) and endogenous controls (GAPDH) was performed with TaqMan Assay-On-Demand Gene Expression Product (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7300 Real-Time PCR System. Relative gene expression was analysed using the delta-delta-Ct method, normalized to *GAPDH* in order to compare results with a reference sample (normal human B cells) from healthy individuals, which was assigned a value of 1.

DNA sequence analysis

DNA was isolated from 3×10^6 cells with Genomic DNA Mini Kit (Geneaid). Exons 5, 9, 10, 11 of the *FBXW7* gene were chosen for sequencing and primers were designed with the Primer 3Plus free software (http://www.bioinformatics.nl/) (Online Resource 1b). Direct sequencing (25 cycles at 51 °C, BigDye Sequencing Kit, Applied Biosystems) was performed after PCR amplification of the *FBXW7* gene (Ready Mix, Thermo Scientific). Products were purified by NucleoSEQ (Macherey-Nagel) and analysed by capillary electrophoresis on a Genetic Analyzer 3500 (Applied Biosystems).

The most frequent mutations of *PIK3CA* were tested by pyrosequencing, using 100 ng template, primers for exon 9 and 20 and AmpliTaqGold360 Master Mix (Applied Biosystems; conditions of amplification were 40 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s – Online Resource 1c), based on the method of Katsuhiko Nosho et al. [30].

Next-generation sequencing was used to analyse the mutational hotspots of 50 oncogenes (Ion AmpliSeq™ Cancer Hotspot Panel v2, Life Technologies) in DNA from HL cell lines. Template preparation and enrichment were similar as described above. Sequencing and alignment to the hg19 human reference genome were performed by KPS Diagnostic Ltd. (Budapest, Hungary).

Statistical analysis

Mean values and SD were calculated from three independent experiments with three or more parallels, depending on the assays used. Statistical analysis of in vivo and in vitro experiments was performed using PAST 3.05 software. Student's *t* test and one-way analysis of variance (ANOVA) with post hoc analysis (Tukey's range test) were used for statistical calculations. $p \leq 0.05$ was considered statistically significant.

Results

Elements of Notch-1 and mTOR signalling are present in Hodgkin lymphoma cells

mRNA expression of *NOTCH1* and *NOTCH2* receptors and *JAG1* (Jagged1), *JAG2* (Jagged2) and *DELTA1* ligands were characteristic for all three HL cell lines. The *NOTCH4* receptor was not present in any of the cells, and *NOTCH3* was expressed only in DEV and L1236 cells (Fig. 1a). At the protein level, cleaved NOTCH1 was detected in all three examined HL cell lines by Western blotting, using different NOTCH1 antibodies directed against NICD or the cleaved (activated) NOTCH1 fragment (c-NOTCH1). The intact full-length NOTCH1 receptor was undetectable in HL cells. In parallel, the presence of both full-length and c-NOTCH1 was shown in NOTCH1 expressing Molt4 and Jurkat control cells (Fig. 1b). Constitutive NOTCH1 activation and the absence of full-length NOTCH1 remained unchanged after GSI treatment in HL cell lines, whereas c-NOTCH1 level was reduced in Jurkat cells (Fig. 1c).

mTOR activity was also investigated in HL cells. The expression of pS6 protein—which indicates mTORC1 activity—was different in the three cell lines: pS6 protein expression was higher in KMH2 than in DEV and L1236 cells. The ratio of Raptor and Rictor proteins was also variable, indicating that mTORC1 and mTORC2 complexes are differently expressed; however, relatively high expression of Raptor and p-S6 suggests that mTORC1 activity is dominant in HL cells (Fig. 2a, b).

C-NOTCH1 was perinuclear and nuclear in Hodgkin and Reed/Sternberg (HRS) cells in human HL biopsies by immunohistochemistry, and it was absent from non-neoplastic lymphoid cells in lymph nodes and tonsils (Fig. 1d). The investigated samples showed high mTORC1 activity, which confirms our previous data [21].

