

Department of Pediatrics and Adolescent Medicine,
University Medical Center, Ulm University

Director: Prof. Dr. Klaus-Michael Debatin

**Targeting of hyperactivated mTOR signaling
in high-risk acute lymphoblastic leukemia as
a novel treatment strategy**

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Md. Nabiul Hasan

Born in Faridpur, Bangladesh

2015

Dean:

Prof. Dr. Thomas Wirth

First Reviewer:

PD Dr. Lüder Hinrich Meyer

Second Reviewer:

Prof. Dr. Peter Gierschik

Day Doctorate Degree Awarded: October 23, 2015

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Abbreviations

4EBP1	4E binding protein 1
7-AAD	7-Aminoactinomycin D
AEIOP-BFM	Associazione Italiana di Ematologia Pediatrica and Berlin-Frankfurt-Münster childhood ALL study group
ALL	Acute Lymphoblastic Leukemia
ALL-BFM	The Berlin Frankfurt Munster acute lymphoblastic leukemia
AKT	AKT8 virus oncogene cellular homolog
AML	Acute myeloid leukemia
APC	Allophycocyanin
ATP	Adenosine triphosphate
BAD	BCL2-associated agonist of cell death
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
BCR-ABL	Gene fusion as a result of reciprocal translocation between chromosome 9 and 22, also known as Philadelphia chromosome
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CLL	Chronic lymphocytic leukemia
cMAP	Connectivity map
CNS	Central Nervous System
CREBP	CREB binding protein
CREMt	cAMP-responsive element modulator t
CRLF2	Cytokine receptor-like factor 2
DDIT4L	DNA-damage-inducible-transcript-4-like
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
ETV6-Runx1/TEL-AML1	Fusion gene as a result of t(12;21)(p13;q22) translocation, previously known as TEL-AML1

EFS	Event free survival
ER α	Oestrogen receptor α
FBS	Fetal bovine serum
FDR	False discovery rate
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FLT3	Fms-related tyrosine kinase 3
FMRP	Fragile X mental retardation 1
FRAP1	FK506 binding protein 12-rapamycin associated protein 1
FRB	FKBP12-rapamycin binding
FSC	Forward scatter
GSK3	Glycogen synthase kinase-3
GTP	Guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
HR	High-risk
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic stem cell transplantation
ICCC	International classification of childhood cancer
IKK β	Inhibitor Of Kappa Light Polypeptide Gene Enhancer In B-Cells
IKZF1	IKAROS family zinc finger 1
IF	Intra-femoral
Ig	Immunoglobulin
IL7R	Interleukin 7 receptor
IP	Intra-peritoneal
IRS	insulin receptor substrate
IT	intra-tibial
IV	intra-venous
JAK	Janus kinase
JMML	Juvenile myelomonocytic leukemia
Ki67	A cellular marker for proliferation, also known as MKI67
MAP43K	MAPK kinase kinase 3
MDM2	Mouse double minute 2 homolog

MFI	Median fluorescence intensity
MLL	Mixed-lineage leukemia
mLST8	Mammalian lethal with Sec13 protein 8
MRD	Minimal residual disease
mTOR	Mammalian Target of Rapamycin
NES	Normalized enrichment score
NGS	Normal Goat Serum
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	Non-obese daibetic
NSG	NOD/SCID/IL2Rcynull
p53	Tumor protein 53
p70S6K	70 kDa ribosomal protein S6 kinase
PAX 5	Paired box 5
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll protein complex
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
PFA	Praformaldehyde
Ph+	Philadelphia-chromosome positive
PI3	Phoaphatidylinositol 3-kinase
PIP2/PtdIns(4,5) P_2	Phosphatidylinositol 4,5-bisphosphate
PIP3/PtdIns(3,4,5) P_3	Phosphatidylinositol (3,4,5)-trisphosphate
PLD	Phospholipase D
PPR	Prednisone good response
PGR	Prednisone poor response
PKC	Protein kinase C
PR	Prednisone response
PRAS40	PROLINE-rich AKT substrate 40 kDa
PTEN	phosphatase and tensin homolog deleted on chromosome 10
p-value	Probability value

q-value	FDR-adjusted p-value
SCID	Severe combined immunodeficiency
SLC	Solute carrier family
RHEB	Ras homologue enriched in brain
S6	Ribosomal protein S6
SDS	Sodium dodecyl sulfate
SKAR	S6K1 Aly/REF-like target
STI	Signal Transduction Inhibitor
SSC	Side scatter
TCR	T cell receptor
TKI	Tyrosine kinase inhibitor
TNF α	Tumor necrosis factor alpha
TOR	Target of rapamycin
TSC	Tuberous sclerosis
TSLP	Thymic stromal derived lymphopoietin
TTL	Time to leukemia
TTR	Time to reoccurrence
VDA	Vincristine, dexamethasone, asparaginase
VDA+R	Vincristine, dexamethasone, asparaginase and rapamycin
WBC	White blood cell
WHO	World Health Organization

1. Introduction

1.1 Leukemia

According to the data presented by the World Health Organization (WHO), worldwide the leading cause of death is cancer; accounting for 8.2 million deaths at 2012 (WHO 2015). Acute lymphoblastic leukemia (ALL) is the most common cancer of children with an incidence rate of about 3 per 100000 populations (Grigoropoulos, Petter et al. 2013). Acute lymphoblastic leukemia is a cancer that starts from white blood cells called lymphocytes in the bone marrow (the soft inner part of the bones, where new blood cells are produced). ALL is characterized by the accumulation of immature progenitor cells in different compartments, e.g. bone marrow, spleen, peripheral blood and central nervous system (CNS) resulting the death of the patient due to anemia, neutropenia, infection, thrombocytopenia and bleeding (Loh and Mullighan 2012). About two-thirds of all acute leukemia cases are in children and ALL is the most frequent cancer in children whereas cancer in central nervous system is in the second place (Redaelli, Laskin et al. 2005) (Figure 1).

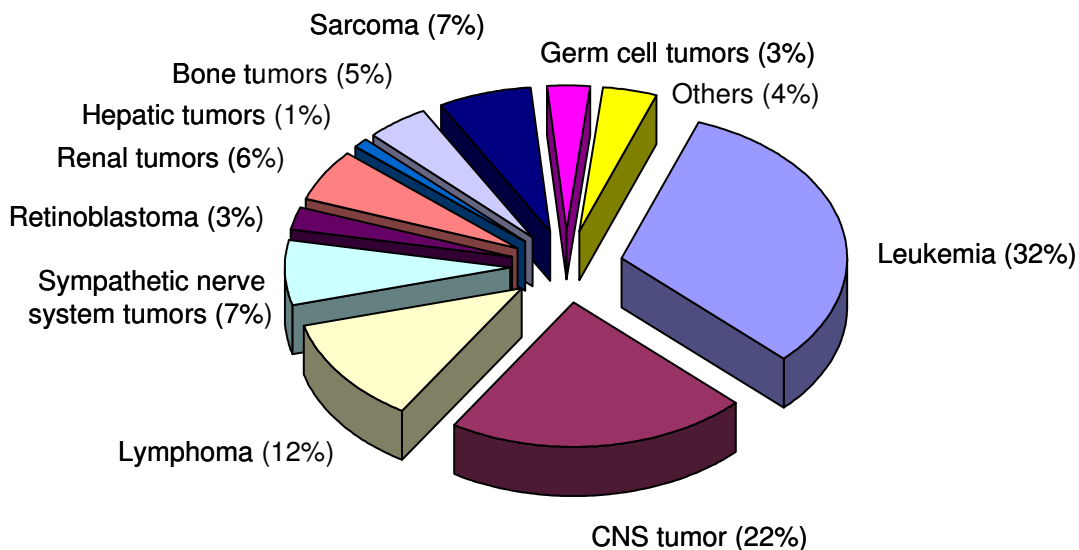


Figure 1: Relative frequencies of malignant diseases in children (Kaatsch 2010)

Distribution of different cancers of children (age 0 -14 years) in Europe according to the International Classification of Childhood Cancer (ICCC) diagnostic groups.

1.1.1 Pathogenesis and molecular epidemiology

The precise pathogenic cause of acute lymphoblastic leukemia is not clear although it is clear that there are a number of factors rather than a single factor involved. Only about 5% cases are associated with inherited and predisposing genetic causes, such as Down's syndrome, Bloom's syndrome, ataxia-telangiectasia and Nijmegen breakage syndrome, or with ionizing radiation or exposure to specific chemotherapeutic drugs (Pui, Robison et al. 2008). *In utero* or prenatal development of ALL has been supported by retrospective identification of leukemia-specific fusion genes, hyperdiploidy, or clonotypic rearrangements of immunoglobulin or T-cell-receptor loci in archived neonatal blood spots (Guthrie cards) and by the studies of leukemia in monozygotic twins (Pui, Robison et al. 2008). Analysis of cord blood cells established the presence of pre-leukemic *TEL-AML1 (ETV6-RUNX1)* clone with altered self renewal and survival properties (Hong, Gupta et al. 2008). But, the modest concordance rate for leukemia in identical twins (5%) and variable latency in the emergence of leukemia suggests that additional postnatal exposure and/or genetic alterations are required for clinically overt leukemia development. Screening of neonatal cord bloods demonstrated the incidence of 1% *TEL-AML1* fusion gene which is approximately 100 times greater than actual disease incidence further suggest the postnatal events in the course of disease development (Mori, Colman et al. 2002).

Another example of postnatal event in ALL development comes from the evidence that rearrangement of the *MLL* gene occurs not only in leukemias of infancy but also in leukemias induced by topoisomerase II inhibitory drugs. Wide range of synthetic and natural compounds including quinolone antibiotics, benzene metabolites, flavonoids present in food and drink, catechins, podophyllin resins, benzene metabolites and oestrogens all are the potential topoisomerase II inhibitors that act both *in vivo* and *in vitro*, and may induce mutations resulting to the development of acute leukemias with *MLL* rearrangements (Pui, Campana et al. 2001). A study has been done linking the exposure of DNA damaging drugs to the development of leukemias with *MLL* gene fusion (Alexander, Patheal et al. 2001). Thus, exposure of topoisomerase II inhibitory drugs to the fetus by different means such as, medical, dietary or environmental exposure and the inability of the fetus or their mother to detoxify these agents could enhance the susceptibility

of leukemia development (Pui, Relling et al. 2004, Pui, Robison et al. 2008) (Figure 2).

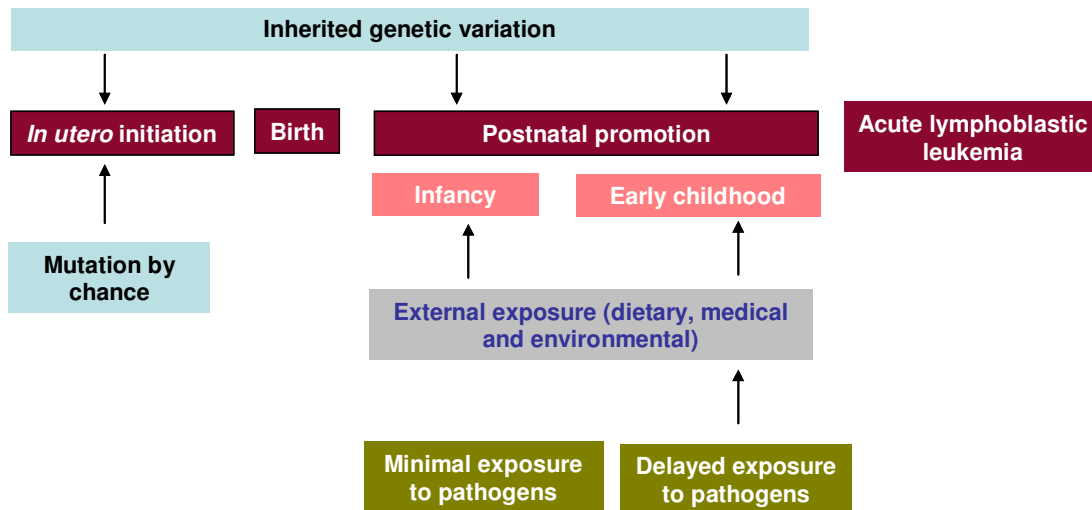


Figure 2: Models of overt leukemia development in childhood

Genetic predisposition, endogenous and exogenous exposure and mutation caused by chance contribute to childhood leukemia development.

1.1.2 Genetic basis of ALL

More than 90% ALL cases are associated with specific genetic alterations in the blast cells (Rubnitz and Pui 1997). Multiple genetic and epigenetic alterations of hematopoietic stem cells (HSCs) or progenitor cells lead to altered cellular differentiation, proliferation, self renewal and normal apoptotic activity resulting in the development of leukemia (Radtke, Mullighan et al. 2009). Advances in the cytogenetics and molecular characterization of ALL facilitated the identification of genetic alterations. About 80% of ALL harbor recurrent genetic alterations which are detected by fluorescence in situ hybridization (FISH), karyotyping and genome wide techniques such as microarrays and next generation sequencing (NSG) (Bacher, Kohlmann et al. 2010, Mullighan 2012). According to Pui (2008), being activated by translocations, transcription factor genes are developmentally regulated and in many cases control cell differentiation rather than cell division. These also frequently encode proteins at the apex of important transcriptional cascades. These master oncogenic transcription factors are expressed aberrantly

in leukemic cells as one gene product or as a unique fusion protein produced by the combination of two elements of two different transcription factors (Pui, Robison et al. 2008). In B cell precursor ALL these genetic alterations includes hypodiploidy with less than 44 chromosomes, hyperdiploidy with more than 50 chromosomes, rearrangements in chromosomes e.g. *ETV6-RUNX1* (*TEL-AML1*), *t(9;22)*, *BCR-ABL1*, *t(12;21)* *t(1;19)* *TCF3-PBX1* (*E2A-PBX1*), and rearrangement of *MLL* at 11q23 to a diverse range of fusion partners (Table 1).

Table 1: Major genetic alterations in B cell precursor acute lymphoblastic leukemia (BCP-ALL)

Genetic lesion	Frequency	Associated clinical relevance	Presumed mechanism of action
Hyperdiploidy (>50)	20%	FLT3 activation; good prognosis	Constitutive tyrosine kinase activation
Hypodiploidy (<44)	1%	Good prognosis	
<i>BCR-ABL</i> , <i>t(9,22)</i>	2%	Associated with IKZF1 or CDKN2A deletion, develops imatinib resistance, poor prognosis	Constitutive tyrosine kinase activation, interacts with RAS, JAK-STAT pathway
<i>MLL</i> rearrangements	6%	Poor outcome	Aberrant DNA and histone methylation
<i>ETV6-RUNX1</i> , <i>t(12,21)</i>	22%	Good prognosis	Parental translocation, chimeric transcription factor, AML1 target gene repression
<i>PAX5</i>	31.7%	Somatic mutation occurs most frequently, not associated with outcome	Impaired DNA binding and transcriptional activation, roles in leukemogenesis
<i>E2A-PBX1</i> <i>t(1,19)</i>	4%	Good prognosis	Chimeric transcription factor
<i>E2A-HLF</i>		Aggressive disease, hypercalcemia, disseminated intravascular coagulation	Dimerization of E2A protein, chimeric transcription factor with activation of anti-apoptotic Snail family zinc finger 2 protein

<i>CRLF2</i>	5-16% in pediatric and adult B-ALL, 50% in DS-ALL	F232C mutation, rearrangements-IGH@CRLF2, P2RY8-CRLF2 fusion gene, resulting CRLF2 over expression; poor outcome	Cause JAK-STAT and mTOR pathway activation
<i>IKZF1</i>	15% of pediatric patients, >80% in BCR-ABL cases	Focal deletion, sequence mutation; poor outcome	Transcription factor needed for lymphoid development, alterations result in functional loss or dominant negative isoforms
<i>JAK1/JAK2</i>	18-35% in Downs syndrome patients	Kinase domain mutation	Causes JAK-STAT pathway activation
<i>CREBP</i>	19% of relapsed B-ALL	Glucocorticoid resistance	Impaired histone acetylation and transcription regulation
<i>TP53</i>	12% in B-ALL cases	Deletions and sequence mutation, poor outcome	Functional loss, dominant negative
<i>IL7R</i>	7% in B-ALL and T-ALL cases	In-frame mutation in transmembrane domain	Receptor dimerization, constitutive IL7 signaling, JAK-STAT signaling

Collected from (Inaba, Greaves et al. 2013, Mullighan 2012, Pui, Relling et al. 2004, Teitell and Pandolfi 2009).

1.1.3 Diagnosis and risk stratification of ALL

Much of the improved treatment outcomes of ALL patients are the result of proper early diagnosis of the disease. During the last two decades the diagnosis of acute lymphoblastic leukemia has been emerged as a bundle of different methods rather than cytomorphology alone (Haferlach, Kern et al. 2005). Although many ancillary laboratory techniques have emerged for the diagnosis of acute leukemia, still morphological examination and cytochemical staining of expertly prepared air-dried blood smears of peripheral blood or bone marrow are critical in the diagnosis and classification of leukemia (Han and Bueso-Ramos 2005). The size of the most typical lymphoblast is small to intermediate with oval or round nucleus containing

dispersed nuclear chromatin, small or absent nucleoli, and small cytoplasm (Kebriaei, Anastasi et al. 2002). Careful morphological examination allows early decision making to adopt certain diagnostic procedure and transport of the bone marrow specimen to the appropriate laboratory to perform further diagnostic work (Han and Bueso-Ramos 2005).

Immunophenotyping of the leukemic blasts by flow cytometry is essential to validate the morphological evaluation of the ALL and to establish correct diagnosis and cell lineage identification. By immunophenotyping ALL can be easily sub-classified into many steps of B and T cell differentiation, but only prognostically important are T-cell, mature B-cell and B-cell precursor phenotype. Chromosomal analysis is an integral part of initial diagnostic work-flow of acute lymphoblastic leukemia. Now, other highly specific and sensitive techniques, for examples, real time PCR, fluorescence in situ hybridization (FISH) and flow cytometry are increasingly used to detect specific fusion transcript, gain or loss of cellular DNA content or specific chromosomal alterations associated with prognostic and therapeutic relevance. Gene expression profiling not only can identify major subtypes of acute lymphoblastic leukemia but also points to single gene or even signaling pathways which is an important determinant of clinical outcome (Pui, Robison et al. 2008).

When first cooperative clinical trials of childhood acute lymphoblastic leukemia were established in the 1950s, all children were treated equally. Analysis of successive trials and registry data led to the identification of infants with unfavorable prognosis aged less than a year indicating the importance of certain clinical features at diagnosis in the prognosis (Kotecha, Gottardo et al. 2014). Today, stratification of the patients into different therapy regimen based on the individual risk for relapse using clinical, biological and treatment response is a common strategy for the treatment of ALL using contemporary treatment protocols. Patients are stratified into different risk-groups (e.g. low-risk, standard-risk and high-risk) based on their individual risk for relapse. According to Carroll (2003), contemporary therapy regimen focused on the intensification of the established therapeutic agents rather than the use of new drugs. Despite this improvement, many children experience poor outcome while many are being over

treated. Current risk-adapted therapy has facilitated augmenting the intensity of the therapy that will be benefited by the intensification while using the standard intensity to those who will be cured without the intensification thereby reducing the toxic side effects. Currently, all the pediatric patients with leukemias are being treated according to the risk-adapted treatment protocol (Carroll, Bhojwani et al. 2003).

1.1.4 Clinical features of acute lymphoblastic leukemia (ALL)

Historically, many clinical variables including age, sex, race, white blood cell (WBC) count, platelet count, hemoglobin, degree of organomegaly and presence of mediastinal mass or extramedullary disease have been used in risk stratification (Teachey and Hunger 2013). Generally, age and the WBC count at diagnosis are the most important clinical factors in predicting risk for relapse (Carroll, Bhojwani et al. 2003). Infants of age < 1year and children > 10 years associated with worse prognosis (Smith, Arthur et al. 1996) (Figure 3). An inverse relationship between age and prognosis exists during infancy with survival of the infants under 3 months is half of the infants 9-12 months old (Teachey and Hunger 2013).

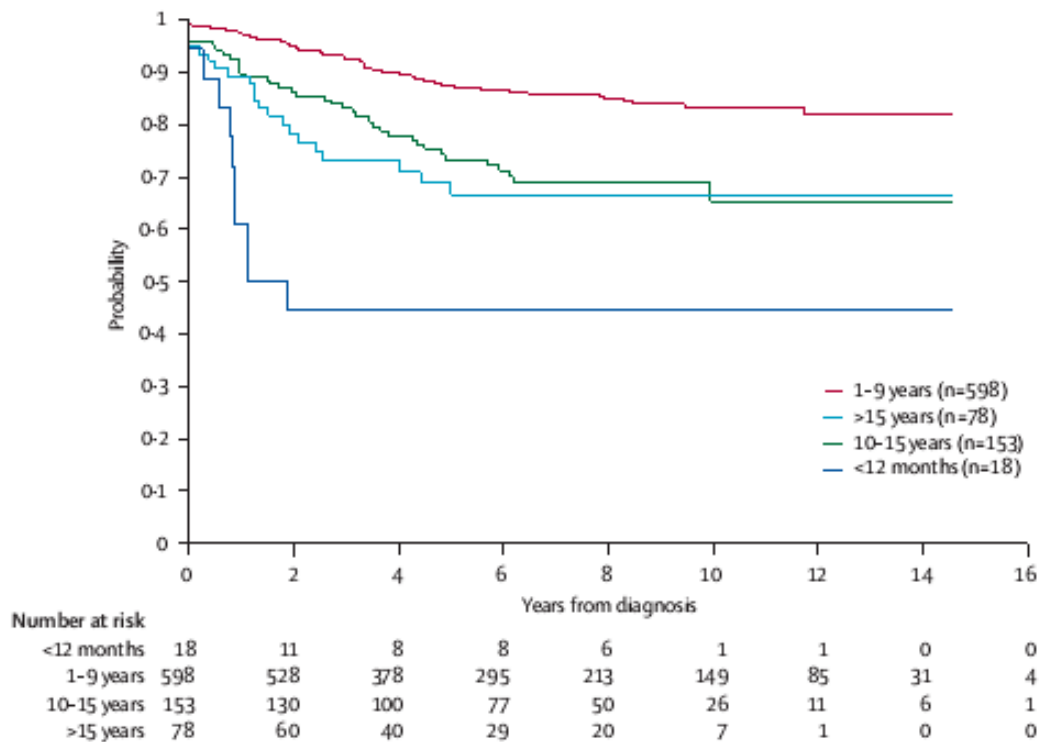


Figure 3: Age in predicting risk for relapse (Pui, Robison et al. 2008, page 1035)

Kaplan-Meier survival curve estimating event free survival (EFS) of children with ALL according to the age at diagnosis. Reprinted with permission from Elsevier.

Leukocyte count at diagnosis is another variable with increasing count associated with poor prognosis (Pui, Robison et al. 2008). An international consensus conference divided leukemia patients into two groups according to their leukocyte count and age at diagnosis. WBC count with $< 50 \times 10^9/l$ and aged between 1.00 to 9.99 years belong to the standard risk (SR) group while WBC count $\geq 50 \times 10^9/l$ and/or age ≥ 10.00 years are in the high-risk (HR) group (Teachey and Hunger 2013). These variables can be measured in all circumstances reliably points to the obvious advantage of these variables as a prognostic marker. However, there is very little prognostic relevance in case of T-ALL. Other clinical factors such as race, gender, obesity proved irrelevant in the prognosis as no concrete data supported their relevance in prognosis (Pui, Robison et al. 2008).