Activated NOTCH1 level is not affected by GSI-DAPT and rapamycin in HL cells

Cell lines were treated by Notch signalling inhibitors (such as GSI-DAPT), Jagged1 ligand, rapamycin and their combinations; however, none of these treatments was able to enhance or reduce the level of NOTCH1 protein expression. Based on these findings, NOTCH1 activation was constitutive in HL cells, which were resistant to the applied treatments (Fig. 3a). NOTCH1 target gene expression was also resistant to rapamycin and Notch signalling inhibitors (DAPT, GSI XII and SAHM1) in vitro. Rapamycin did not influence the expression of *c-MYC* and *HES1*. The expression of these genes remained at a low baseline level in KMH2 and in L1236 (data not shown), and we did not detect statistically significant changes in NOTCH1 target genes—only a slight decrease was detected in the DEV cell line after treatment with different inhibitors (Fig. 3b).

Inhibition of mTORC1 by rapamycin intensively reduced p-S6 expression at several time points (2, 24 and 72 h), which was most pronounced after 2–24-h treatment (0–11.4 % of control samples). However, GSIs did not have a significant effect on pS6 in the examined HL cells: densitometric evaluation showed a slight decrease in p-S6 levels (65.1–90.3 % compared to controls) after 2 h GSI treatment but expression levels were almost similar to controls at 24 or 72 h (85.2–128.5 %). Figure 3c. shows a representative Western blot with pS6 expression in 2 h treated samples.

In vitro effects of NOTCH1 and mTORC1 inhibitors on apoptosis and proliferation in HL cell lines

The effect of gamma-secretase inhibitors (DAPT, GSI XII), the direct inhibitor of the NOTCH1 transcriptional complex (SAHM1) and rapamycin was tested in vitro. Alamar blue assay showed unaffected proliferation after GSI treatment in all HL cell lines. However, SAHM1 was able to inhibit proliferation in these cells and induced apoptosis in L1236 and DEV cells. Rapamycin significantly reduced the proliferation of DEV and KMH2 cells (to 80 %) and induced apoptosis in KMH2 cells after 72 h treatment (Fig. 4a, b). KMH2 was the most sensitive to rapamycin of the three HL cell lines, followed by DEV, with L1236 being the least sensitive in vitro. Long-term rapamycin treatment confirmed these differences: proliferation further decreased in KMH2 cell cultures after 144 h rapamycin treatment (published previously); however, in vitro rapamycin treatment had no further effect on

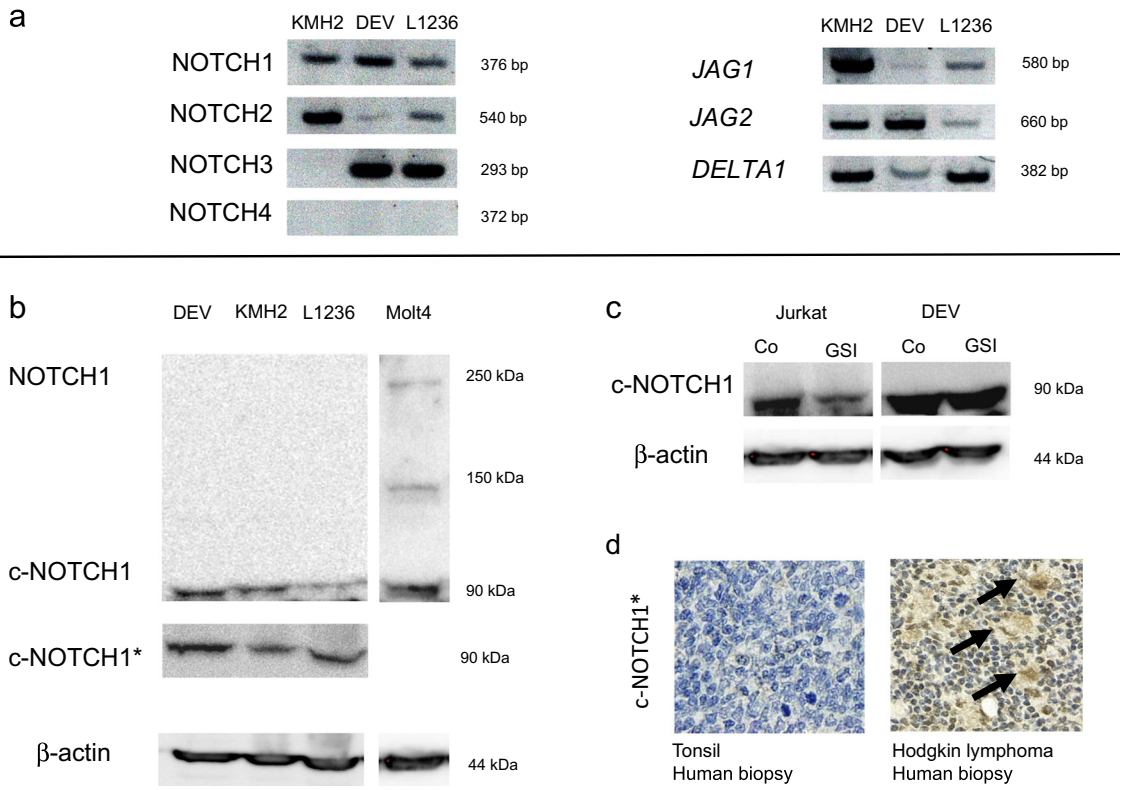


Fig. 1 Expression analysis of Notch signalling elements in HL cell lines (DEV, KMH2, L1236). **a** mRNA expression of *NOTCH1*, *NOTCH2*, *NOTCH3*, *NOTCH4* receptors and *JAG1*, *JAG2, DELTA1* ligands by RT-PCR. **b** Western blot analysis of NOTCH1 and cleaved NOTCH1 protein expression confirm the constitutive activation of NOTCH1. The anti-NOTCH1 antibody detects both the full-length and the activated (cleaved) form of the NOTCH1 receptor (c-NOTCH1), as it is shown in positive control Molt4 cells. The anti-cleaved NOTCH1 antibody (c-NOTCH1*) detects only the cleaved form (corresponding to Gly1753/Val1754 of human NOTCH1). β-actin served as a loading control. **c** Gamma-secretase inhibitor (GSI XII, 4 μM) did not alter the level of cleaved NOTCH1 protein after 72 h treatment in the DEV cell line; however, it reduced the amount of the protein in control GSI treated Jurkat cells where full-length NOTCH1 receptor was also detected (Western blotting). **d** c-NOTCH1* was detected in malignant cells of human HL biopsies by immunohistochemistry (IHC). *Arrows* show positivity in the nuclear and perinuclear area of Hodgkin and Reed/Sternberg (HRS) cells. Non-malignant lymphoid cells were c-NOTCH1* negative both in HL biopsies and in normal tonsil tissues

proliferation in DEV or L1236 cells after 96, 120 or 144 h incubation [21]. Surprisingly, the combination of rapamycin and GSI XII significantly reduced proliferation in all three HL cell lines compared to rapamycin monotreatment; however, it did not induce significant apoptotic response after 72 h treatment (Fig. 4a, b).