1.1.5 Biological factors influences prognosis

Several molecular and cytogenetic factors such as chromosomal ploidy (the number of chromosomes of the ALL), pattern of the specific gene expression and mutation and deletions of the individual gene are of critical importance in the prognosis. Hypodiploidy (chromosome number < 46) or near haploidy (chromosome number 23-29) is associated with poor prognosis with 20-30% survival rate (Margolin 2011). Generally *BCR-ABL* gene fusion also known as Philadelphia positive (Ph+) ALL, t(4;1) translocation with *MLL-AF4* fusion and hypodiploidy (less than 44 chromosomes) all confer to the poor outcome. On the other hand hyperdiploidy (more than 50 chromosomes), *ETV6-RUNX1* fusion, *E2A-PBX1* fusion and trisomy 4, 10 and 17 are associated with favorable prognosis (Pui, Robison et al. 2008). In recent times it has been shown that *IKZF1* deletion and alterations in *CRLF2* are associated with poor patient outcome (Attarbaschi, Morak et al. 2012, Van Der Veer, Zaliouva et al. 2014).

1.1.6 Response to treatment

In addition to the clinical and biological factors prednisone response (PR) is an important prognostic variable which is defined by the cytorreduction (rapid morphological clearance of blasts in peripheral blood) to a 7 days prednisone pre-phase and 1 intrathecal dose of methotrexate on day 1. Less than 1000 blasts/ μ l blood is associated with prednisone good response (PGR) while blasts \geq 1000/ μ l blood is associated with prednisone poor response (PPR) (Dordelmann, Reiter et al. 1999). Several studies have demonstrated the prognostic importance of prednisone response which is used for almost two decades as a tool for risk stratification (Campana 2012, Dordelmann, Reiter et al. 1999, Miller, Coccia et al. 1989, Miller, Leikin et al. 1983).

After about 20 years of development and several prospective clinical trials, the detection of minimal residual disease (MRD) has been emerged as a state-of-art tool to assess the initial therapy response of childhood as well as adult acute lymphoblastic leukemia. Although several methods for MRD detection are available, for ALL two are widely used. One method uses cytometry to detect aberrant expression of leukemia specific antigens while other utilizes quantitative

PCR to detect specific rearrangement of the TCR or immunoglobulin (Ig) gene (Schrappe 2012). According to the MRD, detection of residual leukemia cells at the end of induction therapy indicates poor prognosis and high-risk for relapse as shown by several studies (Conter, Bartram et al. 2010, Flohr, Schrauder et al. 2008, Silverman and Sallan 2003, Steinherz, Gaynon et al. 1996).

However, despite all these efforts in the stratification of patients into different therapy regimens based on individual risk for relapse, the majority of relapsed patients were initially stratified into non-high risk group. In the ALL-BFM 90 study, patients were stratified into standard risk (SR), medium risk (MR) and high risk (HR) according to leukemic cell mass estimate and treatment response but about 67% of relapsed patients were stratified in the non-high risk (non-HR) group (Schrappe, Reiter et al. 2000) (Table 2). In the AIEOP BFM-ALL 2000 study patients were stratified according to MRD criteria but still more than two-thirds of the relapsed patients were stratified into non-high risk group (Conter, Bartram et al. 2010) (Table 3).

Table 2: Treatment outcome of ALL-BFM 90 study (Schrappe, Reiter et al. 2000)

CCR, complete clinical remission

	all patients	CCR	total relapse	relapse (% of total)	% of relapse	% of relapse (non-HR/HR)
Total	2178	1687	385	17.7	100	
SR	636	545	75	11.8	19.5	67.3
MR	1299	1058	184	14.2	47.8	
HR	243	84	126	51.9	32.7	32.7

Table 3: Outcome of AIEOP-BFM ALL 2000 study (Conter, Bartram et al. 2010)

CCR, complete clinical remission

	all patients	CCR	total relapse	relapse (% of total)	% of relapse	% of relapse (non- HR/HR)
Total	3184	2729	387	12.1	100	
MRD-SR	1348	1267	61	4.5	15.8	84.5
MRD-IR	1647	1359	266	16.2	68.7	
MRD-HR	189	103	60	31.7	15.5	15.5

1.1.7 Current therapy and outcome

Intensification of multiagent chemotherapy regimens and advances in supportive care have resulted improved long-term survival of ALL patients with approximately 80% cure rate (Conter, Arico et al. 2010, Pui and Evans 2006, Pui and Jeha 2007, Salzer, Devidas et al. 2010) (Figure 4). But still 20% of ALL patients encounter relapse associated with poor prognosis especially if occurring early (Henze, Fengler et al. 1991). Treatment of acute lymphoblastic leukemia typically consists of remission-induction therapy, an intensification (consolidation) phase followed by continuation therapy to eradicate residual disease. CNS directed therapy is also given early in the clinical course to prevent relapse with leukemic cells sequestered into the CNS (Pui, Robison et al. 2008).

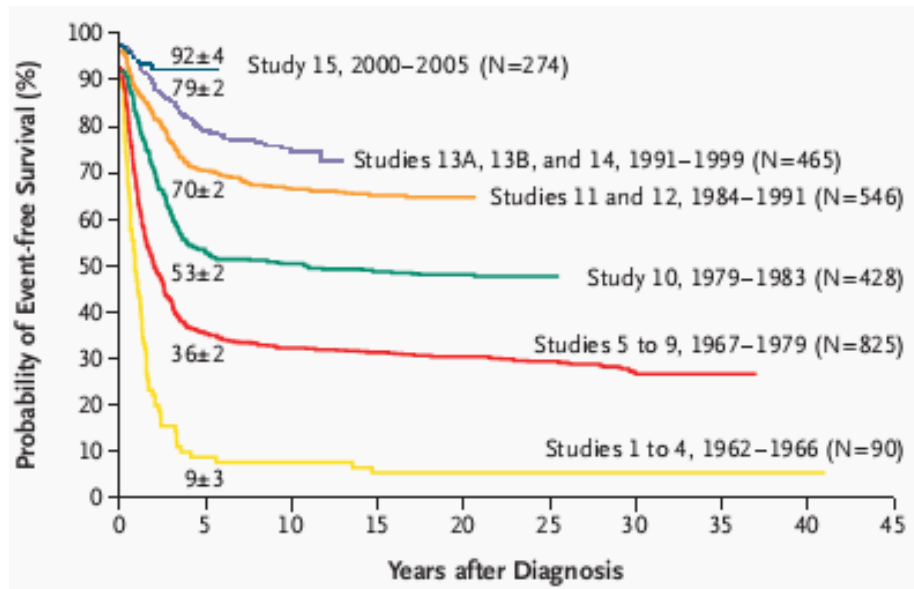


Figure 4: Cure rate of ALL patients during the period of 1962-2005 (Pui and Evans 2006, page 169)

5 year event-free survival in 2628 newly diagnosed ALL patients participated in the St. Jude Children's Research Hospital from 1962 to 2005. Reprinted with permission from (scientific reference citation), Copyright Massachusetts Medical Society.

The goal of remission-induction therapy is to eradicate more than 99 percent of the initial leukemia burden thereby restoring normal hematopoiesis and performance. In most of the studies the goal is achieved in approximately 98% of the patients at the end of remission-induction therapy (Carroll and Raetz 2012, Stanulla and Schrappe 2009). Remission-induction therapy is followed by the consolidation/intensification therapy. The primary goal of the consolidation/intensification therapy is to eradicate the residual leukemic blasts in the patients who have achieved remission. For instance, patients with *ETV6-RUNX1* fusion gene have good outcome in clinical trials of intensive post-remission therapy with corticosteroids, vincristine and asparaginase (Pui, Robison et al. 2008). Without consolidation/intensification therapy, patients achieved remission usually encounter relapses within few months (Stanulla and Schrappe 2009).

As described by Stanulla (2009), for the successful treatment of childhood ALL, CNS directed therapy has emerged as a prerequisite. Before introduction of the CNS directed therapy in the 1960s, more than 50% childhood ALL patients suffered disease recurrence originating from the CNS. The introduction of cranial irradiation, intrathecal chemotherapy with methotrexate alone or in combination with other drugs (cytarabine, hydrocortisone), and systemic application of chemotherapeutics with adequate penetration into the CNS can reduce this recurrence rate to less than 5% (Stanulla and Schrappe 2009). Generally, ALL patients require prolonged continuation treatment due to reasons which is not understood fully (Pui and Evans 2006). In theory, maintenance therapy aims at further stabilization of the remission suppressing the reemergence of resistant clone by continuously reducing the pool of residual leukemic blasts (Stanulla and Schrappe 2009).

1.1.8 Future perspectives/treatment with novel agents

With the advances in the technologies our knowledge of the unique biology of the ALL reaches more precision; future treatment strategies may include additional drugs targeting specific signaling pathways. Many drugs that target important cellular pathways are currently investigated in the clinical trials such as tyrosine kinase inhibitors (TKI), gamma secretase inhibitor, the proteasome inhibitor bortezomib, mammalian target of rapamycin (mTOR) inhibitors such as sirolimus and temsirolimus (Carroll and Raetz 2012). Certain specific ALL patient groups may benefit from these targeted therapies for instance Ph+ ALL. In recent years, patients with Ph+ ALL receiving tyrosine kinase inhibitor imatinib in combination with chemotherapy experienced superior event free survival without any appreciable increase in toxicity compared to patients received no imatinib (Biondi, Schrappe et al. 2012, Schultz, Bowman et al. 2009). Other genetic alterations with potential drug targets are shown in table 4.

Table 4: Genetic alterations with potential drug targets (Harrison 2013)

Abnormality	Agents	Mode of action
<i>BCR-ABL</i> like		
<i>ABL1</i> partner genes	Imatinib or derivatives	Tyrosine kinase inhibitor
<i>PDGFRB</i> partner	Imatinib or derivatives	Tyrosine kinase inhibitor
<i>CRLF2</i> rearrangement	Ruxolitinib	JAK inhibitor
Others		
<i>CREBP</i>		
Mutations in Ras/RTK and PI3K pathway genes	Rapamycin	mTOR inhibitor
<i>MLL</i> rearrangements	EPZ2676, lestaurtinib, PKC412	Histone methyltransferase inhibitor, FLT3 kinase inhibitor

1.2 mTOR pathway

The target of rapamycin (TOR) is a 289-KDa evolutionary conserved serine threonine kinase that forms the catalytic core of two distinct functional complexes; TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Foster andingar 2010, Laplante and Sabatini 2009). These two complexes contains number of proteins with some of them are shared by TORC1 and TORC2 and control a myriad of cellular processes in response to diverse environmental stimuli (Foster andingar 2010). mTORC1 is constituted by five distinct molecules that include mTOR, the catalytic subunit of the complex; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL); proline-rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) (Laplante and Sabatini 2009). On the other hand mTOR complex 2 (mTORC2) is composed of mTOR, the rapamycin-insensitive companion of mTOR (RICTOR), mSin1, Protor (protein observed with Rictor), and mLST8 (Oh and Jacinto 2011). mTORC2 is resistant to rapamycin and its related signaling

mechanism is less understood although it appears to regulate metabolism and survival through activation of AKT, as well as mTORC1, through protein kinase C (PKC), and also plays a role in cytoskeletal organization (Hutt-Cabezas, Karajannis et al. 2013, Oh and Jacinto 2011, Yang, Yang et al. 2008).

1.2.1 Upstream regulation of mTORC1

A variety of upstream signals, cytokines, growth factors, amino acids, mitogens and cell stress regulate the mTORC1 activity. mTORC1 regulation by upstream signaling occurs primarily by two mechanisms: the direct modification of mTORC1 components or the regulation by the small GTPase, RHEB (ras homologue enriched in brain) which directly interacts with and activates mTORC1 in its GTP bound form (Sengupta, Peterson et al. 2010). How different upstream signaling affects mTORC1 function described below.

1.2.1.1 Insulin/PI3K signaling

The insulin/PI3K pathway is one of the best characterized mTOR pathway activator. The binding of insulin to its receptor stimulates autophosphorylation and results in the activation of the insulin receptor intrinsic tyrosine kinase, leading to the recruitment and phosphorylation of members of the insulin receptor substrate (IRS) protein family (IRS1–4), which recruit and activate the PI3K (Magnuson, Ekim et al. 2012, Saltiel and Pessin 2002). According to Courtney (2010), PI3K phosphorylates PIP_2 on the 3'OH position to produce $PI(3,4,5)P_3$. The tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a negative regulator of PI3K signaling which dephosphorylates PIP_3 to PIP_2 , resulting in the termination of PI3K-dependent signaling (Courtney, Corcoran et al. 2010). PIP_3 propagates intracellular signaling by directly recruiting various signaling proteins to the membrane where they are activated (Cantley 2002). Two pleckstrin homology (PH) domain-containing serine/threonine kinases, phosphoinositide-dependent kinase 1 (PDK1) and AKT are brought into close proximity by $PI(3,4,5)P_3$ (Courtney, Corcoran et al. 2010). PDK1 activates AKT by phosphorylating at threonine 308 whereas mTORC2 activates AKT by phosphorylating at serine 473 (Courtney, Corcoran et al. 2010, Magnuson, Ekim et al. 2012). PI3K-AKT signaling promotes cell growth and survival by several

mechanisms. As described by Courtney (2010), AKT promotes cell survival by inhibiting proapoptotic Bcl-2 family members BAD and BAX. AKT also disrupts the negative regulation of the transcription factor NF- κ B, resulting in increased transcription of anti-apoptotic and prosurvival genes. AKT also phosphorylates Mdm2 to antagonize p53-mediated apoptosis, and additionally AKT negatively regulates forkhead transcription factors, resulting in reduced production of cell death-promoting proteins (Courtney, Corcoran et al. 2010). Activated AKT then phosphorylates TSC2 (tuberous sclerosis) in several sites disrupting its interaction with TSC1 resulting in the suppression of inhibitory effect of TSC1-TSC2 complex on mTORC1 thereby activates mTORC1 signaling (Inoki, Li et al. 2002) (Figure 5).

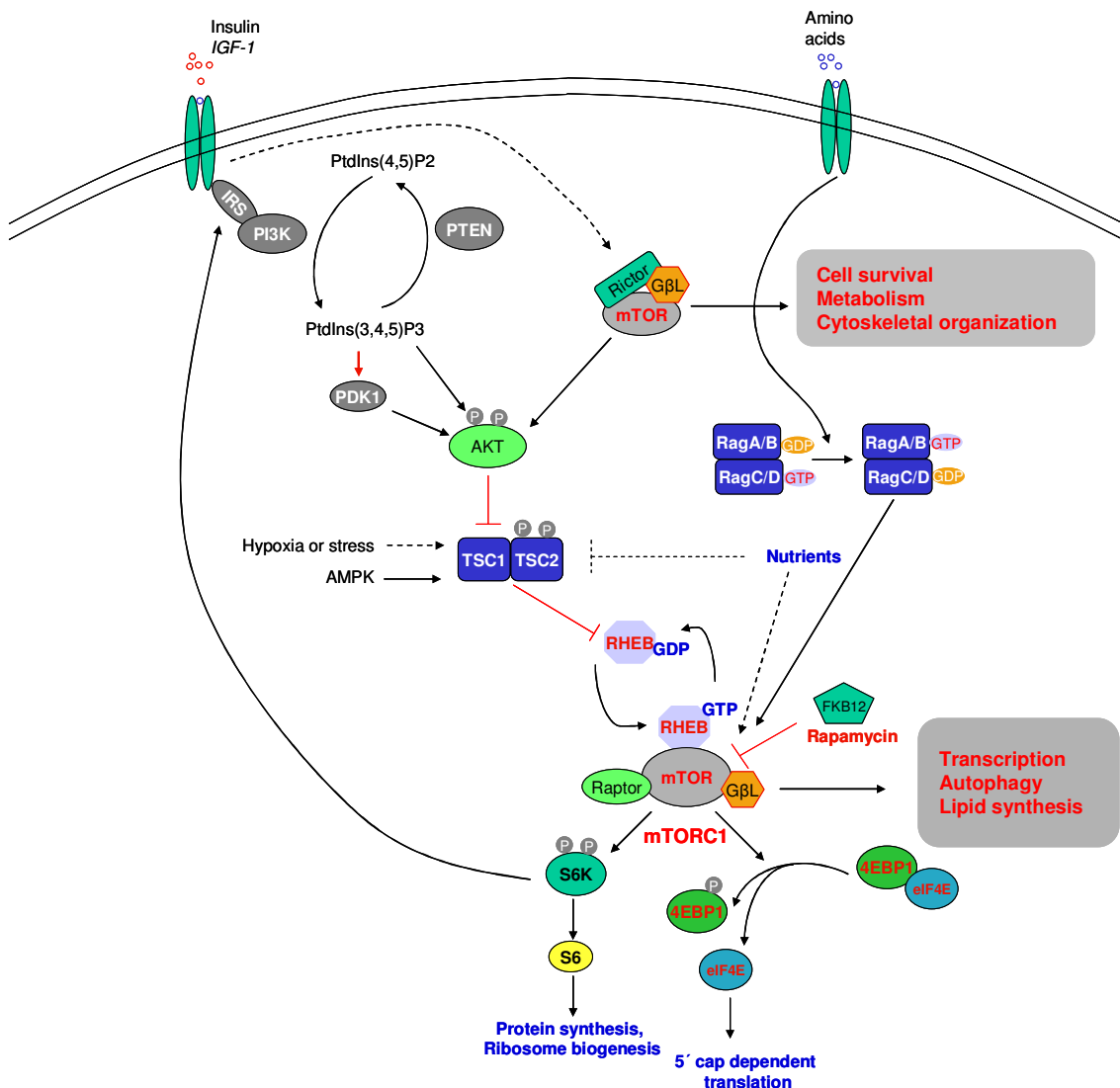


Figure 5: The mammalian target of rapamycin (mTOR) pathway is activated by PI3K-AKT signaling

Growth factors bind to the receptor to recruit phosphatidylinositol 3-kinase (PI3K) to the membrane. At the membrane, PI3K generates PtdIns(3,4,5)P₃ (PIP₃) by phosphorylating PtdIns(4,5)P₂. The tumor suppressor PTEN, a lipid phosphatase, negatively regulates PI3K by dephosphorylating PIP₃. Active PDK triggers the activation of S6K1 and AKT kinases which are key effectors of cell proliferation and survival pathway. Activated AKT phosphorylates TSC to disrupt the TSC1-TSC2 complex which in turn allows the RHEB to activate mTORC1. Activated mTORC1 has two main downstream targets, S6K1 and 4EBP1, the inhibitor of cap dependent translation. Upon activation by mTORC1, S6K1 induces translation and ribosomal biosynthesis. Additionally, S6K1 also acts as a negative feedback activator of PI3K-AKT-mTOR pathway by down-regulation of insulin receptor substrates 1 (IRS-1). mTORC1 phosphorylates 4EBP1 to initiate cap dependent translation.

1.2.1.2 Ras/MAPK signaling

The mitogen-activated Ras/MEK (MAPK/ERK kinase) signaling cascade activates mTOR by converging on TSC1/TSC2 independent of the insulin/PI3K/AKT pathway (Magnuson, Ekim et al. 2012). Mitogen stimuli or oncogenic Ras stimulates the Raf-Ras1/2-Erk1/2 signaling cascade leading to phosphorylation of TSC in multiple sites (Ser540, Ser644 and Ser1789) and subsequent functional inactivation of TSC1-TSC2 complex resulting in RHEB mediated mTORC1 activation (Ma, Chen et al. 2005). Also, the Ras/MAPK pathway activation phosphorylates rapamycin, on proline directed sites (Ser8, Ser696, and Ser863) to activate mTORC1. Raptor is a scaffolding protein that directly binds to mTOR and recruits mTORC1 substrates (Carriere, Cargnello et al. 2008, Carriere, Romeo et al. 2011). Thus, mTORC1 can be activated by Ras/MAPK signaling pathway that phosphorylates TSC2 or raptor, independent of PI3K-AKT pathway activation.

1.2.1.3 Phospholipase D (PLD) signaling

Emerging evidences suggest the involvement of PLD and its product phosphatidic acid (PA) in the regulation of mTOR pathway activity. Phosphatidic acid (PA), a

lipid second messenger produced by PLD mediated hydrolysis of phosphatidylcholine, participates in various intracellular signaling events and regulates a number of signaling proteins, including many phosphatases and protein kinases (English, Cui et al. 1996). PA activates the mTOR signaling by binding to the FKBP12-rapamycin binding (FRB) domain of mTOR protein (Liu, Kach et al. 2013). It has been reported that, PLD plays essential role in the formation of mTORC1 and mTORC2 complexes and its activation (Toschi, Lee et al. 2009). Interestingly rapamycin-FKB12 competes with PA for the binding with mTOR and much higher rapamycin concentration is required for PA-mTORC2 than PA-mTORC1 competition (Magnuson, Ekim et al. 2012, Toschi, Lee et al. 2009). This phenomenon also explains the rapamycin insensitivity of mTORC2 in comparison to mTORC1.

1.2.1.4 Cytokine signaling

Cytokines and growth factors initiate mTOR pathway activity mainly through PI3K/AKT signaling activity (Chiarini, Evangelisti et al. 2014). Interleukin-22 (IL22, a cytokine of IL-10 family) induced proliferation of normal human epidermal keratinocytes (NHEK) cells through PI3K-AKT-mTOR pathway (Mitra, Raychaudhuri et al. 2012). However, inflammatory cytokine such as TNF α activates mTOR pathway in AKT independent manner in certain cells and some cancer cells (Chen, Liu et al. 2010, Dan and Baldwin 2008). TNF α initiates mTORC1 signaling by activating IKK β that phosphorylates TSC1 resulting in TSC1-TSC2 dissociation, mTORC1 activation and insulin resistance (Lee, Kuo et al. 2007).

1.2.1.5 Nutrients regulate mTORC1

Amino acids are the building blocks of proteins that promote cell growth by the synthesis of nucleic acid, glucose and ATP and are absolutely required for mTORC1 activity and can not be compensated by other stimuli (Zoncu, Efeyan et al. 2011). In vertebrates amino acids are sensed intracellularly by the bidirectional amino acid permease SLC7A5-SLC3A2 (SLC, solute carrier family), a system essential for mTORC1 activity (Magnuson, Ekim et al. 2012). The system imports leucine, a key amino acid for mTORC1 activity across the plasma membrane into the cell (Zoncu, Efeyan et al. 2011). Although it is unclear by which biochemical

mechanisms cells sense amino acids, several candidate mediators downstream of amino acids have emerged, including hVPS34, a class 3 lipid kinase known to function in vacuolar sorting and autophagy in yeast, the MAP43K (MAPK kinase kinase 3), the Rag GTPase and the RalA GTPase (Magnuson, Ekim et al. 2012).

1.2.2 Downstream cellular processes of mTORC1

Among many functions protein synthesis is far most the best-characterized process, controlled by mTORC1. mTORC1 plays a very important role in translation control by phosphorylating a number of translation regulators that include 4E(elf4E)-binding protein 1 (4EBP1) and p70S6K1 (Yuan, Xiong et al. 2013). By sensing sufficient nutrients and growth factors, mTORC1 activates S6 kinase activity which phosphorylates ribosomal protein S6, a component of 40s ribosomal subunit. Ribosomal protein S6, being phosphorylated facilitates recruitment and translation of specific mRNA containing a 5'-polypyrimidine tract (5'-TOP) (Carrera 2004). Additionally, both the initiation and elongation phases of translation are also regulated by mTORC1 through phosphorylation of 4EBP1 (Bahrami, Ataie-Kachoei et al. 2014). Phosphorylation of 4EBP1 disrupts its association with cap binding protein eIF4E, thereby releasing it to take part in the formation of eIF4F complex which is required for cap-dependent translation initiation (Laplanche and Sabatini 2012). In addition to protein synthesis, activated mTORC1 plays important role in cell cycle progression, cellular growth, cell motility, gene transcription, adipose differentiation, synaptic plasticity and homeostasis (Bahrami, Ataie-Kachoei et al. 2014) (Figure 6).