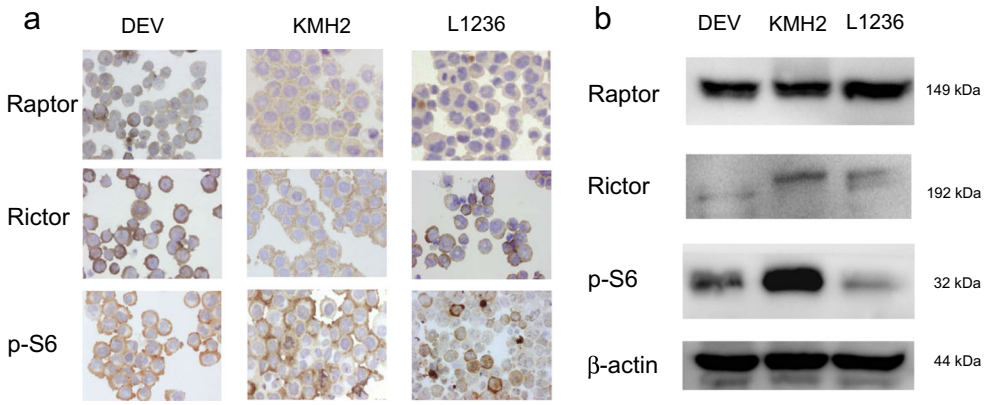


Fig. 2 The expression of mTOR pathway related proteins in HL cell lines. **a** The presence of Raptor (characteristic for mTORC1), Rictor (characteristic for mTORC2) and phosphorylated-S6 (pS6, related to mTORC1 activity) was detected by immunocytochemistry on cytospin slides from HL cell lines. **b** mTOR signalling elements are present in HL cells in variable amounts (Western blotting). Representative results are shown

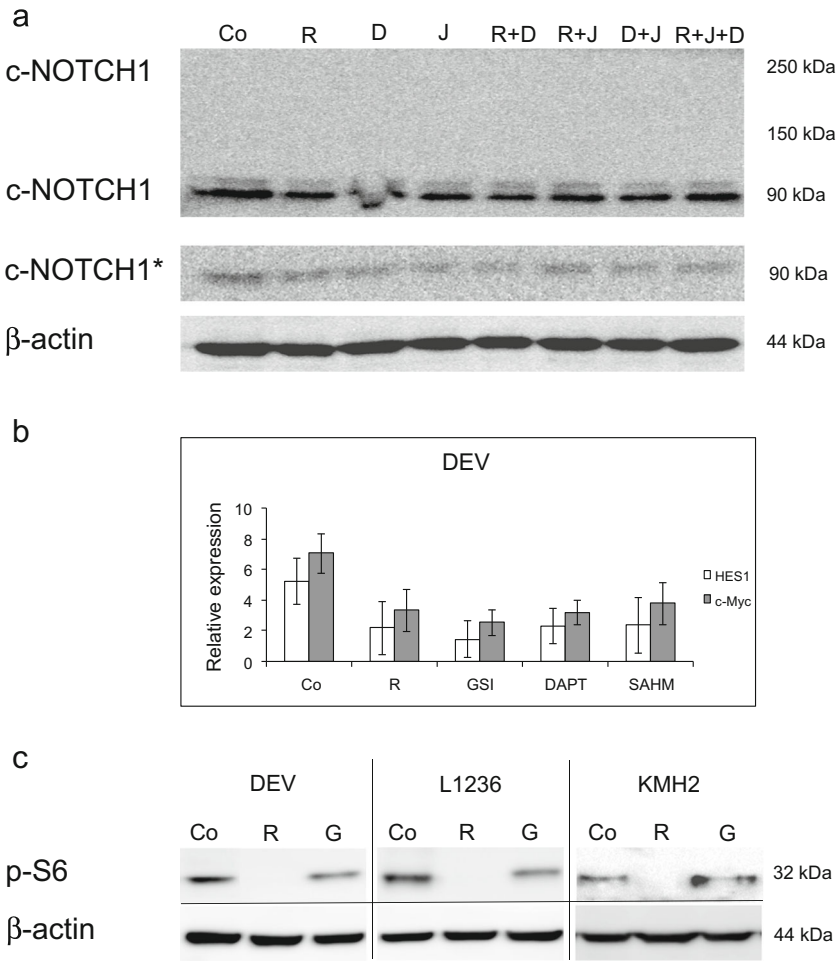


Fig. 3 The effect of signalling modulators on Notch and mTOR signalling activity in HL cell lines. **a** The level of NOTCH1 and cleaved NOTCH1 protein did not change after 2 h treatment with rapamycin (R, 50 ng/ml), gamma-secretase inhibitor DAPT (D, 1 μM), and Jagged-1 (J, 1 μg/ml) in L1236 cells. (c-NOTCH1*: antibody specific for cleaved NOTCH1; Western blotting.) β-actin served as a loading control. **b** The expression of *HES1* (target gene of NOTCH1) and *c-MYC* (target gene of both NOTCH1 and mTOR) is reduced after 2 h treatment with rapamycin (R) or Notch inhibitors (GSI: GSI XII; DAPT, SAHM) in the DEV cell line. However, the alterations were not significant ($p > 0.05$). **c** The amount of phospho-S6 protein (p-S6) is significantly reduced by rapamycin (R) and slightly lowered by GSI (G) treatment (GSI XII) after 2 h treatment (normalized to β-actin)

In vivo effect of NOTCH1 and mTORC1 inhibition on tumour growth in HL xenografts

Inhibition of NOTCH1 and mTORC1 was also investigated in vivo. The growth inhibitory effects of mTORC1 inhibitors were previously shown in several lymphoma types including HL xenografts [21]. Xenografts were established from L1236 cells, a less rapamycin sensitive HL cell line, and mice were treated with Torisel (a rapamycin analogue), GSI and their combination. GSI led to a size reduction of the tumours but this effect was not significant. Torisel and the combination treatment decreased tumour volume and weight significantly in treated mice, without weight loss and any obvious toxic side effects. However, the inhibition of tumour growth was not significantly enhanced by combination treatment, compared to monotreatments (Fig. 5a, b). Morphological and immunohistochemical analysis of tumour tissue sections showed that the reduction of tumour size was associated with an increased number of cleaved-caspase 3 positive (apoptotic) cells in Torisel and Torisel + GSI treated mice. In addition, p-S6 expression related to mTORC1 activity was also reduced after Torisel and combination treatments, in accordance with in vivo results (Fig. 5c).

Activation of NOTCH1 is not caused by known hotspot mutations in common oncogenes

To elucidate the background of constitutive, GSI resistant activation of NOTCH1 signalling in the examined HL cells, mutations of several genes related to NOTCH1 or mTOR deregulation were checked. No mutations were found in the *NOTCH1* receptor, *PIK3CA* and *FBXW7* genes and in other oncogenes included in the Cancer HotSpot Panel (such as *AKT1*; *ALK*; *BRAF*; *CDKN2A*; *CSF1R*; *EZH2*; *FLT3*; *H/N/KRAS*; *IDH1*; *IDH2*; *JAK2/3*; *KIT*; *MLH1*; *MPL*; *NPM1*; *PTEN*; *RB1*; *TP53*; *VHL*) by direct (*FBXW7*, *PIK3CA*) and/or next generation sequencing.

Discussion

Activated NOTCH1 and mTOR signalling and their potential significance were described in the present work in HL cell lines and in HL tissues. Moreover, we confirmed that NOTCH1 signalling activity is ligand and gamma-secretase independent, i.e. constitutive in the examined HL cell lines. These cells showed different mTOR inhibitor sensitivity and