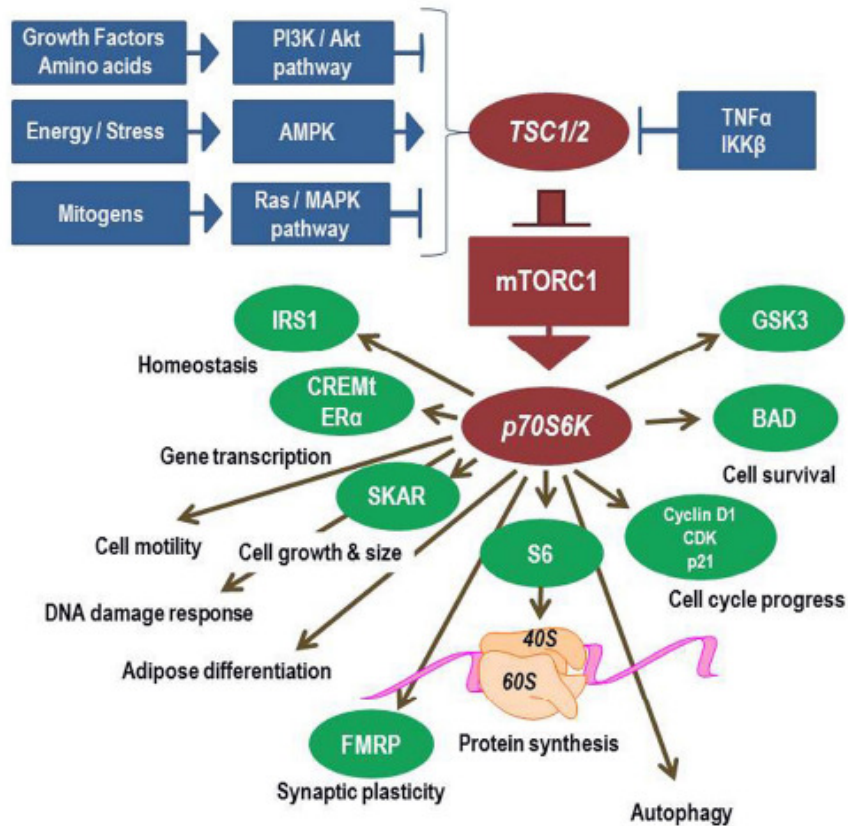


Figure 6: Downstream targets of mTOR C1 (Bahrami, Ataie-Kachoei et al. 2014, page 1021)

mTORC1 regulates a number of cellular processes through the phosphorylation of several proteins. S6K and 4EBP1 is the best-characterized mTORC1 substrate which performs the translation. In addition, S6K promotes a variety of cellular processes. Reprinted with permission from BMJ Publishing Group Ltd.

1.3 NOD/SCID mouse xenograft model for leukemia research

The inability of primary leukemia cells to be propagated *in vitro*, has prompted the scientists to develop a model for leukemia transplantation into mice and progression of leukemia recapitulating many clinical features of the human disease. About 30 years earlier, discovery of the $Prkdc^{scid}$ (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency, abbreviated scid) mutation in CB17 mice led to the successful transplantation (Bosma, Custer et al. 1983). As described by Dick (1992), the homozygous SCID mutation carried on chromosome 16 prevents the production of mature B and T lymphocytes

whereas all other hematopoietic lineages including hematopoietic cells, natural killer (NK) cells, and myeloid progenitor cells are normal. The gene product does not impair normal lymphoid development and although susceptible for infection, the mice harboring SCID mutation has a normal life span and appears normal in all other aspects (Dick 1992). The developed SCID mouse allowed the engraftment of leukemia cells recapitulating the clinical features of the disease without graft rejection (Cesano, O'Connor et al. 1991, Kamel-Reid, Letarte et al. 1989). However, limited engraftment occurred in the SCID mice due to the presence of the adaptive immune system, as known as leakiness, and this phenomenon increases with the increased age of the mice.

The next advance of mouse model was the development of nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice. In 1980 the nonobese diabetic (NOD) strain of mouse was developed in Japan after sib mating of the mice displaying severe glucosuria (Makino, Kunitomo et al. 1980). As described by Shultz (1995), NOD-SCID mice were developed by crossing the mice harboring a SCID mutation with the mice harboring a NOD mutation resulting in more immunodeficient strain due to the multiple defects in adaptive as well as innate immunity resulting from the absence of T cells, lack of functional lymphoid cells and absence of immunoglobulin in the serum with the increase of the age. Additionally, it maintains insulinitis- and diabetes-free state throughout the life (Shultz, Schweitzer et al. 1995). The resulting NOD-SCID strain exhibited superior engraftment over the SCID mice and has been widely used as xenotransplantation model for leukemia (Lock, Liem et al. 2002, Nijmeijer, Mollevanger et al. 2001, Shultz, Schweitzer et al. 1995).

The third breakthrough was the development of NOD/LtSz-scid (NOD/SCID/IL2R γ null, NSG) mouse strain lacking natural killer (NK) cells due to the additional homozygous mutation at the interleukin-2 receptor (IL2-R) γ -chain locus resulting in almost complete absence of the host immunity (Meyer and Debatin 2011, Shultz, Lyons et al. 2005). In addition to this, mature NSG mice survive more than 16 months and do not develop lymphoma even after sublethal irradiation (Shultz, Lyons et al. 2005). The NSG mice were developed by back cross matings of C57BL/6J γ_c^{null} and NOD/shi-scid mice (Ito, Hiramatsu et al.

2002). NSG mice exhibited higher engraftment rate of leukemia samples compared to NOD/SCID mice as shown by different studies (Agliao, Martin-Padura et al. 2008, Diamanti, Cox et al. 2012, Sanchez, Perry et al. 2009).

However, despite the development of more immunodeficient mice, the successful transplantation and the overall engraftment depend not only on the chosen mouse strain but also influenced by a number of factors e.g. (i) source and preconditioning of the leukemic cells, (ii) conditioning of the recipient animal and transplantation procedure, (iii) the recipient and (vi) the definition of leukemia manifestation (Figure 7) (Meyer and Debatin 2011).

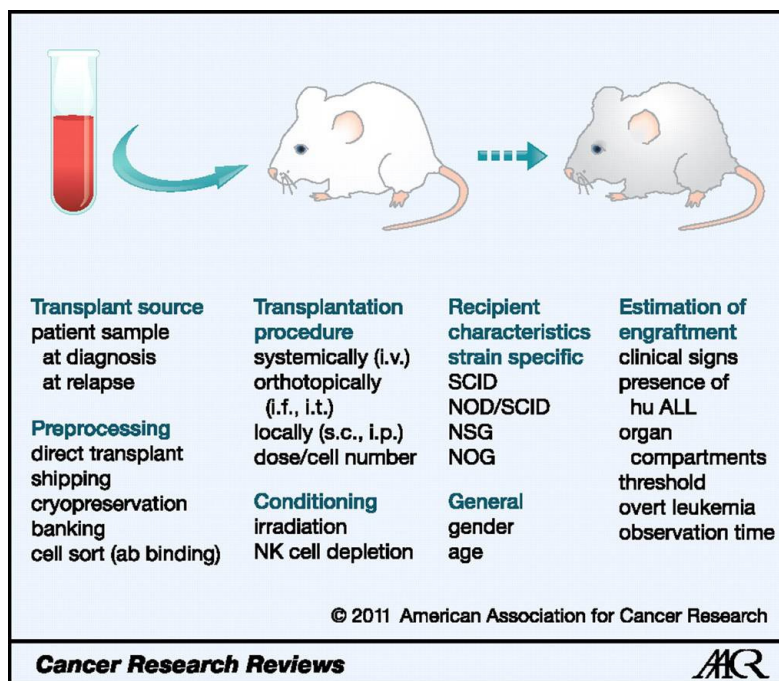


Figure 7: Factors influence the leukemia engraftment into recipient animal (Meyer and Debatin 2011, page 7142)

Primary patient materials are transplanted into recipient animals by different methods. Several factors of the patient material, recipient animal as well as the criteria set to engraftment monitoring influence the leukemia engraftment. Reprinted with permission from American Association of Cancer Research.

Mice as an animal model have been playing a tremendous important role in biomedical research since its first discovery in 1960s. In leukemia research xenotransplantation of leukemic cells into immunodeficient mouse circumvented the bottleneck of limited primary cell proliferation *in vitro*. Xenotransplantation of leukemia cells onto immunodeficient mouse leads to leukemia manifestation recapitulating the human leukemia in the recipient animal thereby providing great opportunity for functional analysis of primary leukemia cells and also preclinical evaluation of novel therapies *in vivo*.

1.4 Aim of the study

Previously, we investigated engraftment properties in a series of primary patient ALL samples transplanted onto NOD/SCID mice and described a short time to leukemia manifestation (time to leukemia short, TTL^{short}) and a longer time to leukemia manifestation (time to leukemia long, TTL^{long}) in the recipient animals. Patients associated with TTL^{short} phenotype had poor patient outcome indicating strong impact of TTL phenotypes for early relapse prognostication (Meyer, Eckhoff et al. 2011). Importantly, this engraftment phenotype is characterized by a specific gene expression profile pointing to pathways regulating cellular growth and proliferation. In particular, this gene signature shows low expression of molecules inhibiting mTOR and high transcript levels of modulators activating mTOR in TTL^{short} signature suggesting increased mTOR signaling activity in this high-risk ALL subgroup.

It is clear that novel therapies targeting key pathways that promote leukemogenesis are necessary to improve the leukemia patient outcome. Based on our previous findings, the aim of this study was:

- (i) Investigation of this key survival pathway functionally by evaluating the constitutive activity of the mTOR pathway.
- (ii) Study the effect of mTOR pathway inhibition using *in vitro*, *ex vivo* and *in vivo* settings.
- (iii) To investigate the mTOR pathway inhibition alone and also in combination with chemotherapy in an *in vivo* preclinical model setting.

2. Material and Methods

2.1 Material

2.1.1 Cell lines

G40	Kindly provided by Dr. Andrew Westhoff, Ulm
Jurkat	DSMZ, Germany
KOPN-8	DSMZ, Germany
Nalm-6	DSMZ, Germany
Primary patient materials and xenografts	Ulm University, Germany

2.1.2 Reagents, chemicals and kits

7-Aminoactinomycin D (7-AAD)	EMD Chemicals, USA
Annexin V	Roche Diagnostics GmbH, Germany
Biocoll Separating Solution	Biochrom GmbH, Germany
Aqueous Mounting medium	Dako North America, Inc., USA
Citrate	Sigma Aldrich, Germany
Chemiluminescence detection kit	Thermo Scientific, Germany
Dako Real Detection System	Dako North America, Inc., USA
Dimethyl Sulfoxide (DMSO)	Serva, Germany
Ethanol	VWR International, Germany
Erythrocyte lysis buffer	Becton Dickinson, Germany
FCS	Gibco Life Technologies, Germany
FACS Flow	Becton Dickinson, Germany
FACS Clean	Becton Dickinson, Germany
FACS Rinse	Becton Dickinson, Germany
HBSS	Gibco Life Technologies, Germany
Glycine	Sigma Aldrich, Germany
Heparin	Ratiopharm, Germany
Hydrochloric Acid	Sigma Aldrich, Germany
Ki67	Becton Dickinson, Germany

L-glutamine 200 mM	Gibco Life Technologies, Germany
Mayer's Hematoxylin	Dako North America, Inc., USA
Methanol	VWR International, Germany
Normal Goat Serum (NGS)	Sigma Aldrich, Germany
NVP-BEZ235	LC laboratories, USA
Paraformaldehyde 16%	Dako North America, Inc., USA
PBS	Gibco Life Technologies, Germany
Penicillin/Streptomycin	Gibco Life Technologies, Germany
Popidium Iodide (PI)	Sigma Aldrich, Germany
Rapamycin	LC laboratories, USA
RNase A	Gibco Life Technologies, Germany
SALSA MLPA P335-A3 ALL- IKZF1 probemix (MLPA kit)	MRC, Holland
Saponin	Sigma Aldrich, Germany
SDS	Sigma Aldrich, Germany
Sodium Azide	Sigma Aldrich, Germany
Sodium Chloride	Sigma Aldrich, Germany
Sodium Hydroxide	Sigma Aldrich, Germany
Staurosporine	Sigma Aldrich, Germany
Tris Base	Sigma Aldrich, Germany
Triton X	Sigma Aldrich, Germany
Trypan Blue	Sigma Aldrich, Germany
Xylol	VWR International, Germany

2.1.3 Reagents used for protein electrophoresis and western blot analysis

Ammoniumpersulfate	Serva, Germany
BCA Protein Assay Reagent Kit	Thermo Scientific, Germany
β -Glycerophosphate	Sigma Aldrich, Germany
Bovine Serum Albumin	PAA, Austria
DTT	Sigma-Aldrich, Germany
Enhanced Chemiluminescence	Thermo Scientific, Germany
Hybond ECL Nitrocellulose membrane	GE Healthcare, Germany
Hyperfilm ECL	GE Healthcare, Germany
Milk powder	Roth, Germany
Polyacrylamid Rotiporese 30	Merck KGaA, Germany
Protease inhibitor cocktail	Roche Diagnostics, Germany
Protein Marker PageRuler	Thermo Scientific, Germany
Sodiumorthovanadate	Sigma-Aldrich, Germany
Temed	Serva, Germany

2.1.4 Antibodies

2.1.4.1 Primary antibodies used for western blots

Rabbit p-S6	Cell Siganling, USA
Mouse-S6	Cell Siganling, USA
Rabbit p-AKT (T308)	Cell Siganling, USA
Rabbit p-AKT (S473)	Cell Siganling, USA
Mouse AKT	Becton Dickenson, Germany
mTOR	Cell Siganling, USA
Rabbit LC3	Thermo Scientific, Germany
Rabbit Caspase 3	Cell Siganling, USA
Mouse β -Actin	Sigma-Aldrich, Germany
Rabbit-mTOR	Cell Siganling, USA

2.1.4.2 Secondary antibodies used for western blots

Goat anti-mouse IgG conjugated to HRP	Santa Cruz Biotechnology, USA
Goat anti-rabbit IgG conjugated to HRP	Santa Cruz Biotechnology, USA

2.1.4.3 Phosphoflow antibodies

Alexa Flour 647 conjugated p-S6	Cell Siganling, USA
Alexa Flour 488 conjugated p-4EBP1	Cell Siganling, USA
Alexa Flour 488 conjugated p-AKT (T308)	Cell Siganling, USA
Alexa Flour 647 conjugated p-STAT5	Cell Siganling, USA

2.1.4.4 Antibodies used for monitoring leukemia engraftment

APC-Human CD19	Becton Dickinson, Germany
PerCP-Human CD45	Becton Dickinson, Germany
FITC-Human CD7	Becton Dickinson, Germany
PE-Mouse CD45 (Ly5)	Becton Dickinson, Germany

2.1.4.5 Immunohistochemistry antibodies

Caspase 3 polyclonal antibody	Abcam, UK
CD19	Dako, USA
Goat- α -Rabbit-AP secondary antibody	JIR, USA
Ki67	Dako, USA

2.1.5 Consumables

Cell culture flasks	Sarstedt, Germany
Falcons (15 ml, 50 ml)	Becton, Dickenson, Germany
FACS Tube	Sarstedt, Germany
Needle	B. Braun, Germany
Nunc Cryotube	Sarstedt, Germany
Nylon Cell Sieves	Becton Dickenson, Germany
Pasteur pipette (glass)	VWR international, Germany
Pipette tips	Sarstedt, Germany
Safe-lock tubes	Eppendorf, Germany

Serological pipettes (5, 10, 25 ml)	Sarstedt, Germany
Syringe 1.0 ml	BD Plastipak, Germany

2.1.6 Instruments

ABI-3130 Genetic Analyzer	Applied Biosystems, Italy
Centrifuge 5417R	Eppendorf, Germany
Centrifuge Varifuge 3.0 R	Heraeus, Germany
Developer Optimax	Protec medical Systems, Germany
Electrophoresis Power Supply EPS 300	Pharmacia Biotech, Sweden
Elisa Reader EL800	BioTek, USA
GS Junior 454 next generation sequencer	454 Sequencing, USA
LSSR II flow cytometer	Becton Dickinson, Germany
Incubator	Heraeus, Germany
Laminar Air Flow Hera Safe	Heraeus, Germany
Microscope	Leica, Germany
Microscope	Keyence, Germany
Neubauer Improved Counting Chamber	Marienfeld Superior, Germany
Pipetboy Pipette Controller	VWR International, Germany
Pipettes 1 – 1000 µl	Gilson, Germany
qRT-PCR LightCycler 2.0	Roche Diagnostics GmbH, Germany
Thermomixer comfort	Eppendorf, Germany
Transblot SD	BioRad, Germany
Water bath	Julabo, Germany

2.1.7 Software

Adobe Illustrator CS3	Adobe Systems Incorporated, USA
Coffalyser.Net	MRC, Holland
Cytobank.org	Cytobank, Inc., USA
Excel 2003	Microsoft, USA
FACSDIVA™	BD Bioscience, USA
Flowjo 8.7	TreeStar Inc. USA
GS Amplicon variant analyzer	454 Sequencing, USA
GS Rub Browser	454 Sequencing, USA
Image J 1.45s	NIH Image, USA
SPSS 19.0	IBM, Germany

2.2 Methods

2.2.1 Analysis of gene expression data

Gene set enrichment analysis (GSEA) was applied to the gene expression data of individual patient derived 5 TTL^{short} and 7 TTL^{long} xenograft samples (Meyer, Eckhoff et al. 2011) (<http://www.ncbi.nlm.nih.gov/geo>; GSE13576). Gene set enrichment analysis (GSEA, v2.0.14; <http://www.broadinstitute.org/gsea>) (Mootha, Lindgren et al. 2003, Subramanian, Tamayo et al. 2005) was performed using the 189 gene sets belonging to the “C6 oncogenic signatures” database within the Molecular Signature Database (MSigDB) collection. Only gene sets with NOM p-value ≤ 0.05 and FDR q-value ≤ 0.05 were considered significant. Connectivity Map analysis (cmap build 02, <http://www.broadinstitute.org/cmap>) (Lamb 2007, Lamb, Crawford et al. 2006) of our previously generated TTL signature was performed using the top 398 genes ranked according to q-value ≤ 0.25 . As a result, the CMAP provides a “permuted results” table that showed the “perturbagens” positively or negatively connected with the uploaded signature ranked according to a connectivity score. A high positive connectivity score indicates that the corresponding perturbagen induced the expression of the query signature. A high negative connectivity score indicates that the corresponding perturbagen reversed the expression of the query signature.

2.2.2 Patient sample characteristics

2.2.2.1 Patient characteristics

Leukemia samples of pediatric patients diagnosed with de novo BCP-ALL were obtained after informed consent in accordance with the institution's ethical review board. Leukemic cells from pediatric patients at diagnosis were used to establish xenografts according to the previously described method (Meyer, Eckhoff et al. 2011, Queudeville, Seyfried et al. 2012). Animal experiments were conducted upon approval by respective authority (Regierungspräsidium Tübingen, Versuch-Nr. 980). After transplantation, time to leukemia (TTL) was calculated for each patient in weeks from the date of transplantation to the overt leukemia. Xenograft ALL cells were isolated from splenic cell suspensions obtained from leukemia bearing animals at disease onset and further transplanted onto subsequent recipients in order to amplify cells for *ex vivo* analyses or for *in vivo* evaluation in a preclinical model setting. Characteristics of patients and xenografts are summarized in Table 8.

2.2.2.2 Detection of leukemia engraftment

After transplantation of xenograft leukemia cells onto NOD/SCID mice, blood samples were collected from the mouse once in a week and evaluated for the presence of human leukemia cells. Approximately 50 µl of peripheral blood were taken from the tail vein and mixed with heparin (500 IU per 50 µl blood) in the FACS tube. Blood samples were stained with allophycocyanin (APC)-conjugated anti-human B-cell antigen (CD19), Percp conjugated human leukocyte common antigen (CD45) and phycoerythrin (PE)-conjugated anti-murine leukocyte common antigen (Ly-5) for 20 minutes at room temperature followed by the addition of the lysis buffer to lyse the erythrocytes. Cells were washed with BSA supplemented PBS, and analyzed by BD LSR II multiparameter flow cytometry. The proportion of human CD19-positive BCP-leukemia cells was calculated reflecting the leukemic burden in the mouse.

2.2.2.3 Manifestation of leukemia

After transplant the general condition and well-being of the mice were examined regularly. Manifestation of disease was assessed by clinical signs of overt

leukemia such as severely impaired general condition, lethargy, ruffled fur and impaired posture. Upon clear evidence for leukemia related morbidity, mice were killed and autopsy was performed. At autopsy cell suspensions from spleen and bone marrow were prepared. Spleen tissue was minced and passed through nylon cell sieves (70 μ m) followed by ficoll gradient centrifugation for 20 minutes at 1300 rpm at room temperature. Bone marrow cells were isolated flushing the femoral cavity with HBSS. Cell suspension from spleen and bone marrow were stained with allophycocyanin (APC)-conjugated anti-human B-cell antigen (CD19), Percp conjugated human leukocyte common antigen (CD45) and phycoerythrin (PE)-conjugated anti-murine leukocyte common antigen (Ly-5) for 20 minutes at 4° C followed by washing with BSA washing solution. Blood staining was done according to the same protocol described in the previous section. Cells have been acquired by BD LSRII multiparameter flow cytometry. Time to leukemia (TTL) was determined for each patient sample transplanted as weeks from the date of transplant to the date of clinical manifestation of the disease.

2.2.3 Cell culture

Human BCP-ALL leukemia cell lines Nalm-6, KOPN8 and T-ALL cell line Jurkat were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1 mmol/L L-glutamine. All cells were maintained in a 37° C incubator with 5% CO₂ and harvested in the logarithmic phase of growth.

2.2.4 Analysis of protein phosphorylation by flow cytometry

Isolated leukemia cells from the spleen of leukemia bearing animal was inoculated either with 100 nM rapamycin and 100 nM NVP-BEZ235 or DMSO as vehicle control. The level of phosphorylated ribosomal protein S6 (p-S6) was measured by flow cytometry according to the published method (Krutzik and Nolan 2003). Briefly, cells were fixed by paraformaldehyde (PFA, 1.5% final concentration) and permeabilized by 70% methanol. Staining of the phospho-proteins was performed using fluorochrome conjugated p-S6 (Ser235/236) and p-4EBP1 (T37/38), p-AKT (Thr308) and p-STAT5 (Y694) antibodies. Flow cytometry was performed in BD

LSRII flow cytometry and data were analyzed using FlowJow 8.7 version software (TreeStar Inc, USA) and cytobank.org (Kotecha, Krutzik et al. 2010).

2.2.5 Cell cycle analysis and detection of apoptosis and proliferation

Cell cycle analysis was done staining the cells with 7-Aminoactinomycin D (7-AAD). Cells were fixed in 100% methanol on ice for 30 minutes. 50 µl of RNase (100 µg/ml) was added and incubated at room temperature for 15 minutes in the dark followed by the addition of 200 µl 7-AAD (25 µg/ml) and 15 minutes incubation at room temperature (RT). Detection of apoptotic cells by flow cytometry was performed using Annexin V according to the manufacturer's instructions. Briefly, cells were rinsed with ice-cold binding buffer and 100 µl of Annexin V solution was added to the cells and incubated for 15 min at RT followed by immediate acquisition. Cell proliferation was detected by ki67 and human CD19 co-staining of the isolated leukemia cells. Cells were stained with APC-CD19 followed by fixation with 4% PFA and permeabilization with 0.2% saponin. Cells were stained with Ki67 antibody for 30 minutes at room temperature followed by washing with PBS.