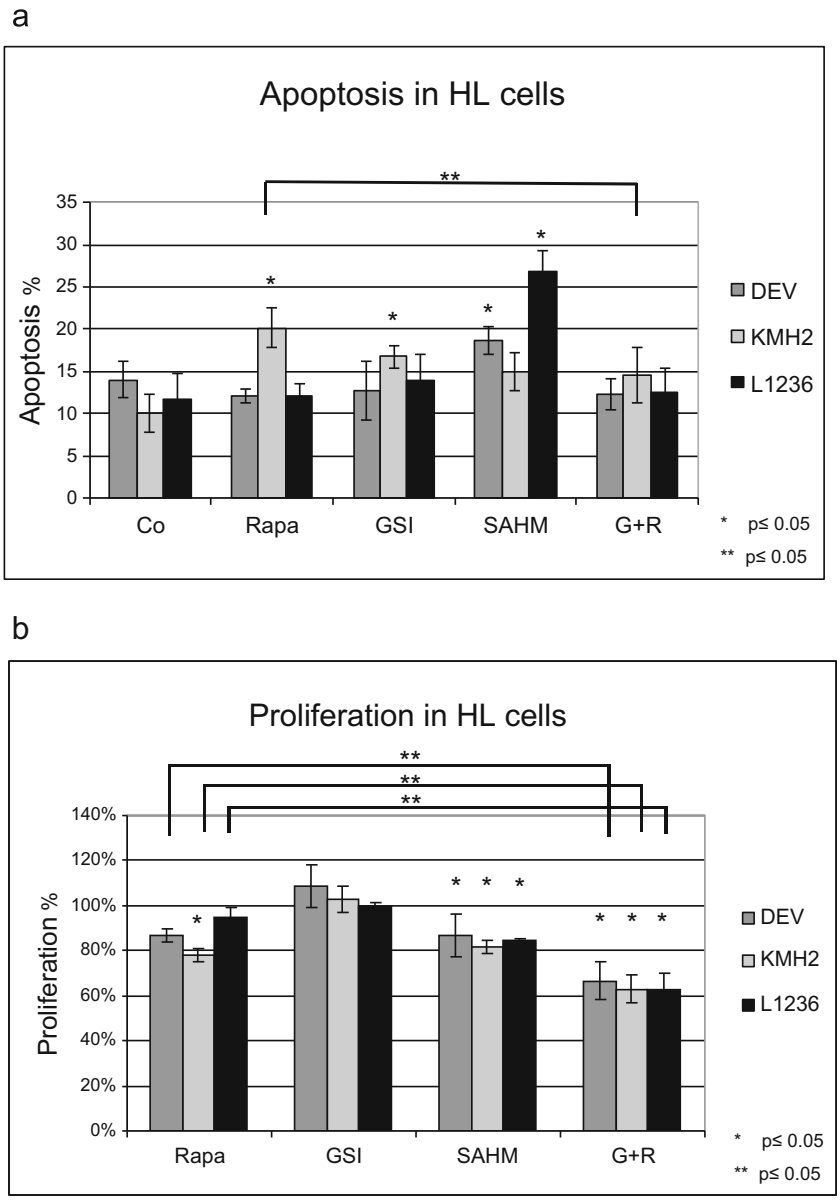


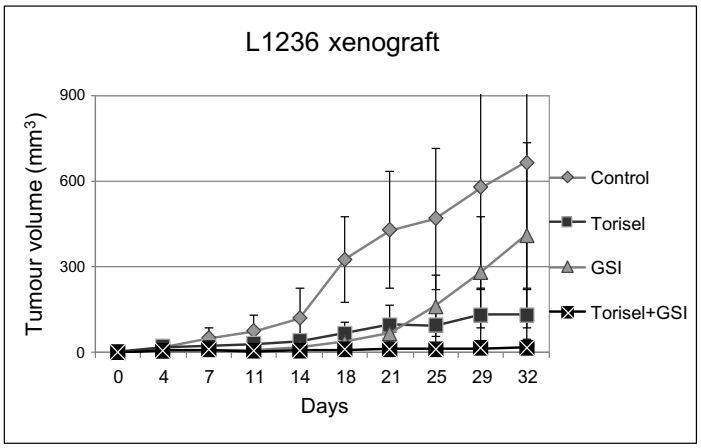
Fig. 4 In vitro effect of Notch and mTOR inhibitors in HL cell lines. **a** The percentage of apoptotic cells in HL cell cultures treated with rapamycin (R, 50 ng/ml), GSI XII (GSI, 4 μM), direct NOTCH1 transcriptional inhibitor (SAHM1, 5 μM) and combined GSI XII + rapamycin (G + R) for 72 h. (flow cytometry). **b** Rate of proliferation in HL cell lines treated with rapamycin (R, 50 ng/ml), GSI XII (GSI, 4 μM), SAHM1 (5 μM) and combined GSI XII + rapamycin (G + R) for 72 h. The percentage of proliferation is given relative to untreated control cells (100 %)

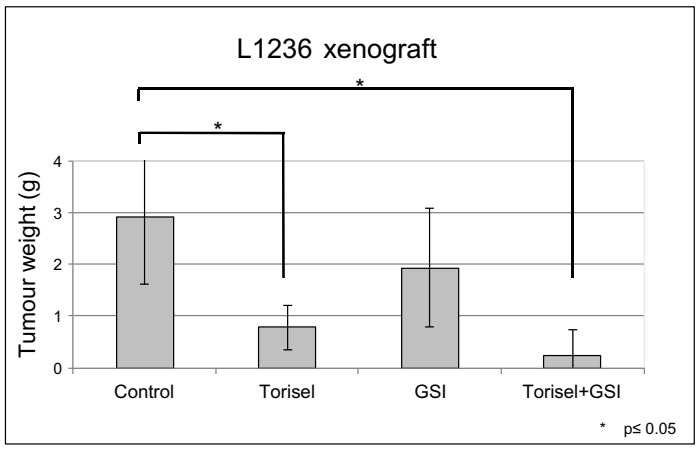
GSI resistance in vitro; however, Torisel (an mTORC1 inhibitor) was very effective and inhibited tumour growth in vivo.