2.2.6 Western blot

2.2.6.1 Protein extraction and concentration determination

Protein extraction and quantification was performed on ice. After harvesting, cells were washed with PBS and resuspended with protein lysis buffer containing 30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X 100, 10% glycerol, 2 mM DTT and 200 µM PMSF and incubated for 15 minutes on ice. Whole cell lysates were centrifuged at 14000 rpm for 20 minutes at 4°C and the supernatant was collected to a fresh eppendorp tube. Quantification of the whole protein lysate was performed using the BCATM Protein Assay Reagent Kit according to the manufacturer's instruction.

2.2.6.2 Western blot

To perform western blot proteins were separated using SDS polyacrylamide gel electrophoresis. 6X protein loading dye (60 mM Tris/HCl pH 6.8, 1% SDS, 5%

glycerol, 0.01 mg/ml bromphenolblue and 0.34 M β -Mercaptoethanol) was added to 20 μ g whole protein lysate and denatured at 95°C for 15 minutes. Proteins were loaded to 10-15% polyacrylamide gels and were separated by applying 120 volts using a running buffer containing 125 mM TrisBase, 1.25 M Glycin and 0.1% SDS. Molecular weight protein marker was used to distinguish the proteins according to their molecular weight. After the separation, semidry blotting method was used to transfer the proteins into ECL nitrocellulose membrane. Membrane and the Whatman paper were cut according to the size of the gel and soaked in the blotting buffer containing 48 mM Tris Base, 39 mM Glycin, 0.037% SDS and 20% Methanol. Then two Whatman papers, ECL membrane, gel and again two Whatman papers were piled together and constant current of 1 mAmp/cm² of the membrane was applied for 60 minutes, except for LC3; 1.5 mAmp/cm² for 30 minutes. After the blotting, the membrane was blocked with 5% nonfat dry milk solution in PBS containing 0.1% Tween for 1 hour at room temperature. Then membranes were washed three times for 15 minutes with the washing buffer containing PBS and 0.1% Tween. Membranes were incubated overnight at 4°C with primary antibody diluted in PBS containing 0.1% Tween, 2% BSA and 0.02% Sodium Azide. After washing three times with washing solution, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody diluted into 5% milk solution followed by washing for three times. After that membrane was incubated with ECL solution and developed using Hyperfilm in a Protec optimax developer. Densitometric quantification of the protein bands was performed using ImageJ 1.45s software.

2.2.7 Immunohistochemistry

Immunostaining for Ki67 and CD19 was performed according to standardized protocols using the Dako Real Detection system based on alkaline phosphatase and permanent red chromogen for detection of bound antibody (Dako, CA, USA) as described previously (Barth, Martin-Subero et al. 2003). Briefly, deparaffinized serial sections of decalcified bone tissue were fixed in buffered formalin for 10 minutes. For antigen retrieval, slides were heated in EDTA (pH 8.0) and incubated with the primary anti CD19 and Ki-67 antibody (Dako, CA, USA). Immunostaining for active caspase 3 was performed using previously described method (Hipp,

Christner et al. 2014). Briefly, deparafinized tissue sections were pressure cooked in 10 mM citrate buffer (pH 6.0) and probed with active caspase 3 (Abcam, UK) antibody.

2.2.8 *In vivo* treatment

All experimental animal studies were conducted according to the national animal welfare law and were approved by the appropriate authority. Establishment of the xenograft and leukemia progression monitoring were done according to the previously described method (Meyer, Eckhoff et al. 2011). When the proportion of human CD19+ cells reached approximately 5% in mouse peripheral blood, mice were randomized into four groups and treated with (a) rapamycin, (b) vincristine, dexamethasone, asparaginase [VDA], (c) vincristine, dexamethasone, asparaginase, rapamycin [VDA+R] and (d) DMSO as vehicle control. All drug administration was done by intraperitoneal injection, and consisted of vincristine (0.075 mg/kg, once in a week), dexamethasone (2.5 mg/kg, 5 days in a week), asparaginase (500 IU/kg, 5 days in a week), rapamycin (2.5 mg/kg, 5 days in a week). All drugs were dissolved in 75% DMSO and treatment was continued for 2 weeks. Mice were monitored regularly for the clinical signs of leukemia and were sacrificed upon leukemia manifestation. Upon sacrifice, high leukemia infiltration was confirmed detecting ALL cells in bone marrow, spleen and peripheral blood by flow cytometry. Time to reoccurrence (TTR) was determined in weeks from the date of treatment to the date of leukemia related morbidity.

2.2.9 Detection of *CRLF2* alterations

CRLF2 transcript over expression (20-fold above median expression of cohort of 464 BCP-ALL patients) and the detection of *P2RY8-CRLF2* fusion transcript were performed according to the previously described method (Palmi, Vendramini et al. 2012). *IGH@CRLF2* translocation was analyzed by PCR using primers designed in the first exon of *IGH* (5'-AATACTTCCAGCACT-3') and the third exon of *CRLF2* (5'-GTCCCATTCTGATGGAGAA-3'). PCR conditions were as follows: initial denaturation at 94° C for 2 min; 30 PCR cycles: denaturation at 94° C for 30 sec, annealing at 59° C for 30 sec, extension at 72° C for 1min and final extension at

72° C for 10 min. *JAK2* (R683G) and *CRLF2* (F232C) mutation were analyzed by JS Junior 454 platform (454 Sequencing, USA) as described previously (Kohlmann, Klein et al. 2011).

2.2.10 Detection of *IKZF1* deletion

Detection of *IKZF1* was performed by Dr. Chiara Palmi of Monza, Italy. Multiplex Ligation-dependent Probe Amplification (MLPA; SALSA MLPA P335-A3 ALL-*IKZF1* probemix, MRC-Holland, Amsterdam, The Netherlands) was used according to the manufacturer's instruction to detect *IKZF1* deletion. DNA of pediatric ALL patients at remission was used as wild type control. The separation of the fragments was performed on ABI-3130 Genetic Analyzer instrument. Data were analyzed using Coffalyser.Net software.

2.2.11 Statistical analysis

Statistical analyses were carried out using SPSS19.0 software (IBM, Munich, Germany) using the respective tests indicated. *P* values of <0.05 were considered significant in all the tests performed in this study. Unless otherwise stated, Mann-Whitney U test was performed to compare difference between two independent groups. Survival analyses were performed by Kaplan-Meier method and log-rank test was used to compare therapy response.

3. Results

3.1 Analysis of gene set enrichment and chemical genomics point to mTOR signaling

Previous study of our group reported differential expression of transcripts coding for regulators of the mTOR. In this study, gene set enrichment analysis of previously generated gene expression data (Meyer, Eckhoff et al. 2011) (<http://www.ncbi.nlm.nih.gov/geo>; GSE13576) was performed in order to investigate associations of pathway annotated gene sets with the TTL profile. GSEA is a tool that helps linking prior knowledge to newly generated data to uncover the collective behavior of genes in disease (Subramanian, Tamayo et al. 2005). Interestingly, two mTOR annotated gene sets (Majumder, Febbo et al. 2004, Wei, Twomey et al. 2006) were identified to be significantly associated (FDR q-value $\leq .01$) with the TTL phenotype, emphasizing the significance of mTOR signaling in leukemia engraftment on NOD/SCID/huALL model system and patient outcome (Figure 8A, Table 5).

Additionally, a chemical genomics approach (connectivity map analysis, CMAP) was used to analyze similarities of TTL signature with genome wide expression profiles obtained by CMAP. CMAP is a hypothesis generating tool (<http://www.broadinstitute.org/cmap/>) allowing finding connections between the gene expression modifications caused by 164 different small molecules (i.e. perturbagens) in 4 different cancer cell lines and diseases (Lamb 2007, Lamb, Crawford et al. 2006). Among the top-ranked compounds with negative connectivity score, mTOR inhibitor sirolimus (rapamycin) and a PI3K inhibitor LY-294002 were identified. This indicates the potential of mTOR inhibition to revert the TTL^{short} phenotype into TTL^{long} phenotype (Figure 8B, Table 6).

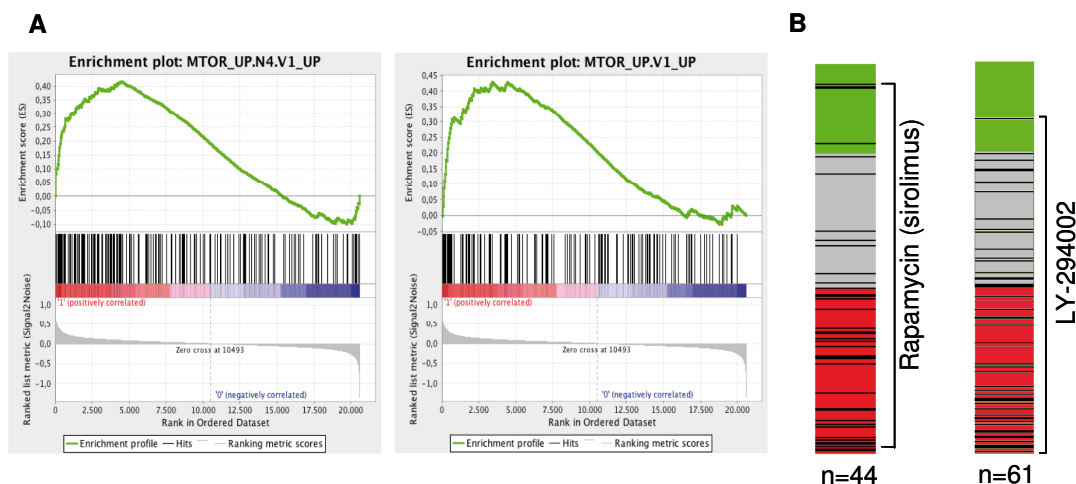


Figure 8: The association of TTL expression profile with mTOR signaling and potential of mTOR inhibition

(A) Two mTOR annotated gene sets in the TTL^{short} profile (Gene Set Enrichment Analysis, FDR q-value $\leq .01$) are enriched significantly. Green lines depict the enrichment score. Members of the annotated gene sets (black vertical lines) appear in the enrichment plot along the gene list ranked from TTL^{short} (red) to TTL^{long} (blue). (B) Connectivity map analysis (CMAP) shows negative connection of the genes of TTL^{short} phenotype with sirolimus (rapamycin, mTOR inhibitor) and LY-294002 (PI3K inhibitor) induced gene sets. In the bar view, each drug treatment instances is represented by the black lines (sirolimus, n=44 or LY-294002, n=61) which are ordered by their corresponding connectivity score as depicted by different colors (green, positive; gray, null; red, negative).

Originally published in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget **6**(3): 1382-1395 (2015).

Table 5: The TTL profile is significantly enriched in mTOR pathway annotated gene sets

Gene set enrichment analysis using our previously generated TTL gene expression data set. Three gene sets with a FDR q-value of $\leq .01$ are identified including two mTOR pathway annotated data sets (Molecular Signatures Database, MSigDB; collection 6: oncogenic signatures). NES, normalized enrichment score; NOM p-val, nominal p-value; FDR q-val, false discovery rate q-value.

Gene set	NES	NOM p-val	FDR q-val
IL2_UP.V1_UP	1.915	0.000	0.007
<i>MTOR_UP.V1_UP</i>	<i>1.792</i>	<i>0.000</i>	<i>0.010</i>
<i>MTOR_UP.N4.V1_UP</i>	<i>1.817</i>	<i>0.000</i>	<i>0.014</i>
LTE2_UP.V1_DN	1.693	0.000	0.020
CSR_LATE_UP.V1_UP	1.641	0.000	0.029
IL15_UP.V1_UP	1.592	0.002	0.040
STK33_NOMO_DN	1.440	0.007	0.136
BCAT_GDS748_UP	1.605	0.007	0.039
RPS14_DN.V1_UP	1.475	0.007	0.107
CSR_LATE_UP.V1_DN	1.483	0.008	0.114
STK33_SKM_UP	1.399	0.009	0.148
HOXA9_DN.V1_UP	1.405	0.011	0.163
RELA_DN.V1_UP	1.377	0.013	0.158
HOXA9_DN.V1_DN	1.403	0.016	0.154
STK33_UP	1.343	0.016	0.175
E2F1_UP.V1_DN	1.391	0.017	0.147
CRX_NRL_DN.V1_DN	1.371	0.029	0.149
P53_DN.V1_DN	1.330	0.034	0.188
BMI1_DN_MEL18_D	1.358	0.036	0.160
N.V1_UP			
SIRNA_EIF4GI_UP	1.372	0.038	0.155
RAF_UP.V1_DN	1.327	0.040	0.184

Originally published in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget **6**(3): 1382-1395 (2015).

Table 6: The TTL signature is negatively connected with mTOR inhibitor induced gene sets

Connectivity map analysis employing our previously identified TTL expression profile. Within the top four gene sets showing significant association with the TTL profile (top three with negative connection), two gene sets induced by sirolimus (rapamycin) and the PI3K inhibitor LY-294002 were identified to be negatively connected indicating potential to revert the TTL^{short} phenotype.

Rank	CMap name	Mean connectivity score	Number of instances	p-value	Specificity
1	adiphenine	0.743	5	0.000	0.000
2	tanespimycin	-0.455	62	0.000	0.261
3	LY-294002	-0.436	61	0.000	0.319
4	sirolimus	-0.366	44	0.000	0.295
5	finasteride	0.568	6	0.000	0.014
6	Prestwick-692	0.703	4	0.000	0.012
7	etiocholanolone	0.459	6	0.000	0.000
8	pargyline	-0.736	4	0.001	0.000
9	PHA-00745360	0.380	8	0.001	0.027
10	podophyllotoxin	0.673	4	0.001	0.029
11	calcium folinate	0.668	5	0.001	0.017
12	vancomycin	0.674	4	0.001	0.000
13	biperiden	0.645	5	0.001	0.018
14	trifluoperazine	-0.485	16	0.002	0.164
15	metyrapone	-0.731	4	0.002	0.005
16	GW-8510	-0.670	4	0.002	0.176
17	fludrocortisone	0.323	8	0.002	0.065
18	alpha-estradiol	-0.451	16	0.002	0.093
19	trazodone	-0.718	3	0.002	0.023
20	promazine	-0.664	6	0.003	0.000
21	phthalylsulfathiazole	-0.669	5	0.003	0.035
22	meticrane	-0.692	5	0.003	0.015
23	colistin	0.668	4	0.003	0.006
24	CP-320650-01	0.292	8	0.003	0.041
25	sulfadimethoxine	0.550	5	0.004	0.040

26	2-aminobenzenesulfonamide	0.600	4	0.004	0.006
27	quinostatin	-0.817	2	0.004	0.092
28	8-azaguanine	-0.787	4	0.004	0.014
29	metrifonate	-0.627	5	0.004	0.005
30	apigenin	-0.714	4	0.004	0.038
31	ceforanide	0.664	4	0.004	0.028
32	famprofazone	-0.628	6	0.004	0.012
33	oxybuprocaine	-0.650	4	0.005	0.011
34	Prestwick-983	0.650	3	0.005	0.012
35	furazolidone	0.565	4	0.005	0.039
36	diloxanide	0.605	4	0.005	0.017
37	isometheptene	0.617	4	0.005	0.012
38	vinblastine	0.629	3	0.005	0.039
39	ginkgolide A	-0.692	4	0.005	0.020
40	nadolol	0.472	4	0.006	0.071
41	nortriptyline	-0.649	4	0.006	0.029
42	levonorgestrel	-0.561	6	0.006	0.066
43	isocarboxazid	0.463	5	0.006	0.006
44	tobramycin	-0.602	4	0.007	0.006
45	dapsone	0.498	5	0.007	0.011
46	lycorine	0.356	5	0.007	0.124
47	gentamicin	0.429	4	0.007	0.019
48	lisuride	0.362	5	0.007	0.084
49	practolol	-0.621	4	0.007	0.000
50	atractyloside	0.383	5	0.007	0.117
51	thioridazine	-0.395	20	0.007	0.323
52	ribavirin	0.429	4	0.008	0.019
53	karakoline	0.352	6	0.008	0.005
54	clomipramine	-0.647	4	0.009	0.061
55	chlorpromazine	-0.415	19	0.009	0.032
56	ethisterone	0.358	6	0.009	0.013
57	guanadrel	0.561	5	0.009	0.052

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Thus, these findings indicate the relevance of mTOR signaling in rapid *in vivo* leukemia engraftment resulting in TTL^{short} phenotype and the potential of mTOR pathway inhibition by signal transduction inhibitors e.g. rapamycin to effectively target high-risk ALL.

3.2 Patient-derived ALL xenograft samples

Individual patient-derived fourteen B-cell precursor (BCP) ALL xenograft samples (TTL^{short} , $n=7$, S1-7; TTL^{long} , $n=7$, L1-7) were investigated in this study utilizing the NOD/SCID/huALL xenotransplant mouse model. All the xenografts were derived from *de novo* diagnosed pediatric B-cell precursor ALL. After transplantation recipient animals were monitored regularly for the onset of leukemia assessing human CD19+ cells in murine peripheral blood and were sacrificed at leukemia manifestation. Upon sacrifice high leukemia infiltration was confirmed in the peripheral blood, bone marrow and spleen detecting human CD19+ cells by flow cytometry indicating advanced human ALL in the mice. The time to leukemia (TTL) for individual ALL was calculated from the date of transplantation to the date of leukemia manifestation (Figure 9).

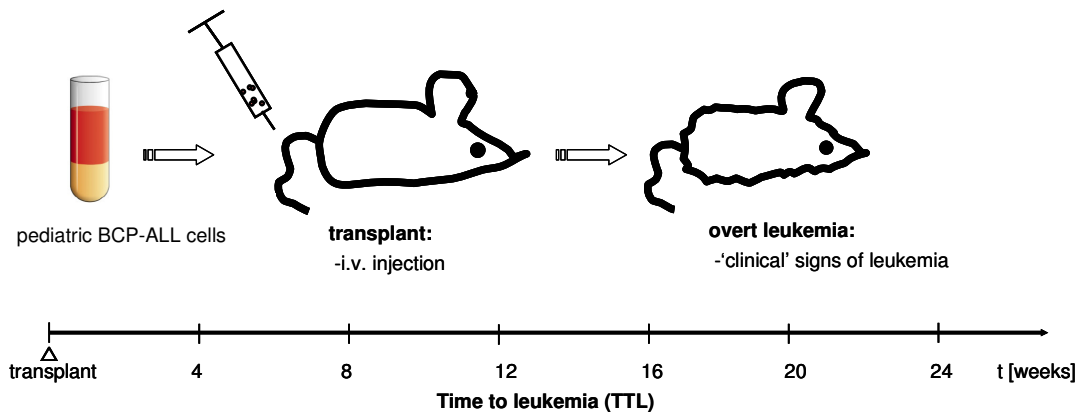


Figure 9: Schematic representation of NOD/SCID/huALL xenotransplant model

Pediatric BCP-ALL cells at diagnosis are transplanted into NOD/SCID mice intravenously and leukemia progression is monitored regularly. When recipient animals show clinical signs of leukemia manifestation, they are sacrificed and leukemia infiltration in different compartments is confirmed by flow cytometry. Time to leukemia (TTL) for each BCP-ALL patient is calculated from the date of transplant to the date of leukemia related morbidity.

The TTL of all the ALL samples investigated in this study were consistent with the previous passages (Table 7). Most importantly, short NOD/SCID/huALL engraftment was associated with inferior relapse free patient survival in the patient cohort used in this study (log rank test $p = .013$) (Figure 10).

Table 7: Consistency of TTL phenotype

Time to leukemia (TTL) of individual patient derived xenografts in 1st recipient (P0) and in the passage (P) used in this study. TTL, time to leukemia; P, passage.

Leukemia	TTL of 1 st recipient (P0) (weeks)	Passage (P) used in this study	TTL of passages used in this study (weeks)
S1	8,8	P3	6
S2	9,1	P3	7
S3	9,1	P3	8
S4	9	P8	6
S5	9	P9	5,5
S6	10	P4	7,5
S7	8,6	P4	8
L1	27,7	P1	11,5
L2	26,9	P0	27
L3	36,6	P0	36,5
L4	16,9	P2	17
L5	11,9	P3	12,5
L6	19,1	P4	15,5
L7	22	P5	25

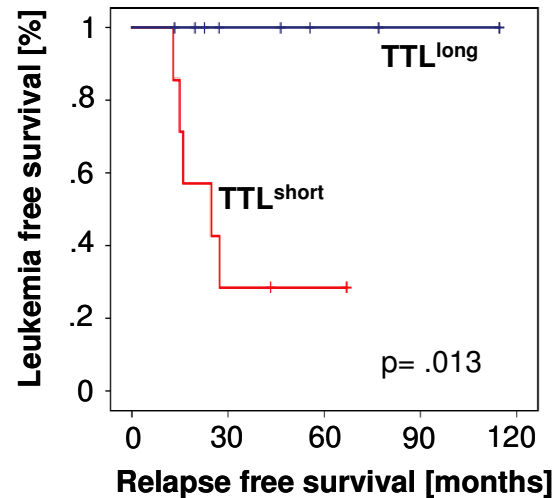


Figure 10: Poor outcome of patients associated with TTL^{short} phenotype

Patients associated with long engraftment phenotype (TTL^{long}, n=7) show superior relapse free survival in contrast to the patients associated with short engraftment phenotype (TTL^{short}, n=7); Kaplan-Meier analysis; log rank test; p, significance.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

The TTL phenotypes of the leukemias used in this study were not associated with intrinsic leukemia characteristics and prognostic subgroups. Most of the samples used in this study carried no known genetic alterations. An *MLL/AF4* gene fusion was carried by one TTL^{short} leukemia and an *ETV6/RUNX1* gene fusion was carried by one TTL^{long} leukemia. Recently, alterations of cytokine receptor-like factor 2 (*CRLF2*) have been described to be associated with hyperactivated mTOR signaling (Tasian, Doral et al. 2012). Alterations in *CRLF2* include *P2RY8/CRLF2* gene fusions or *IGH@/CRLF2* translocation and point mutations (*CRLF2*, F232C) (Chen, Harvey et al. 2012). Altered *CRLF2* alone or in combination with Janus kinase 2 mutations (*JAK2*, R683G) are frequently associated with *CRLF2* over expression resulting in aberrant PI3K/mTOR signaling (Chapiro, Russell et al. 2010, Hertzberg, Vendramini et al. 2010, Mullighan, Collins-Underwood et al. 2009, Russell, Capasso et al. 2009, Tasian, Doral et al. 2012). Importantly, no alterations in *CRLF2* and *JAK2* genes or high

CRLF2 transcript or TSLPR protein expression was detected in any of the samples analyzed in this study (Table 8).