Mechanisms leading to constitutive NOTCH1 activation are well known in T cell acute lymphoblastic leukaemias and in certain solid tumours [2, 5]. However, none of the known oncogenic *NOTCH1* or *FBXW7* mutations, translocations or amplifications was detected by sequencing and routine cytogenetic karyotyping in the examined HL lymphoma cell lines [31].

The *PIK3CA* gene is mutated in less than 10 % of diffuse large B cell lymphomas, but the loss of *PTEN* is a frequent event in a number of tumours; however, these events are uncommon in other lymphomas, including HL [32, 33]. In spite of this rare mutation incidence, a high level of AKT phosphorylation was shown in 63 % of Hodgkin lymphomas [24]. This corresponds well with our previous findings, which demonstrate that high mTOR activity is characteristic for HL cells, but at the same time no mutations can be detected in well-known oncogenes (Cancer HotSpot Panel) in the examined cell lines. It has been suggested that high mTOR activity may be induced by other signals from the microenvironment (for example through different receptors such as CD40, BCR) [26, 34]. High mTOR activity could be related to constitutive NOTCH1 activation, as well [35].

Transcriptional targets of NOTCH1—*HES1* and *c-MYC*—were reported to regulate *PTEN* expression through promoter binding in T-ALL cells. HES1 inhibits *PTEN* expression; in contrast, c-MYC can induce it, therefore Notch signalling alters AKT/mTOR activity in opposite ways. The inhibitory effects of HES1 are usually more dominant, even though this





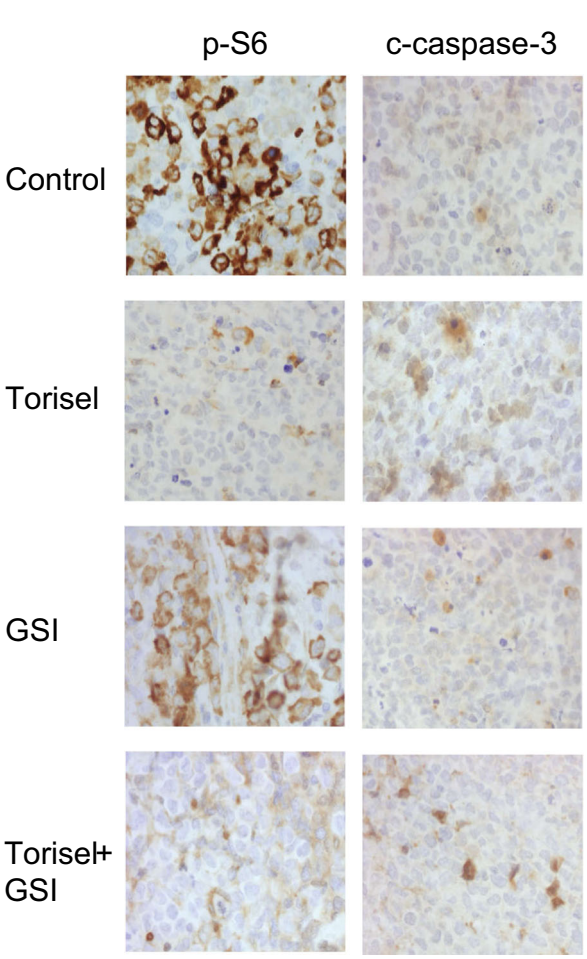


Fig. 5 In vivo treatment with Notch and mTOR inhibitors. **a** L1236 HL xenografts were treated with mTOR inhibitor (Torisel, 10 mg/kg), gamma-secretase inhibitor (GSI XII, 4 mg/kg) and their combination for 32 days in vivo. **b** Both tumour volume and tumour weight were reduced by the treatments, with Torisel and GSI XII acting synergistically. *Statistically significant ($p < 0.05$). **c** Phospho-S6 protein expression was reduced and the number of cleaved caspase-3 labelled apoptotic cells was increased after Torisel and GSI + Torisel treatment in mice with L1236 xenografts. Representative tissue sections are shown. (IHC; 400X)

regulatory loop depends on the actual type and condition of cells [36, 37].