Table 8: Characteristics of patients and derived ALL xenografts

		total		TTL ^{short}		TTL ^{long}	
		N	%	N	%	N	%
Total		14	100	7	100	7	100
Gender	Female	6	43	2	29	4	57
	Male	8	57	5	71	3	43
Age	1-9 years	10	71	4	57	6	86
	0-1 and >9 years	4	29	3	43	1	14
Immunophenotype	pro-B ALL	1	7	1	14	0	-
	c-ALL	10	72	3	43	7	100
	pre-B ALL	3	21	3	43	0	-
Genetic alterations	<i>ETV6/RUNX1</i>	1	7	0	-	1	14
	<i>MLL/AF4</i>	1	7	1	14	0	-
	<i>BCR/ABL</i>	0	-	0	-	0	-
	<i>P2RY8/CRLF2</i>	0	-	0	-	0	-
	<i>IGH@/CRLF2</i>	0	-	0	-	0	-
	<i>CRLF2F232C</i>	0	-	0	-	0	-
	<i>JAK2R683G</i>	0	-	0	-	0	-
	<i>IKZF1</i> deletion	2	14	02	29	0	-
<i>CRLF2</i> /TSLPR expression	<i>CRLF2</i> transcript high	0	-	0	-	0	-
	TSLPR positive (FACS)	0	-	0	-	0	-
Hyperleukocytosis	<50 (1000/ μ l)	7	50	4	57	3	43
	>50 (1000/ μ l)	7	50	3	43	4	57
Prednisone response day 8	Good	13	93	7	100	6	86
	Poor	1	7	0	-	1	14
Remission at day 33	Yes	14	100	7	100	7	100
MRD risk group	MRD-HR	0	-	0	-	0	-
	MRD-non-HR	13	93	7	100	6	86
	No MRD available	1	7	0	-	1	14
final risk group	Non-HR	12	86	6	86	6	86
	HR	2	14	1	14	1	14

Originally published in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.3 Higher mTOR pathway activity in TTL^{short} leukemia

3.3.1 Higher mTOR expression in TTL^{short} leukemia compared to TTL^{long} leukemia

In our previous study, the TTL^{short} engraftment phenotype has been characterized by expression profiling pointing to differential expression of molecules (*FRAP1*, *RHEB* and *DDIT4L*) regulating mTOR signaling. Since *FRAP1* encodes for mTOR, the central molecule to form the mTORC1 complex, mTOR protein expression was assessed in a cohort of TTL^{short} and TTL^{long} phenotype. Primary leukemia cells were isolated from the leukemia bearing recipients at disease onset and proteins were extracted to perform western blot analysis. Higher mTOR protein expression was observed in TTL^{short} leukemia compared to TTL^{long} leukemia (Figure 11).

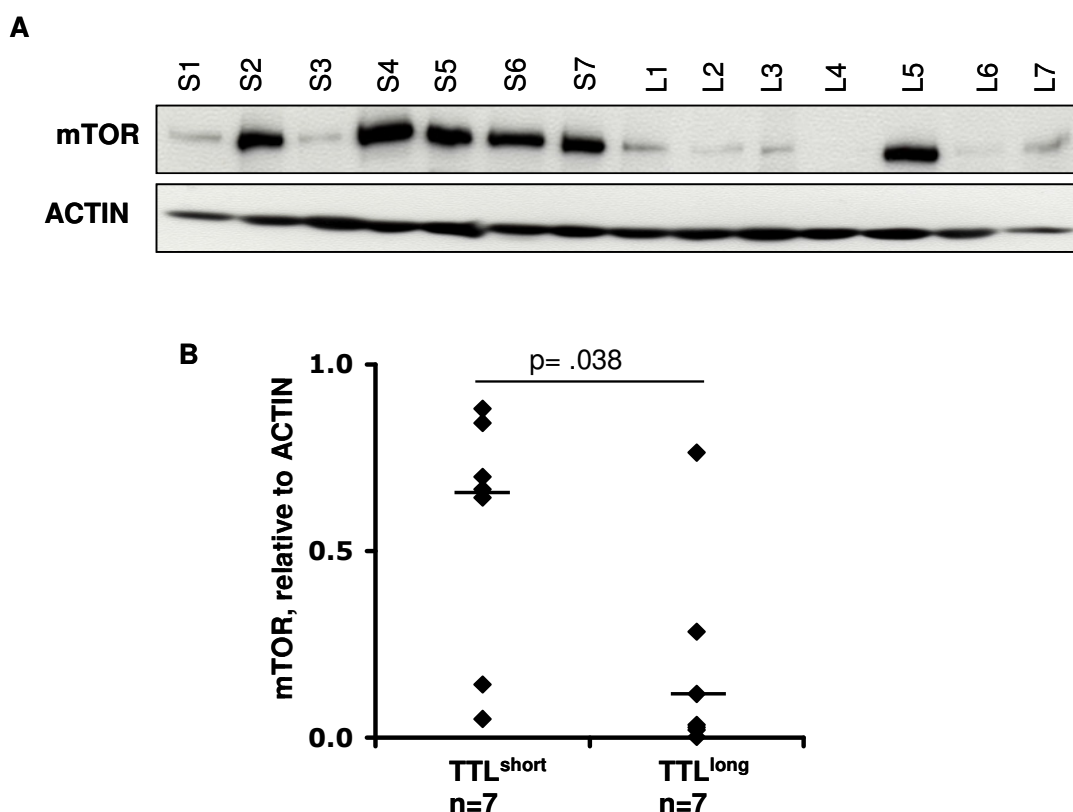


Figure 11: Higher mTOR protein expression in TTL^{short} ALL compared to TTL^{long} ALL

(A) Analysis of mTOR protein expression by western blot showing higher mTOR expression in TTL^{short} (S1-7) leukemia compared to TTL^{long} (L1-7) leukemia. (B) Significantly higher mTOR expression in TTL^{short} ALL compared to TTL^{long} ALL quantified by ImageJ densitometric analysis. Each data point represents mTOR expression relative to ACTIN; Mann-Whitney U test; p, significance.

In our previous study, higher mTOR transcript was detected in TTL^{short} ALL compared to TTL^{long} ALL. Concomitantly, in this study, higher mTOR protein expression was detected in TTL^{short} ALL indicating necessity for functional analysis of mTOR signaling activity.

3.3.2 Higher constitutive p-S6 in TTL^{short} ALL compared to TTL^{long} ALL

To identify constitutive activation of the mTOR pathway phosphorylation of ribosomal protein S6, a molecule downstream of mTOR that facilitates recruitment and translation of mRNA (Carrera 2004) was assessed. Leukemia cells were isolated from the spleen of leukemia bearing mice at disease onset containing at least 80% leukemic blasts and p-S6 was measured by phospho flow cytometry. Higher S6 phosphorylation was detected in TTL^{short} leukemia in contrast to low p-S6 level in TTL^{long} leukemia pointing to higher constitutive mTOR activity in TTL^{short} leukemia (Figure 12A, B, C). S6 phosphorylation was also assessed by western blot to validate phospho flow cytometry technique using the same cohort of TTL^{short} and TTL^{long} leukemia. In line with phospho flow cytometry data, higher S6 phosphorylation was detected in TTL^{short} ALL compared to TTL^{long} ALL confirming the phospho flow cytometry data (Figure 12D, E).

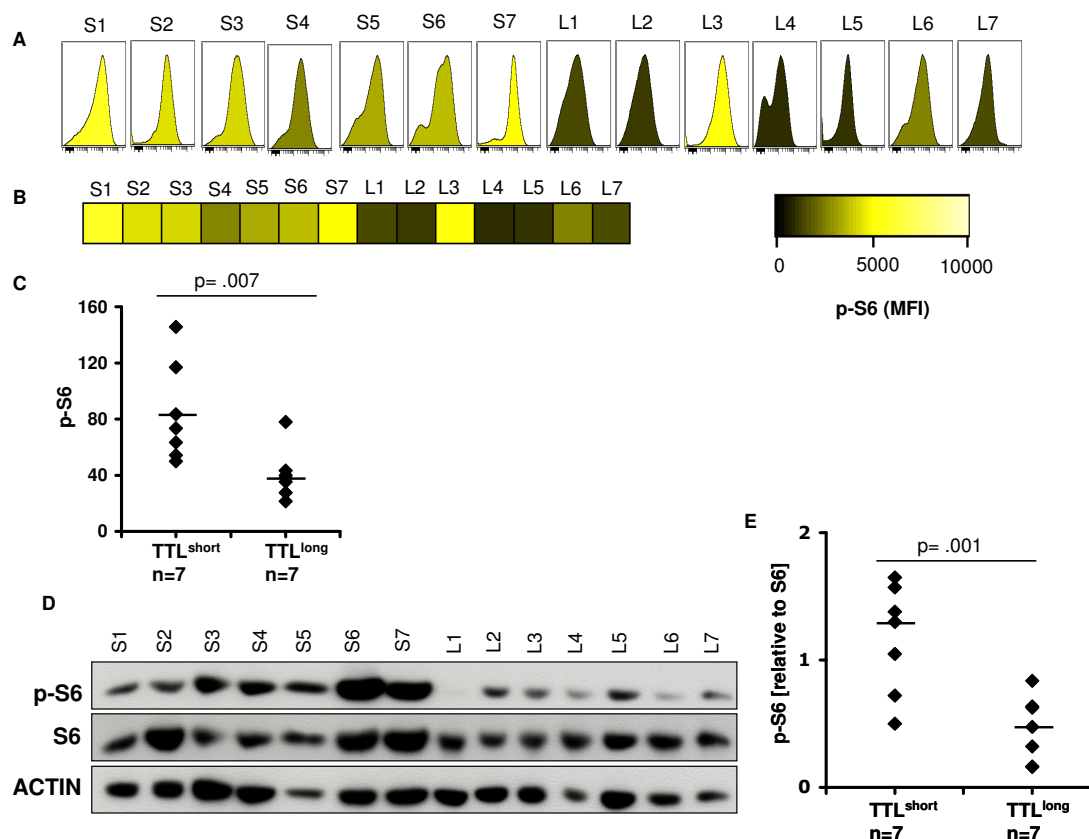


Figure 12: Higher p-S6 level in TTL^{short} compared to TTL^{long} ALL

(A, B) High constitutive S6 phosphorylation in TTL^{short} (S1-7) compared to TTL^{long} (L1-7) individual patient derived xenograft ALL cells. Histogram represents median fluorescence intensity (MFI) where black and yellow in colorimetric scale represents low and high MFI respectively. (C) Higher p-S6 in TTL^{short} compared to TTL^{long} xenograft ALL. Diagram represents MFI relative to cellular autofluorescence of p-S6 and each data point represents mean values of triplicate measurement of each sample. Bars represent median values of TTL^{short} and TTL^{long} samples; Mann-Whitney U test; p, significance. (D) Western blot analysis showing higher constitutive S6 phosphorylation in TTL^{short} (n=7) compared to TTL^{long} (n=7) ALL. (E) Significantly higher S6 phosphorylation in TTL^{short} ALL compared to TTL^{long} ALL as quantified by ImageJ densitometric analysis; relative to S6. Bars represent median values of TTL^{short} and TTL^{long} samples, Mann-Whitney U test; p, significance).

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.3.3 Similar constitutive 4EBP1 phosphorylation among TTL^{short} and TTL^{long} ALL

mTOR exerts its effect to initiate 5'-cap dependent mRNA translation by phosphorylating 4EBP1 resulting in the release of eIF4E to initiate translation (Carrera 2004). To investigate the translation initiation activity by 4EBP1 in TTL^{short} and TTL^{long} ALL, 4EBP1 phosphorylation was also assessed by phospho flow cytometry using the same subset of ALL samples used to assess S6 phosphorylation. In contrast to S6 activity, no significant difference in the constitutive p-4EBP1 level was observed among the TTL phenotypes (Figure 13).

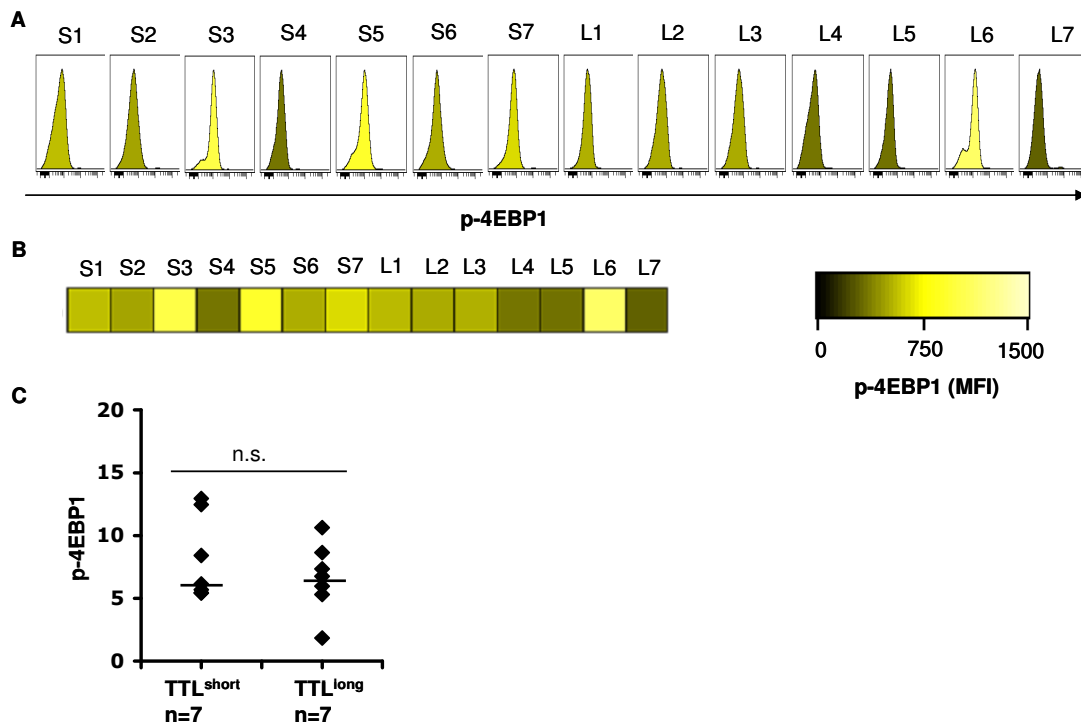


Figure 13: Similar constitutive p-4EBP1 level in TTL^{short} compared to TTL^{long} ALL

(A, B) Similar constitutive 4EBP1 phosphorylation in TTL^{short} (S1-7) compared to TTL^{long} (L1-7) individual patient derived xenograft ALL cells. Histogram represents

median fluorescence intensity (MFI), yellow and black in colorimetric scale represents high and low MFI respectively. Diagram represents MFI relative to cellular autofluorescence of p-4EBP1 and each data point represents mean values of triplicate measurement of each sample. Bars represent median values of TTL^{short} and TTL^{long} samples; Mann-Whitney U test; n.s., not significant.

3.3.4 Higher mTOR signaling activity in TTL^{short} ALL in contrast to TTL^{long} ALL after *ex vivo* culture

In addition to the constitutive mTOR pathway activation assessing p-S6 and p-4EBP1, also S6 and 4EBP1 phosphorylation of the xenograft ALL cells was assessed after *ex vivo* culture in serum containing medium. Freshly isolated ALL cells were cultured for 3 hours and p-S6 and p-4EBP1 levels were assessed by phospho flow cytometry. Interestingly, high levels of p-S6 were detected in TTL^{short} ALL in contrast to low p-S6 in TTL^{long} ALL after *ex vivo* culture (Figure 14). Consistent with constitutive p-4EBP1 levels, no significant difference in 4EBP1 phosphorylation was observed among TTL phenotypes although slightly higher p-4EBP1 was detected in TTL^{short} ALL (Figure 15).

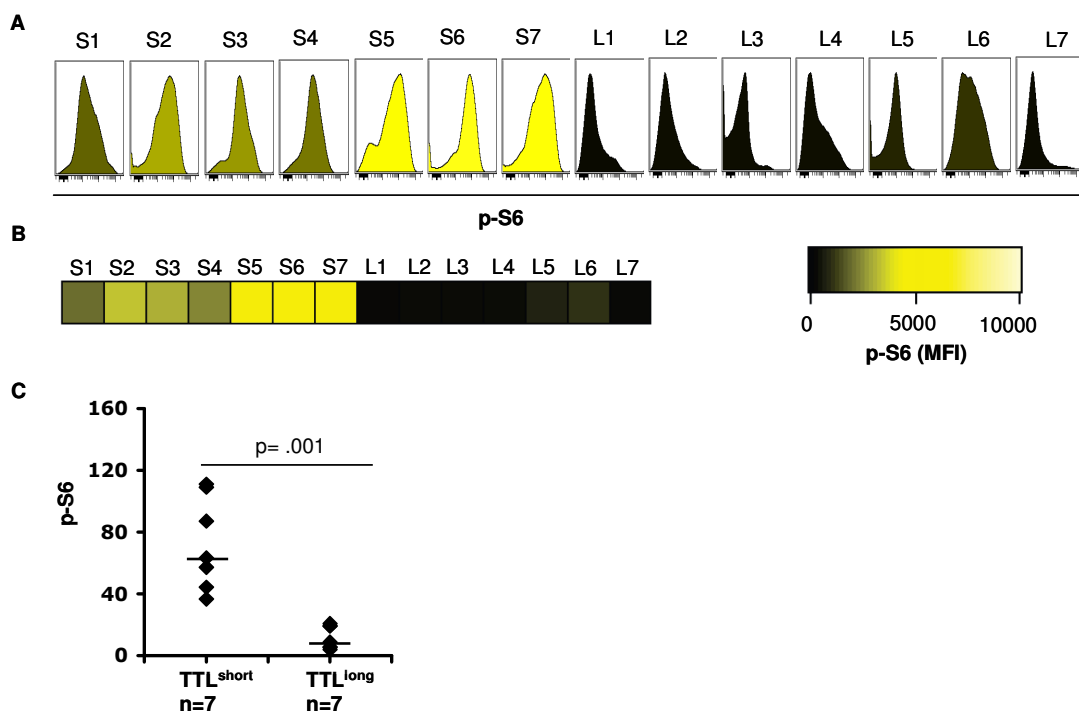


Figure 14: Higher p-S6 level in TTL^{short} compared to TTL^{long} ALL after *ex vivo* culture

(A, B, C) High S6-phosphorylation maintained in TTL^{short} (S1-7) ALL upon *ex vivo* culture in contrast to low p-S6 in TTL^{long} (L1-7) individual patient derived ALL. Histogram represents median fluorescence intensity (MFI), yellow and black in colorimetric scale represents high and low MFI respectively. Diagram represents MFI relative to cellular autofluorescence of p-S6 where each data point represents mean values of triplicate measurement of each sample. Bars represent median values of TTL^{short} and TTL^{long} samples; Mann-Whitney U test, p, significance.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

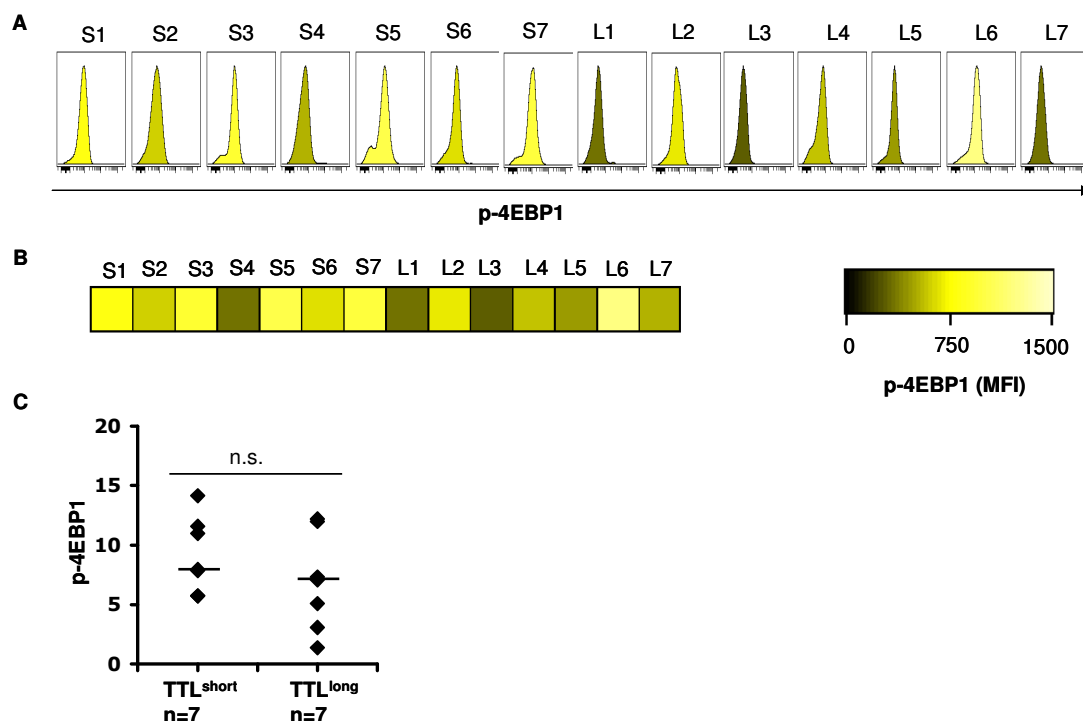


Figure 15: Similar 4EBP1 activity in TTL^{short} and TTL^{long} ALL after *ex vivo* cultures

(A, B, C) 4EBP1-phosphorylation maintained in both TTL^{short} (S1-7) and TTL^{long} (L1-7) ALL upon *ex vivo* culture of individual patient derived xenograft cells. Histogram represents median fluorescence intensity (MFI), yellow and black in colorimetric scale represents high and low MFI respectively. Diagram represents MFI relative to cellular autofluorescence of p-4EBP1 where each data point represents mean values of triplicate measurement of each sample. Bars represent median values of TTL^{short} and TTL^{long} samples; Mann-Whitney U test, n.s., not significant.

3.3.5 No constitutive AKT activation in either TTL^{short} or TTL^{long} ALL

In addition to assessing activity of the downstream molecules of the mTOR signaling, AKT activity was also assessed. Being activated by Phosphatidylinositol 3-kinase (PI3K), AKT triggers the mTOR signaling. In many cancers, the mTOR pathway is activated via the PI3K-AKT pathway. AKT phosphorylation was assessed from freshly isolated xenograft ALL cells by phospho flow cytometry

showing very low levels of AKT phosphorylation in both TTL^{short} and TTL^{long} ALL samples. Importantly, there was no difference in p-AKT levels between TTL^{short} and TTL^{long} ALL (Figure 16A, B, C). Phospho flow cytometry data was validated using western blot technique for the detection of AKT phosphorylation showing absent AKT phosphorylation in all the ALL samples regardless of their engraftment phenotype confirming the phospho flow cytometry data (Figure 16D).

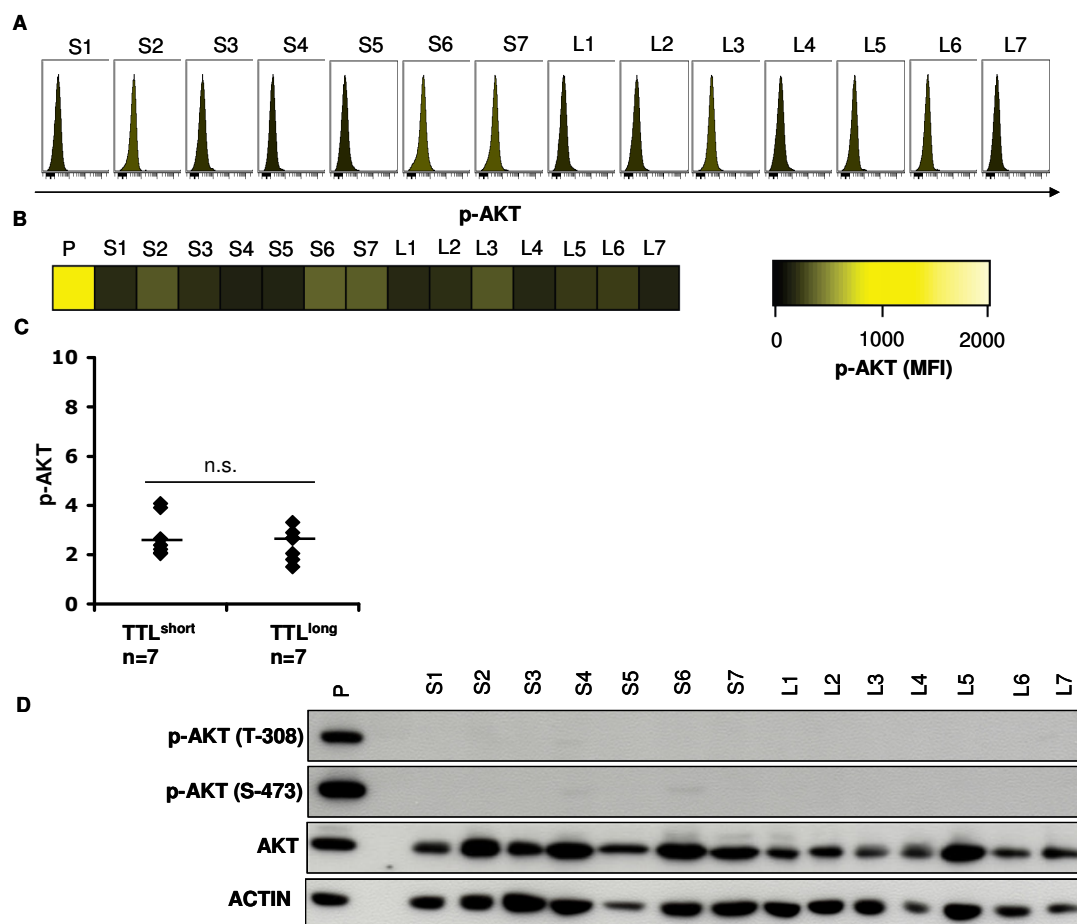


Figure 16: No constitutive AKT activation in TTL^{short} and TTL^{long} xenograft ALL

(A, B, C) No constitutive AKT-phosphorylation in both TTL^{short} (S1-7) and TTL^{long} (L1-7) individual patient derived xenograft ALL cells. Histogram represents median fluorescence intensity (MFI), yellow and black in colorimetric scale represents high and low MFI respectively. Diagram represents MFI relative to cellular autofluorescence of p-AKT where each data point represents mean values of

triplicate measurement of each sample. Bars represent median values of TTL^{short} and TTL^{long} samples; Mann-Whitney U test, n.s., not significant. (D) Western blot analysis of p-AKT showing no AKT phosphorylation in either TTL^{short} (n=7) or TTL^{long} (n=7) ALL; P, pervanadate treated cells (positive control).

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget 6(3): 1382-1395 (2015).

3.3.6 AKT is not activated after *ex vivo* culture

In addition to constitutive AKT phosphorylation, AKT phosphorylation was also assessed after *ex vivo* culture in serum containing medium providing a general growth stimulus. Consistent with absent constitutive AKT phosphorylation, p-AKT was detected in neither TTL^{short} nor TTL^{long} ALL xenografts upon culture (Figure 17).

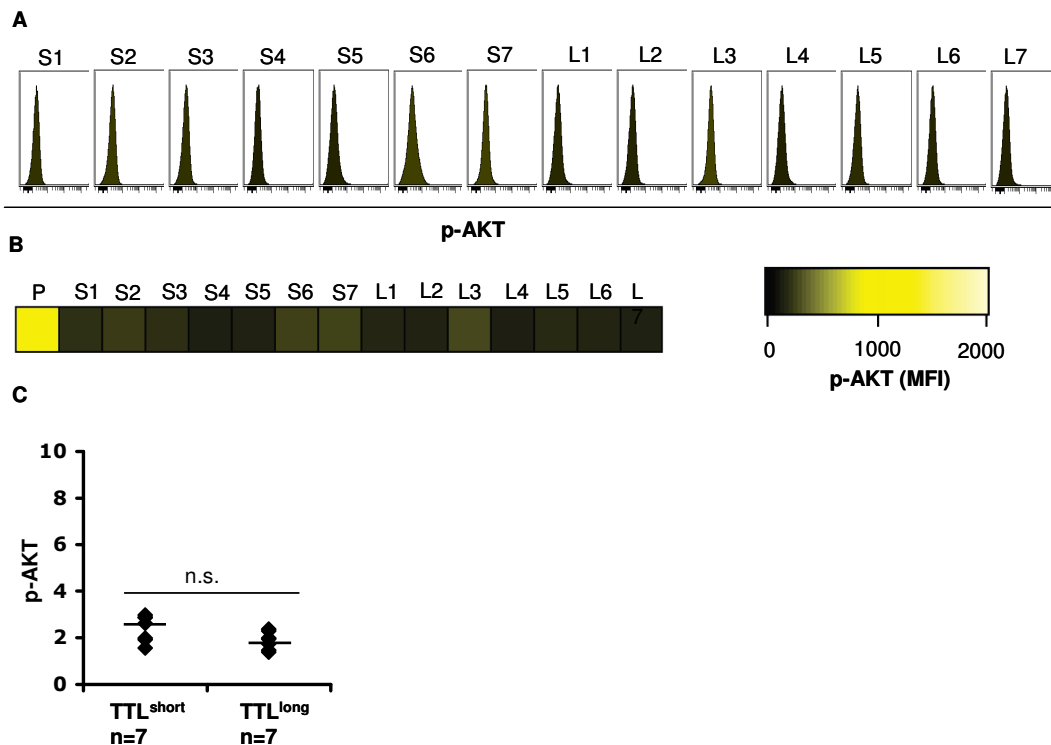


Figure 17: No AKT activation in TTL^{short} and TTL^{long} xenograft ALL upon *ex vivo* culture

(A, B, C) AKT-phosphorylation is also absent in both TTL^{short} (S1-7) and TTL^{long} (L1-7) individual patient derived xenograft ALL cells after *ex vivo* culture. Histogram represents median fluorescence intensity (MFI), yellow and black in colorimetric scale represents high and low MFI respectively. Diagram represents MFI relative to cellular autofluorescence of p-AKT where each data point represents mean values of triplicate measurement of each sample. Bars represent median values of TTL^{short} and TTL^{long} samples; Mann-Whitney U test, n.s., not significant.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.4 Assessment of JAK-STAT5 pathway activation

In many hematological malignancies the mTOR pathway is activated via PI3K/AKT pathway. But, none of the ALL samples used in this study the mTOR signaling is not activated via this commonly attributed mTOR activator, the PI3K/AKT signaling. A recent study based on genomic profiling of BCP-ALLs revealed the association between mTOR and JAK-STAT signaling (Roberts, Li et al. 2014). In this context, STAT5 phosphorylation of xenograft ALL cells was assessed by phospho flow cytometry constitutively and also after *ex vivo* culture in serum containing medium. STAT5 is constitutively active in all the xenografts but there was no significant difference between the TTL^{short} and TTL^{long} xenograft ALL cells (Figure 18). Additionally, STAT5 activity was retained after *ex vivo* culture and there was no difference in STAT5 phosphorylation between TTL^{short} and TTL^{long} ALL (Figure 19).

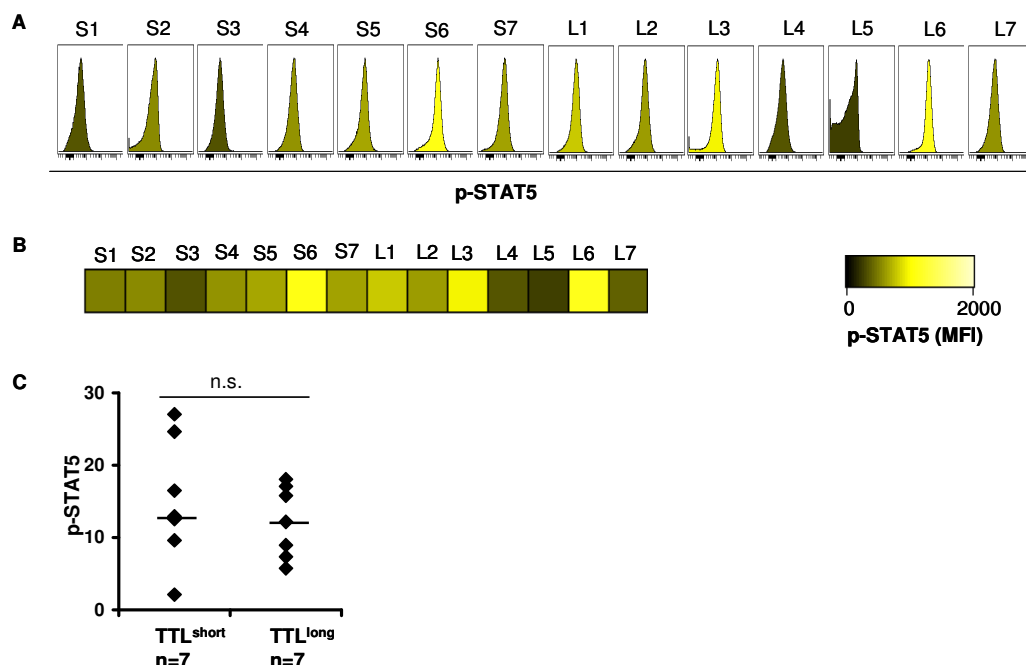


Figure 18: Similar JAK-STAT5 signaling activity in TTL^{short} and TTL^{long} ALL

(A, B, C) Similar constitutive STAT5 phosphorylation in TTL^{short} (S1-7) compared to TTL^{long} (L1-7) xenograft ALL cells. Histogram represents median fluorescence intensity (MFI), yellow and black in colorimetric scale represents high and low MFI respectively. Diagram represents MFI relative to cellular autofluorescence of p-STAT5 where each data point represents mean values of triplicate measurement of each sample. Bars represent median values of TTL^{short} and TTL^{long} samples; Mann-Whitney U test, n.s., not significant.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget 6(3): 1382-1395 (2015).

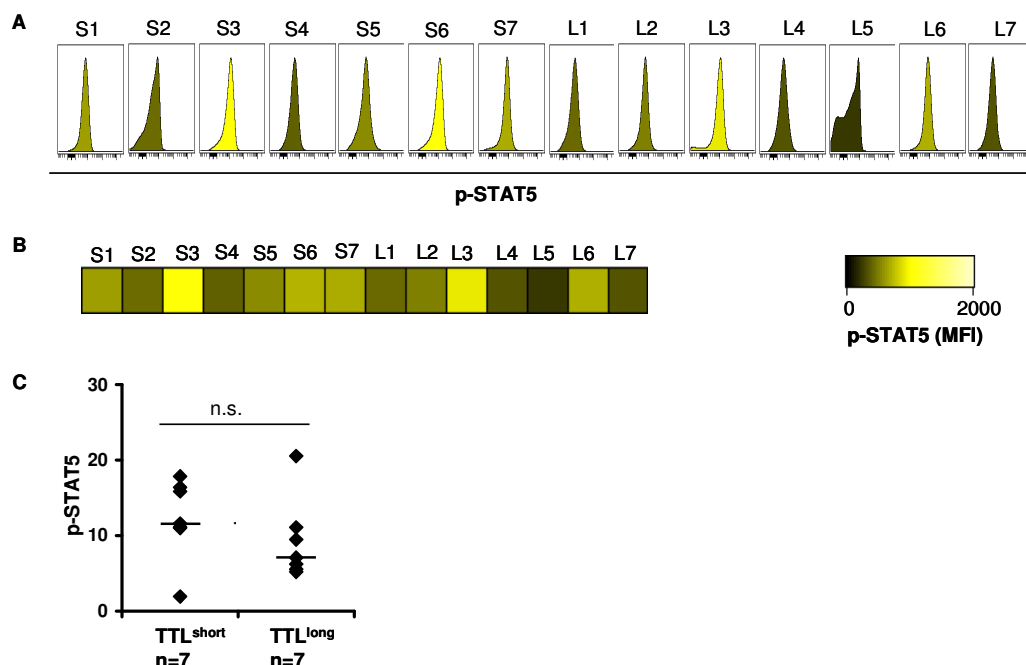


Figure 19: Similar JAK-STAT5 signaling activity in TTL^{short} and TTL^{long} ALL after *ex vivo* culture

(A, B, C) Similar STAT5 phosphorylation in TTL^{short} (S1-7) compared to TTL^{long} (L1-7) individual patient derived ALL after 3 h *ex vivo* culture. Histogram represents median fluorescence intensity (MFI), yellow and black in colorimetric scale represents high and low MFI respectively. Diagram represents MFI relative to cellular autofluorescence of p-STAT5 where each data point represents mean values of triplicate measurement of each sample. Bars represent median values of TTL^{short} and TTL^{long} samples; Mann-Whitney U test, n.s., not significant.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

Taken together, a constitutively highly activated mTOR pathway is a characteristic feature of TTL^{short}/high risk leukemias where the mTOR pathway activity is maintained upon *ex vivo* culture in contrast to TTL^{long} leukemias associated with constitutively low mTOR activity which diminish further upon *ex vivo* culture. No PI3K-AKT pathway activation was detected constitutively or after *ex vivo* culture

suggesting that mTOR activation in the ALL samples used in this study is not activated by upstream PI3K-AKT signaling. Additionally, in line with absent *JAK* mutation, no significant difference in the activity of JAK-STAT5 pathway between TTL^{short} and TTL^{long} phenotypes was observed.

3.5 Effective inhibition of mTOR pathway

3.5.1 mTOR pathway activity associated with TTL^{short} ALL can be inhibited *ex vivo* by mTOR inhibitors

Since the results indicate that TTL^{short} leukemia is associated with hyperactivated mTOR signaling, PI3K and mTOR signaling activity was addressed in response to pathway inhibition by two different inhibitors; rapamycin, an allosteric mTOR inhibitor and the dual PI3K/mTOR inhibitor NVP-BEZ235, which is currently evaluated in clinical studies on different malignancies. Freshly isolated xenograft ALL cells were incubated for 3 hours with rapamycin and BEZ235 and DMSO as vehicle control and p-S6 and p-4EBP1 were assessed by phospho flow cytometry. S6 phosphorylation in TTL^{short} leukemias were clearly reduced in response to both inhibitors while p-S6 levels in TTL^{long} leukemias remained unaffected. Interestingly, parallel inhibition of the PI3K and mTOR by NVP-BEZ235 did not show superior repression of pathway activity compared to repression by rapamycin (Figure 20A, B). To validate the phospho flow cytometry data for p-S6 measurement, S6 phosphorylation of the same sample cohort was also assessed using western blot which showed reduced S6 phosphorylation upon mTOR inhibition in TTL^{short} leukemia confirming our phospho flow cytometry data (Figure 20C). On the other hand, BEZ235 reduced 4EBP1 phosphorylation to similar extent in both TTL^{short} and TTL^{long} leukemias. In contrast rapamycin had no effect on p-4EBP1 activity (Figure 21).

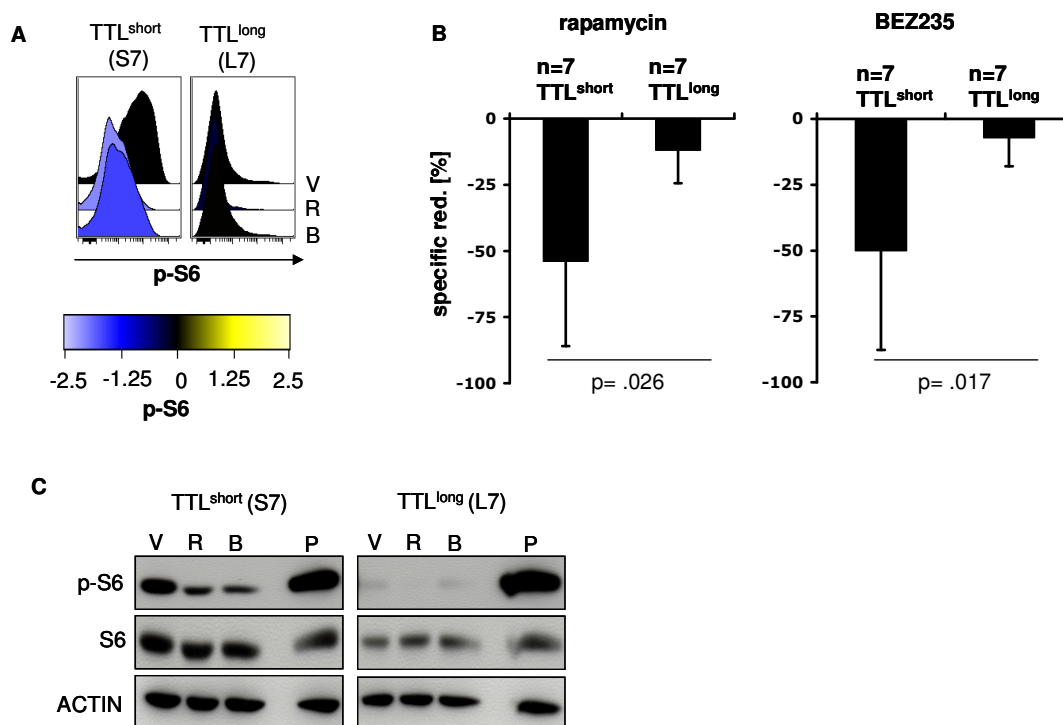


Figure 20: Effective inhibition of hyperactivated mTOR signaling in TTL^{short} ALL

(A) Rapamycin (R) and NVP-BEZ235 (B) reduce S6-phosphorylation with equal effectivity in TTL^{short} but not TTL^{long} ALL as shown by representative histogram; V, vehicle. Histograms represent median fluorescence intensities (MFI), arcsinh-transformed data and reduced (negative, blue) phosphorylation according to the colorimetric scales. (B) Diagrams show specific reduction of S6 phosphorylation of TTL^{short} (n=7) or TTL^{long} (n=7) samples. Columns represent mean values of triplicate measurements of each sample with corresponding standard deviations; Mann-Whitney U test; p, significance. (C) Western blot analysis of representative TTL^{short} and TTL^{long} leukemia showing reduction of S6 phosphorylation upon mTOR inhibition in TTL^{short} leukemia; V, vehicle; R, rapamycin; B, BEZ235; P, pervanadate incubated ALL cells (positive control).

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget 6(3): 1382-1395 (2015).

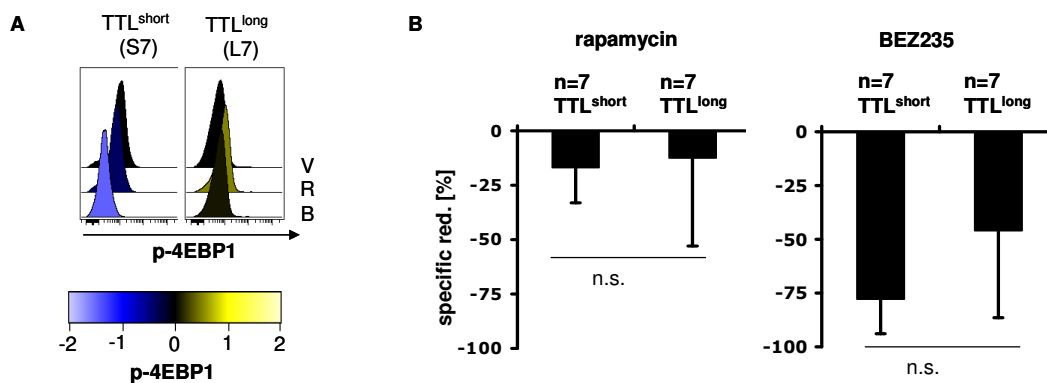


Figure 21: Reduction of 4EBP1 phosphorylation by BEZ235

(A) ATP competitive inhibitor NVP-BEZ235 (B), not the allosteric inhibitor rapamycin (R) reduces 4EBP1-phosphorylation in TTL^{short} and TTL^{long} ALL as shown by representative histogram; V, vehicle. Histograms represent median fluorescence intensities (MFI), arcsinh-transformed data, reduced (negative, blue) phosphorylation according to the colorimetric scales. (B) Diagrams show specific reduction of 4EBP1 phosphorylation of TTL^{short} (n=7) or TTL^{long} (n=7) samples. Columns represent mean values of triplicate measurements of each sample with corresponding standard deviations; Mann-Whitney U test; n.s., not significant.

3.5.2 No AKT activation upon mTOR pathway inhibition

Previously, it has been shown that the PI3K-AKT pathway is activated by feedback activation in response to mTOR inhibition (O'reilly, Rojo et al. 2006, Rodrik-Outmezguine, Chandarlapaty et al. 2011). To investigate feedback activation of the AKT in response to mTOR inhibition, AKT phosphorylation was assessed upon mTOR inhibition by rapamycin and NVP-BEZ235. Corresponding to inactive upstream PI3K-AKT signaling, rapamycin or BEZ235 did not affect p-AKT in both TTL phenotypes, therefore confirming the absence of feedback activation upon mTOR inhibition in these ALL samples and also explaining equal effectivity of the mTOR inhibitor rapamycin with dual PI3K-mTOR inhibitor BEZ235 (Figure 22).

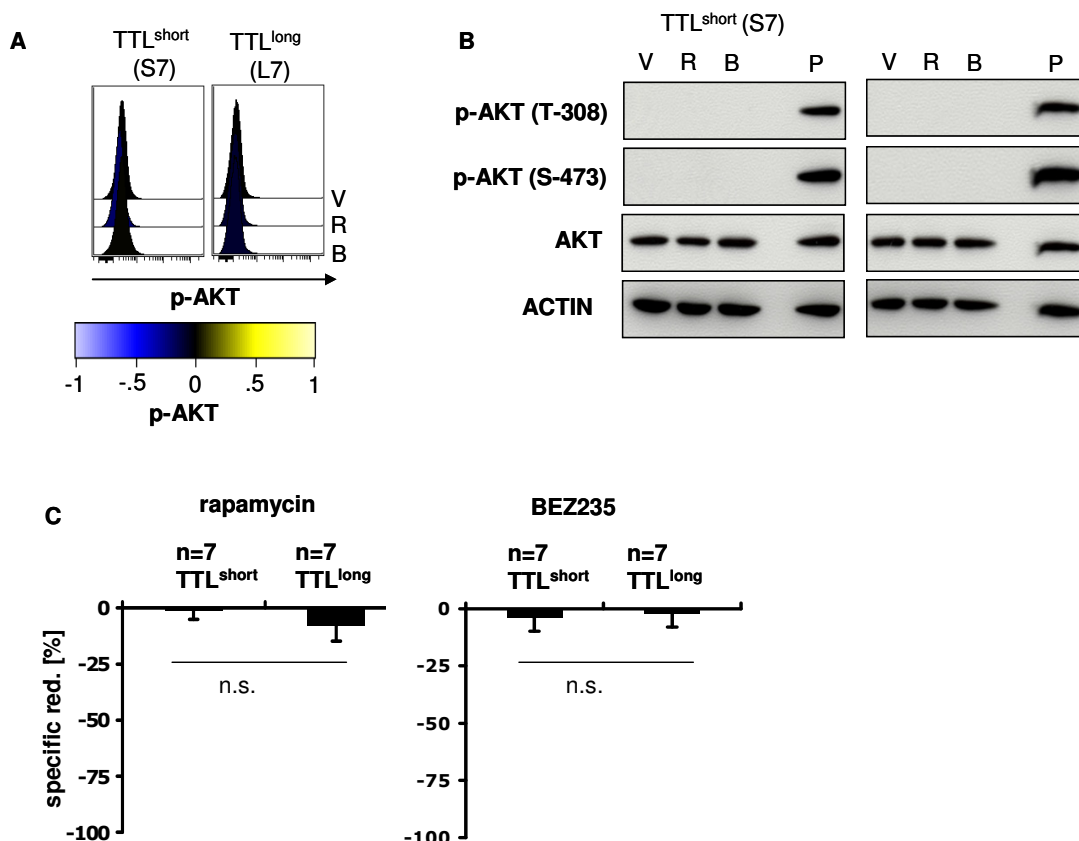


Figure 22: No feedback AKT activation or reduction of its activity upon mTOR inhibition

(A, B) Neither rapamycin (R) nor NVP-BEZ235 (B) reduces AKT activity or activates it by feedback mechanism in TTL^{short} and TTL^{long} ALL as shown by representative histogram and western blot; V, vehicle; R, rapamycin; B, BEZ235; P, pervanadate incubated ALL cells (positive control). Histograms represent median fluorescence intensities (MFI), arcsinh-transformed data; blue color (negative value) indicates reduced phosphorylation according to the colorimetric scales. (C) Diagrams show specific reduction of AKT phosphorylation of TTL^{short} (n=7) or TTL^{long} (n=7) samples. Columns represent mean values of triplicate measurements of each sample with corresponding standard deviations; Mann-Whitney U test; n.s., not significant.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.5.3 Effect of mTOR inhibition on BCP-ALL cell lines and xenograft cells *ex vivo*

3.5.3.1 mTOR inhibition reduces S6 phosphorylation *in vitro*

Two BCP-ALL cell lines (Nalm-6, KOPN-8) were used to address effects of mTOR inhibition on cell proliferation, cell cycle and apoptosis *in vitro* since it is difficult to culture and expand primary cells *ex vivo*. To check whether mTOR inhibition *in vitro* also abrogate signaling activity, S6 phosphorylation was assessed in response to mTOR inhibition by phospho flow cytometry and western blot using Nalm-6 and KOPN-8 cells. Consistent with the findings in primary xenograft ALL samples, rapamycin and NVP-BE235 reduced S6 phosphorylation in both cell lines while constitutive p-AKT was absent and remained unaffected upon treatment. To show AKT activation in a system using our methodology the T-ALL cell line (Jurkat) was treated in the same way as was done for other two BCP-ALL cell lines. In all the cell lines, mTOR inhibition by rapamycin and dual PI3K-mTOR inhibitor BEZ235 reduced the p-S6 levels whereas only BEZ235 reduced AKT phosphorylation in Jurkat cells (Figure 23).