In our experiments, only one HL cell line showed some (but not significant) changes in *c-MYC* and *HES1* mRNA levels after different treatments, which indicates that rapamycin and Notch-inhibitors could not influence constitutive NOTCH1 activation, and this is not related to high mTOR activity in these HLs. GSI did not alter proliferation, spontaneous apoptosis and the level of mTOR or NOTCH1 signalling activity. mTOR activity was unaffected after GSI treatment in xenografts as well. However, SAHM1 was able to reduce proliferation and increase apoptosis in HL cell lines in vitro. GSIs enhanced the inhibitory effect of rapamycin in vitro and in vivo, although this was not significant in vivo because Torisel and Torisel + GSI had almost the same growth inhibitory effect in xenografts. The results of our experiments with KMH2 and DEV HL xenografts, as well as xenografts from other B cell non-Hodgkin lymphoma cell lines indicate that rapamycin has a greater inhibitory effect on tumour growth in vivo than in vitro (data not shown). This may be explained by the long-term treatment period (~1 month) in vivo and by the complex formulation of drugs used for in vivo treatments. It is also well known that rapalog treatment can reverse resistance to available negative regulators in the tissue microenvironment [19]. Moreover, continuous mTORC1 inhibition can interfere with the assembly of mTORC2, and it can inhibit the translation of many other proteins (e.g.: cyclinD1, cyclinD3 and $p27^{Kip1}$) [16, 38]. GSI treatment may also modify the microenvironment of tumour cells in vivo, which may explain why GSI and mTORI combination was more effective in vivo [26].

Our results show that mTOR inhibitor treatment can inhibit tumour growth in different HL cell lines regardless of constitutive NOTCH1 activity. One possibility is that constitutive NOTCH1 activity increases proliferation and survival through mTOR activation in HL cells. Our findings—along with those of other work groups—suggest that mTOR inhibitors may be useful in the therapy of refractory or relapsed HLs [21, 39]. Our in vivo results are supported by recent studies which showed effectivity of mTOR inhibitors in end stage Hodgkin lymphoma [22]. mTOR inhibitors (or their combination) may overwrite the effects of Notch signalling, or cause a central regulatory failure in the signalling network, thereby inhibiting HL cell growth. Moreover, our data suggests that rapalog treatment can also be considered in other GSI resistant malignancies with aberrant NOTCH1 and mTOR signalling, such as ALL or solid tumours (e.g. breast, gastric or prostate cancers) [40].

In the last decades, there have been no major changes in the therapy of HL, apart from qualitative changes in radiation therapy, and the reduction of total dose and toxicity [41]. Compared to other tumour types, the relatively successful current treatment protocols for HL may be the reason for the absence of new therapeutic agents. However, nearly 15 % of patients have relapsed or refractory disease, and no therapeutic solutions have been developed for them. Based on our results, constitutive NOTCH1 or mTORC1 activity may be considered as a therapeutic target in the early and final stages of cases with potentially poor prognosis, after histological target validation [42]. Ligand and gamma-secretase independent activation of NOTCH1 does not alter mTOR inhibitor sensitivity in HL cell lines, which was shown in our study. This suggests that mTOR inhibitors may be used successfully in other tumours as well, regardless of Notch activity and constitutive NOTCH1 activation.

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Compliance with ethical standards In vivo experiments were approved by the Institutional Ethical Review Board (PEI/001/2457-6/2015) and the Institutional Animal Care Laboratory, with official permissions (PEI/001/1733-2/2015). Immunohistochemical studies on human tissue biopsies were approved by the Institutional Ethical Review Board (TUKEB no. 7/2006).

Conflicts of interest None

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