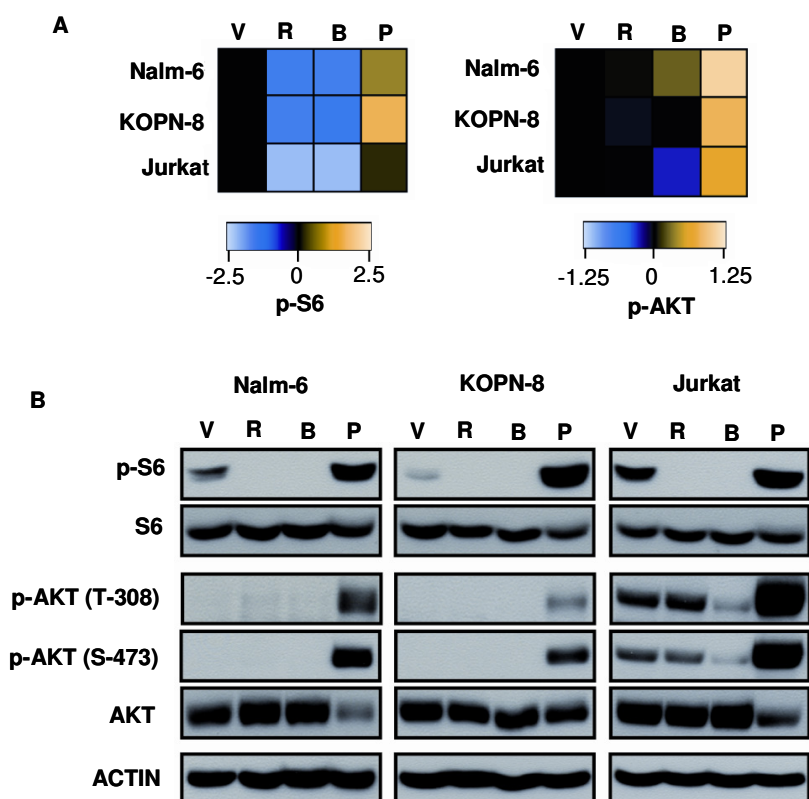


Figure 23: Rapamycin and BEZ235 inhibits mTOR pathway *in vitro*

(A, B) Nalm-6, KOPN-8 and Jurkat cells were treated with rapamycin and BEZ235 for 3 hours and p-S6 and p-AKT level was assessed by phospho flow cytometry and western blot. Constitutive S6 phosphorylation but no AKT phosphorylation in BCP-ALL cell lines Nalm-6 and KOPN-8 in contrast T-ALL cells (Jurkat) show constitutive AKT phosphorylation. Rapamycin (R) and BEZ235 (B) reduce p-S6 but not p-AKT in Nalm-6 and KOPN-8. In Jurkat cells, p-AKT is reduced by BEZ235; V, vehicle; P, pervanadate treated cells (positive control). Heatmaps represent median fluorescence intensities (MFI); arcsinh-transformed data; yellow represents increased phosphorylation and blue represents decreased phosphorylation according to the colorimetric scales.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.5.3.2 *In vitro* mTOR inhibition suppresses cell proliferation

To assess the effect of mTOR inhibition on cell proliferation, two BCP-ALL cell lines (Nalm-6 and KOPN-8) were treated with mTOR inhibitors and proliferation was assessed counting the cells by trypan blue exclusion and intracellular Ki67 staining which is a marker for cell proliferation (Kee, Sivalingam et al. 2002, Urruticoechea, Smith et al. 2005). Both inhibitors suppressed the proliferation as shown by the reduced cell number and low Ki67+ cells in response to rapamycin and NVP-BEZ235 treatment (Figure 24A, B). Additionally, cell cycle was analyzed by staining the DNA with 7-Aminoactinomycin D (7-AAD). Both inhibitors arrested the cell cycle in G0/G1 phase as demonstrated by an increase in G0/G1 cell population upon mTOR inhibition (Figure 24C).

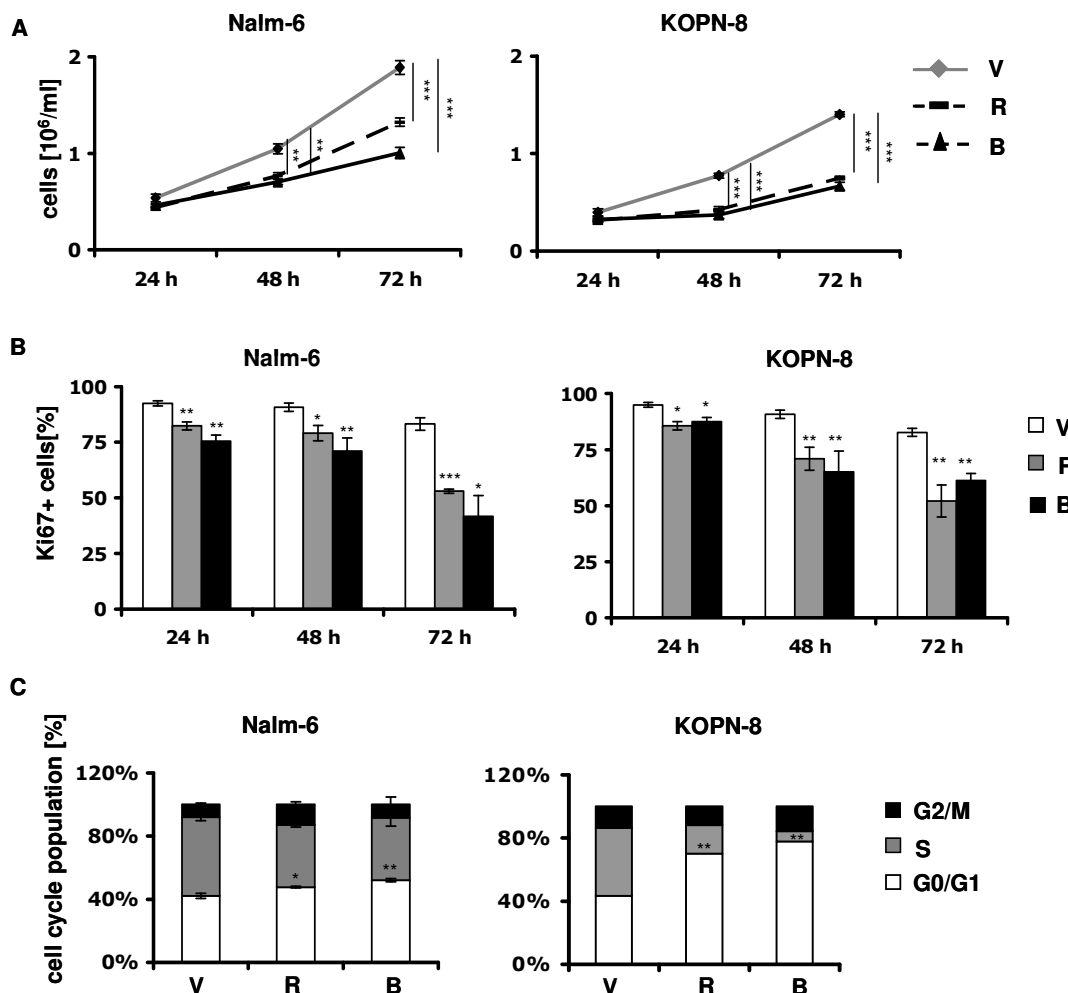


Figure 24: *In vitro* mTOR inhibition suppresses cell proliferation

Nalm-6 and KOPN-8 cells were treated with rapamycin and BEZ235 for 3 hours and cell proliferation was assessed counting the cells by trypan blue exclusion method and intracellular Ki67 staining. (A) Decreased cell growth and (B) decreased Ki67+ cells upon rapamycin (R) and BEZ235 (B) treatment; V, vehicle. (C) Rapamycin and BEZ235 arrest cell cycle in G0/G1 phase in both the cell lines; V, vehicle; R, rapamycin; B, BEZ235. Data points and columns represent mean values of triplicate measurement with corresponding standard deviations; Student's T test compared to vehicle; * < .05, ** < .01, *** < .001.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget 6(3): 1382-1395 (2015).

3.5.3.3 mTOR inhibition does not induce cell death or apoptosis *in vitro* or *ex vivo*

Cell death was assessed using TTL^{short} xenograft ALL cells in response to mTOR inhibition *ex vivo*. No cell death was observed in xenograft ALL cells as determined by forward scatter/side scatter (FSC/SSC) criteria (Figure 25A). Additionally, to assess whether mTOR inhibition induces apoptosis, xenograft ALL cells, Nalm-6 and KOPN-8 cells were treated with rapamycin and NVP-BEZ235 and the cell death/apoptosis was assessed by annexin V/Propidium Iodide (PI) staining. Consistently, no apoptosis was detected in both the cell lines as well as TTL^{short} xenograft cells in response to both the inhibitors (Figure 25B). Proteins were extracted from the same experiments and caspase 3 activity was assessed by western blot. No caspase 3 activation was detected in response to mTOR inhibition by rapamycin and BEZ235 whereas active caspase 3 was detected upon staurosporine treatment (Figure 25C). These data indicate that, despite reduction in cellular proliferation, mTOR inhibition does not induce cell death or apoptosis *in vitro*.

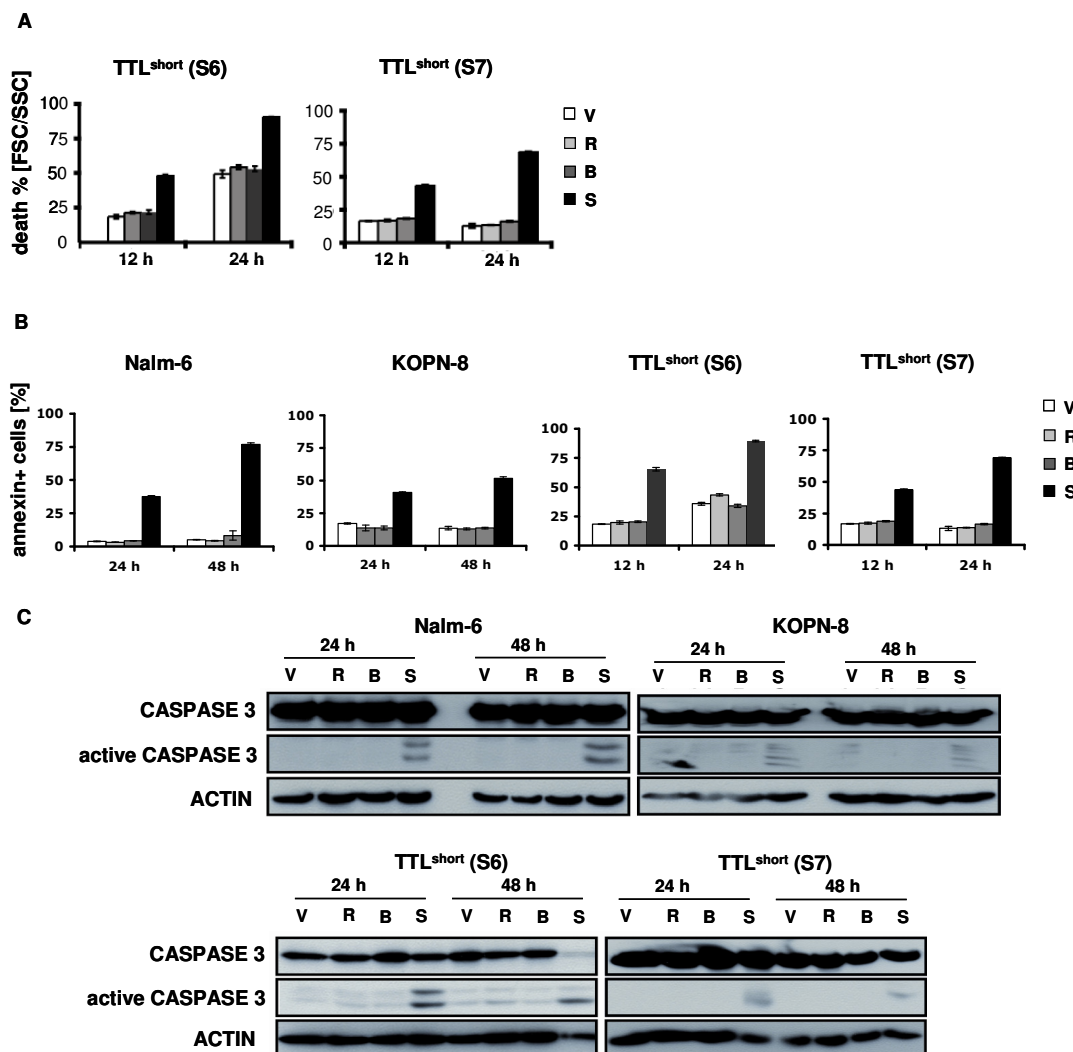


Figure 25: mTOR inhibition does not induce apoptosis *in vitro* or *ex vivo*

(A) No induction of cell death upon rapamycin (R) and NVP-BEZ235 (B) treatment in xenograft ALL cells *ex vivo* as determined by forward scatter/side scatter (FSC/SSC) criteria; V, vehicle. Staurosporine (S) treated cells were used as a positive control for cell death induction. (B) Rapamycin (R) and NVP-BEZ235 (B) do not induce cell death in Nalm-6 and KOPN-8 cell lines and also in xenograft ALL cells (S6 and S7) *ex vivo* as indicated by the absence of annexin V positivity. Staurosporine (S) treated cells were used as a positive control for apoptosis induction; V, vehicle. Data points and columns represent mean values of triplicate measurement with corresponding standard deviations. (C) No apoptosis upon mTOR inhibition shown by the absence of active caspase 3. Staurosporine (S) treated cells were used as a positive control for apoptosis induction; V, vehicle.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.5.3.4 *In vitro* mTOR inhibition does not induce autophagy

Earlier reports showed that mTOR inhibition led to autophagy induction in different cancer cell lines (Crazzolara, Cisterne et al. 2009, Fan, Cheng et al. 2010, Neri, Cani et al. 2014). To assess autophagy induction upon mTOR inhibition Nalm-6 and KOPN-8 cells were treated with rapamycin and NVP-BEZ235 for indicated times and conversion of LC3I to LC3II was detected by western blot. No conversion was LC3 was detected in response to mTOR inhibition indicating no autophagy induction upon mTOR inhibition in BCP-ALL cell lines whereas G40 glioblastoma cells showed autophagy upon mTOR inhibition. In line, no autophagy was detected in TTL^{short} (S5, S6, and S7) xenograft ALL cells upon *ex vivo* mTOR pathway inhibition (Figure 26).

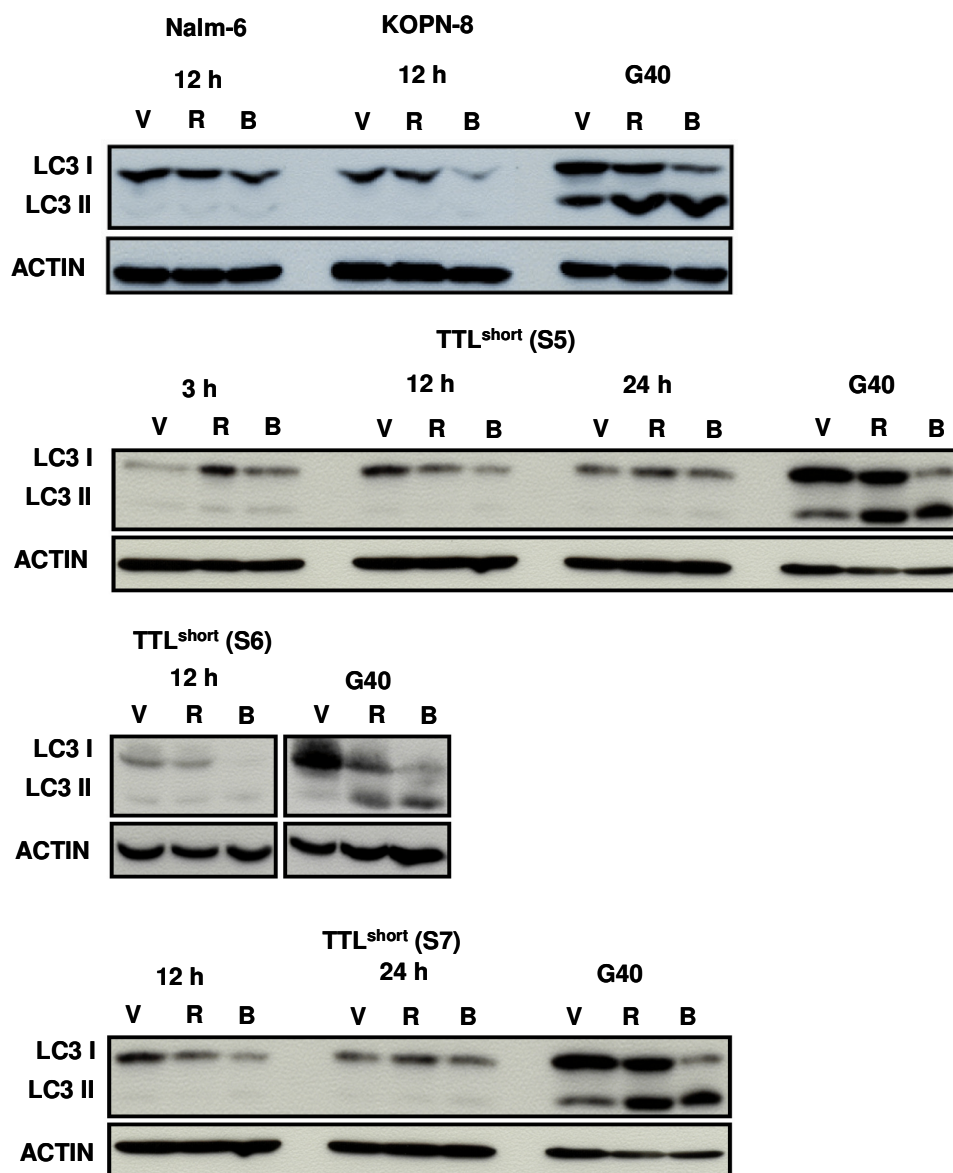


Figure 26: mTOR inhibition does not induce autophagy *in vitro* or *ex vivo*

Inhibition of mTOR signaling for indicated time with Rapamycin (R) and NVP-BEZ235 (B) does not induce autophagy in Nalm-6 and KOPN-8 cell lines *in vitro* and also in xenograft ALL cells (S5, S6 and S7) *ex vivo* as indicated by the absence of LC3 conversion. G40 glioblastoma cells were used as positive control for autophagy induction upon mTOR inhibition.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget 6(3): 1382-1395 (2015).

Taken together, both inhibitors showed an anti-proliferative effect on BCP-ALL cell lines *in vitro* but no induction of cell deaths either by apoptosis or autophagy was detected. Interestingly, the dual PI3K-mTOR inhibitor NVP-BEZ235 did not show a superior anti-proliferative effect compared to the mTOR inhibitor rapamycin and no feedback activation of the PI3K-AKT signaling was detected. These findings prompted us to use rapamycin in further *in vivo* testing.

3.5.4 Effects of *in vivo* mTOR pathway inhibition in high-risk ALL

To assess the *in vivo* effectivity of rapamycin on high-risk ALL, mice were transplanted with a TTL^{short} (S7) leukemia cells and treatment was initiated when leukemia cells reached 5% in the mouse peripheral blood. Mice were treated with rapamycin or DMSO as vehicle control for five consecutive days and mice were sacrificed on day 6. Leukemia cells were isolated from the spleens of the recipient animals and used for further *ex vivo* analyses.

3.5.4.1 *In vivo* rapamycin treatment reduces mTOR activity

Upon *in vivo* rapamycin treatment a significant reduction in S6 phosphorylation was observed compared to vehicle treated mice. However, very low levels of constitutive p-AKT were found in both treatment groups and likewise no reduction in AKT activity was found upon rapamycin treatment (Figure 27). These findings are consistent with the previous observation of absent PI3K-AKT activity in all the xenografts used in this study and BCP-ALL cell lines.

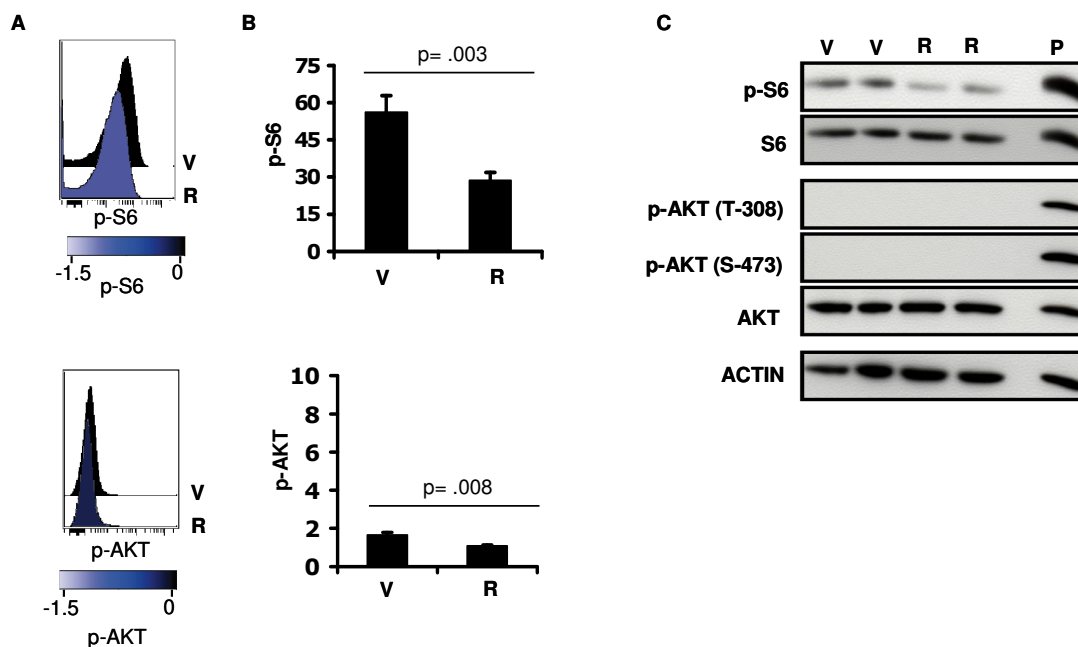


Figure 27: *In vivo* rapamycin treatment reduces mTOR signaling activity in *TTL^{short} ALL*

(A, B, C) High constitutive p-S6 is reduced upon rapamycin (R) treatment, while p-AKT remains low constitutively and no reduction upon rapamycin treatment. Assessment of protein phosphorylation by cytometry (n=3/group) and western blot (n=2/group); P, pervanadate. Histograms represent median fluorescence intensities (MFI) of p-S6 and p-AKT, arcsinh-transformed data; reduced phosphorylation according to the colorimetric scale as depicted by blue color. Diagrams show MFI relative to cellular autofluorescence of p-S6 or p-AKT. Data points and columns represent mean values of triplicate measurement with corresponding standard deviations. Student's T test compared to vehicle; p, significance.

Originally published in *Oncotarget*; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* **6**(3): 1382-1395 (2015).

3.5.4.2 *In vivo* rapamycin treatment reduces cell proliferation

In order to assess the effect of rapamycin treatment on the cellular proliferation *in vivo*, the cells isolated from the splenic cell suspension were co-stained with Ki67 and human CD19 followed by flow cytometric analysis. Significantly decreased

Ki67 positive cells were observed in rapamycin treated mice compared to vehicle treated mice indicating anti-proliferative effect of rapamycin treatment *in vivo* (Figure 28A). Additionally, immunohistochemical staining for Ki67 and human CD19 of bone marrow sections of rapamycin and vehicle treated mice were performed. Consistent with the results obtained with the splenic cells, bone marrow sections of rapamycin treated mice had less Ki67 positive cells compared to the vehicle treated mice although in both treatment groups CD19 positive cells were present to a similar extent of high leukemia infiltration (Figure 28B). Additionally, cell cycle analysis of the splenic cells showed significant increase in the G0/G1 population in rapamycin treated mice compared to vehicle treated mice; in line with the results of the less proliferating cells in rapamycin treated mice (Figure 28C).

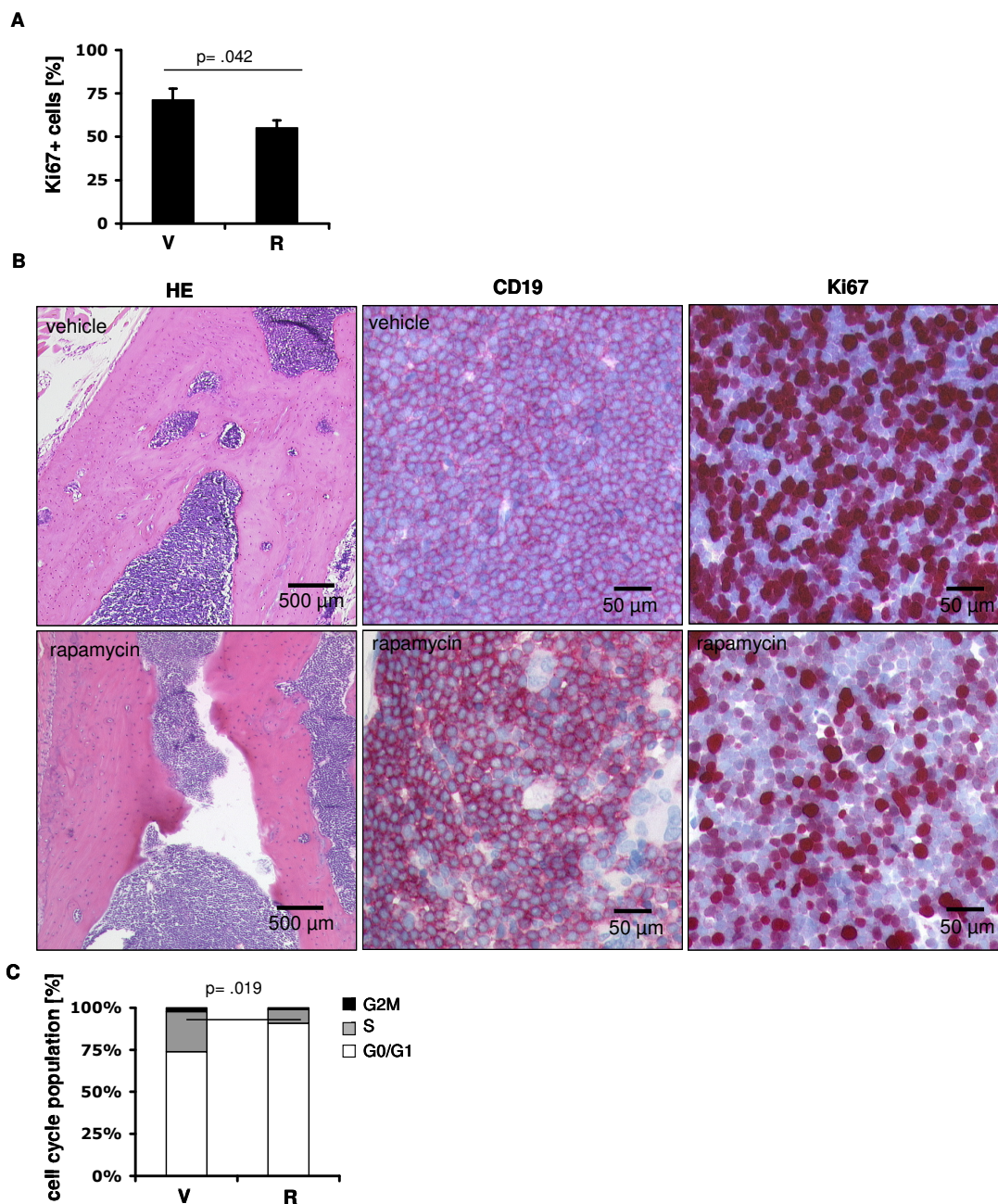


Figure 28: *In vivo* rapamycin treatment stops cellular proliferation

TTL^{short} leukemia (S7) bearing mice were treated with rapamycin (R) or vehicle (V) for 5 days. (A) Decreased Ki67-positive cells in the spleen of rapamycin treated mice compared to vehicle treated mice. (B) Hematoxylin/eosin (HE), human CD19 (CD19), and Ki67 staining of bone marrow sections of vehicle (upper row) or rapamycin treated (lower row) ALL bearing recipients, showing reduced Ki67-positivity upon *in vivo* rapamycin treatment. (C) Increased proportions of cells in

G0/G1 phase in rapamycin treated mice compared to vehicle treated mice. V, vehicle; R, rapamycin; columns represent mean values of triplicate measurements with corresponding standard deviations, Student's T test compared to vehicle; p, significance.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.5.4.3 *In vivo* rapamycin treatment does not induce cell death/apoptosis

Previous experiments demonstrated no cell death or apoptosis induction upon *in vitro* or *ex vivo* mTOR inhibition. Here, apoptosis induction was assessed *in vivo* by annexin V staining of spleen cells and detection of caspase 3 cleavage by western blot. No difference in annexin positivity or caspase 3 activities between rapamycin and vehicle treatment was observed indicating no apoptosis induction upon rapamycin treatment *in vivo* (Figure 29A, B). Detection of apoptosis *ex vivo* might be influenced due to the loss of apoptotic cells during the isolation of leukemia cells by ficoll gradient centrifugation and subsequent washing. To circumvent this problem, immunohistochemical stainings for active caspase 3 of spleen sections of rapamycin and vehicle treated mice were performed. No caspase 3 cleavage were observed in the rapamycin or vehicle treatment group whereas strong caspase 3 cleavage was observed in chemotherapy treated mice used as positive control for apoptosis induction (Figure 29C). These results indicate that rapamycin treatment does not induce apoptosis *in vivo*.

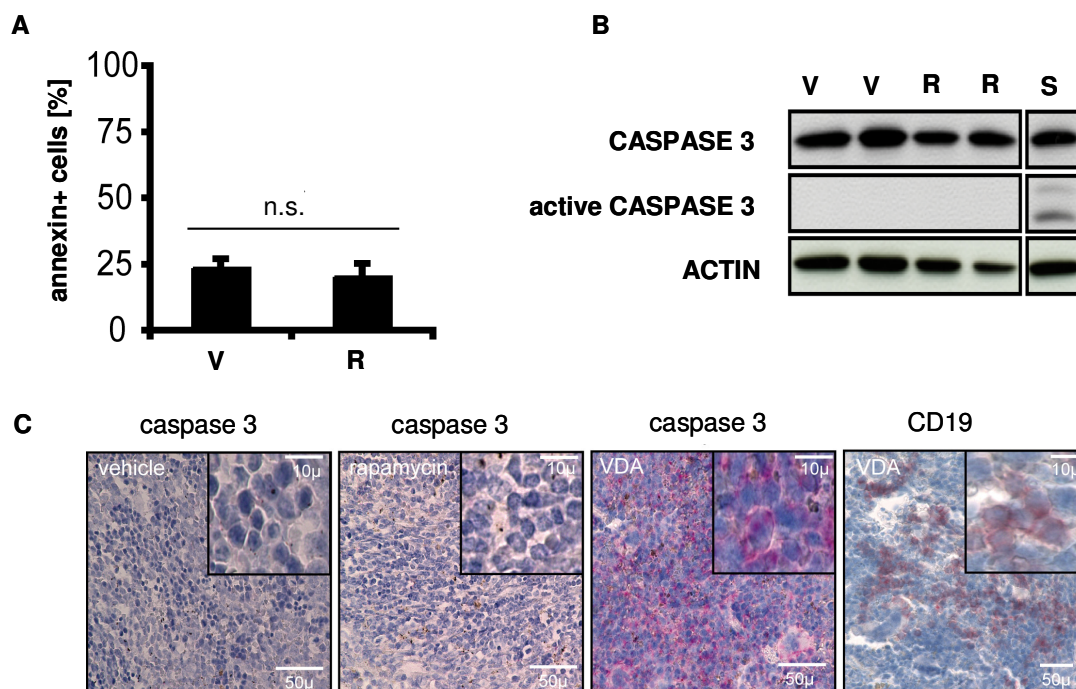


Figure 29: mTOR inhibition does not induce apoptosis *in vivo*

(A, B) No difference in annexin V positivity and caspase3 activity between rapamycin and vehicle treated mice indicating no apoptosis induction upon *in vivo* rapamycin treatment. Staurosporin (S) treated cells were used as positive control for caspase 3 activation; V, vehicle; R, rapamycin. Columns represent mean values of triplicate measurements with corresponding standard deviations, Student's T test; n.s., not significant. (C) Spleen sections of ALL bearing recipients treated with vehicle, rapamycin or remission induction chemotherapy (vincristine, dexamethasone asparaginase, VDA; one dose) stained for cleaved caspase 3. Presence of cleaved caspase 3 indicates apoptosis induction upon remission induction chemotherapy but not rapamycin treatment *in vivo*.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget 6(3): 1382-1395 (2015).

3.5.4.4 mTOR inhibition does not induce autophagy *in vivo*

In addition to apoptosis, autophagy induction upon mTOR inhibition *in vivo* was assessed. Proteins were isolated from splenic cell suspension of two rapamycin and two vehicle treated mice and conversion of LC3I to LC3II was detected by

western blot. No conversion of LC3 was detected in response to mTOR inhibition in contrast clear LC3 conversion detected in G40 glioblastoma cells upon rapamycin and NVP-BEZ235 treatment, used as a positive control for autophagy induction (Figure 30).

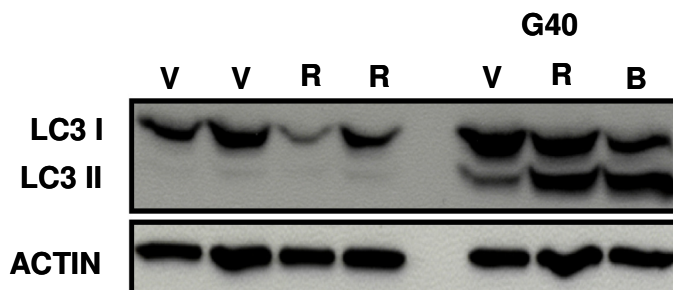


Figure 30: No autophagy induction upon *in vivo* rapamycin treatment

No LC3 conversion in TTL^{short} ALL upon *in vivo* rapamycin treatment but clear LC3 conversion in G40 glioblastoma cells upon mTOR inhibition (n=2/group); V, vehicle; R, rapamycin; B, BEZ235.

Originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. Oncotarget 6(3): 1382-1395 (2015).

3.6 mTOR inhibition as a targeted therapy for the TTL^{short}/early relapse ALL in a preclinical setting

By gene expression analysis it was previously shown that molecules of the mTOR pathway were differentially regulated among TTL phenotypes indicating potential association of activated mTOR pathway in high-risk/TTL^{short} leukemia. Additionally, *ex vivo* data also showed that leukemia with a rapid engraftment phenotype was characterized by a hyperactivated mTOR pathway which can be inhibited by mTOR inhibitors. Hence, the potential effectivity of the mTOR inhibition on TTL^{short} and TTL^{long} leukemia was addressed in a preclinical *in vivo* setting. Leukemia cells were transplanted into NOD/SCID mice intravenously and leukemia progression was monitored weekly in the peripheral blood (PB) of recipient mice detecting huCD19⁺ cells by flow cytometry. Treatment was initiated when leukemia cells reached approximately 5% in mouse PB and treatment was

continued for 10 days in two weeks. Mice were monitored regularly for the clinical signs of leukemia and sacrificed upon leukemia onset. High infiltration of human leukemia cells were detected in mouse peripheral blood, bone marrow and spleen. Time to reoccurrence (TTR) was calculated for each recipient animal from the date of treatment to the date of leukemia related morbidity.

3.6.1 Dose optimization for *in vivo* mTOR inhibition

In order to optimize the amount of rapamycin administration, different doses of rapamycin were tested *in vivo*. A TTL^{short} leukemia (S6) was transplanted and treated with 5 mg/kg and 2.5 mg/kg of rapamycin for 2 weeks and TTR was calculated for both doses. No superior delay in post treatment leukemia growth with high dose as compared to the lower dose of rapamycin was observed (difference of median survival between vehicle and rapamycin treated groups, 1.75 weeks for both the doses) (Figure 31). This result prompted us to use the lower dose (2.5 mg/kg) for the future *in vivo* experiments since higher dose might induce toxicity especially if combined with other agents.

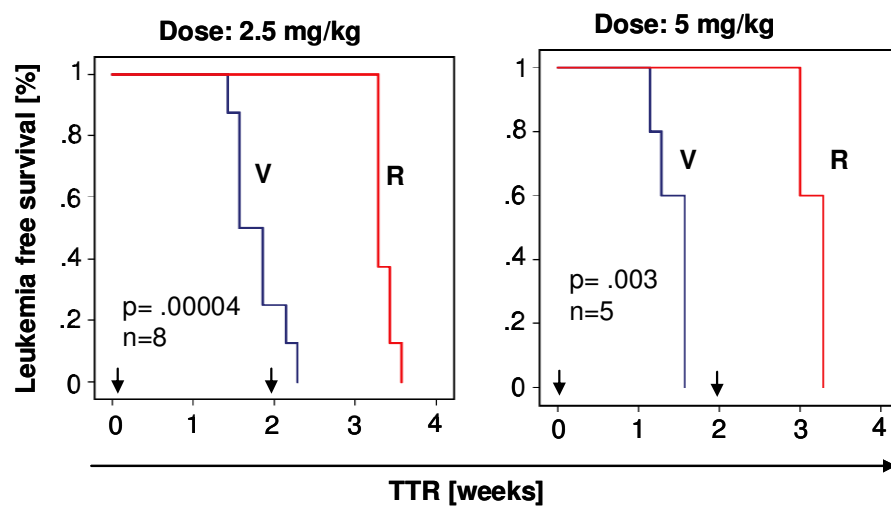


Figure 31: Dose optimization for the targeting of mTOR hyperactivation *in vivo* by rapamycin

In TTL^{short} (S6) leukemia, lower dose of rapamycin (R) showed similar post treatment leukemia free survival of the recipient animals as compared to the higher dose of rapamycin. Arrows indicate treatment intervals (2 weeks); 5 to 8

animals per treatment group; TTR, time to leukemia reoccurrence; Kaplan Meier analysis, log rank test; p, significance.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.6.2 *In vivo* mTOR inhibition by rapamycin delays leukemia onset in TTL^{short}/early relapse ALL

Three individual patient derived TTL^{short} leukemias (S5, S6 and S7) and two TTL^{long} leukemias (L6 and L7) were investigated to analyze mTOR signaling inhibition by rapamycin *in vivo*. Upon manifestation of leukemia as determined by the presence of 5% ALL cells in peripheral blood, mice were treated either with rapamycin (R) and vehicle (V) for two weeks and time until leukemia reoccurrence (Time to reoccurrence, TTR) was calculated for each recipient animal. Upon rapamycin administration, in all the three TTL^{short} ALL a significant post treatment delay of leukemia growth was observed. In contrast, rapamycin treatment did not show significant post treatment delay of leukemia growth in the TTL^{long} leukemias. Only a very small delay of post treatment leukemia growth observed in one TTL^{long} ALL (Figure 32, Table 9).

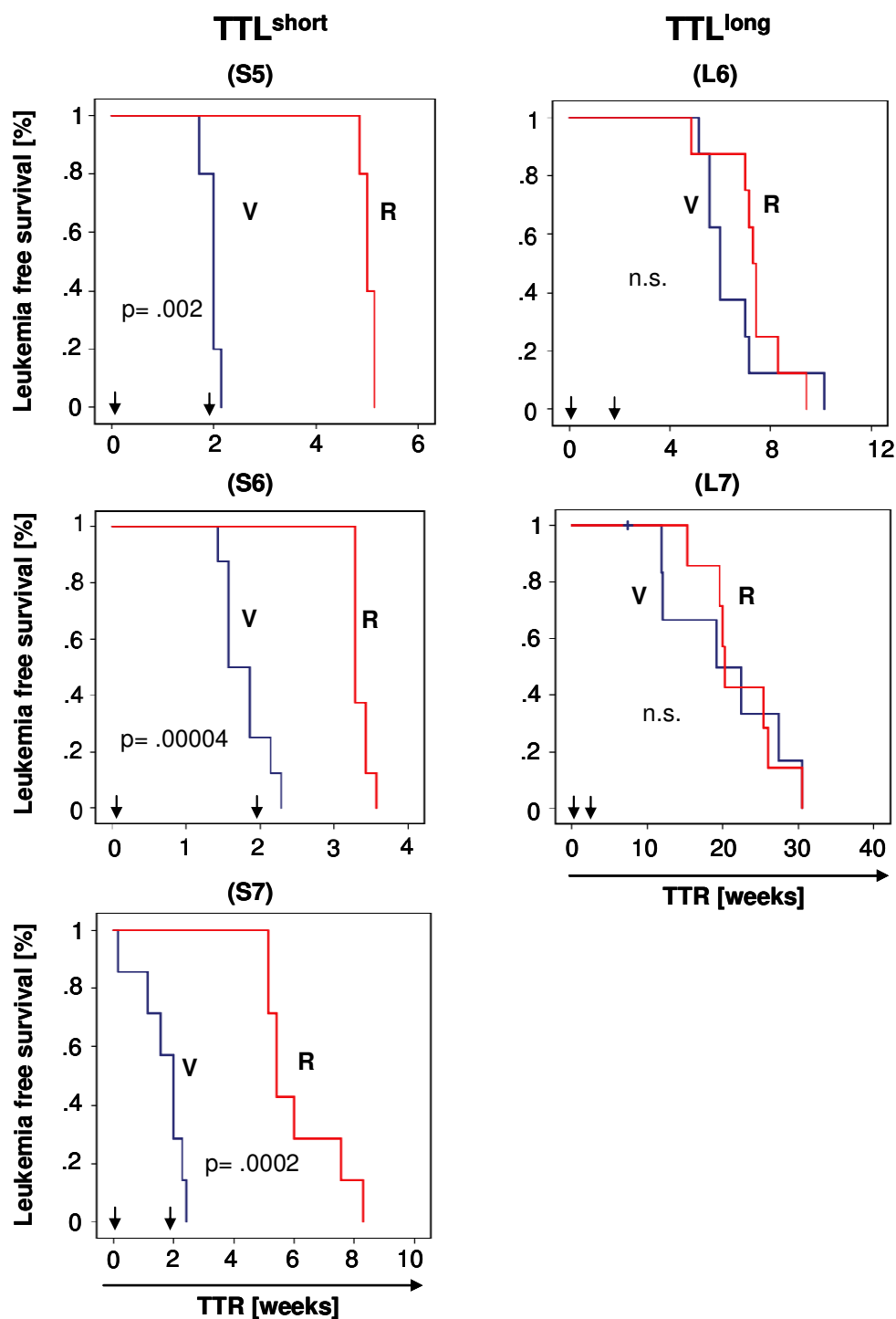


Figure 32: Targeting of hyperactivated mTOR signaling *in vivo* by rapamycin prolongs survival of mice transplanted with TTL^{short} ALL

Superior leukemia free survival of recipient animals transplanted with TTL^{short} (S5, S6, and S7) leukemia, but not TTL^{long} leukemia (L6 and L7) in response to *in vivo*

rapamycin (R) treatment compared to vehicle (V) treatment. Two weeks treatment interval is indicated by arrows; 5 to 8 animals per treatment group; TTR, time to leukemia reoccurrence; Kaplan Meier analysis, log rank test; p, significance; n.s., not significant).

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* **6**(3): 1382-1395 (2015).

Table 9: Probability of leukemia free survival of recipient animals engrafted with TTL^{short} or TTL^{long} ALL in response to rapamycin treatment.

LFS, leukemia free survival; SE, standard error; CI, confidence interval; p, significance (log rank test); and -, not applicable.

Leukemia	Treatment	Total	Censored	Events	Median LFS [weeks]	SE	CI	p
S5	vehicle	5	0	5	2.00	.085	1.83-2.16	
	rapamycin	5	0	5	5.00	.078	4.85-5.15	.002
S6	vehicle	8	0	8	1.57	.12	1.33-1.8	
	rapamycin	8	0	8	3.29	-	-	.00004
S7	vehicle	7	0	7	2.00	.26	1.49-2.50	
	rapamycin	7	0	7	5.43	.18	5.06-5.79	.0002
L6	vehicle	8	0	8	6.00	.29	5.42-6.57	
	rapamycin	8	0	8	7.28	.13	7.02-7.5	.34
L7	vehicle	7	1	6	19.14	6.38	6.62-31.66	
	rapamycin	7	0	7	20.28	.37	19.55-21.02	.9

Originally published in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* **6**(3): 1382-1395 (2015).

3.6.3 mTOR inhibition in combination with chemotherapy results in superior leukemia free survival of mice engrafted with TTL^{short}/early relapse ALL

The effectiveness of rapamycin in combination with chemotherapy was investigated since in a clinical setting it is unlikely to treat leukemia with a single

agent. Vincristine, dexamethasone and asparaginase are the agents generally used in remission induction therapy for the treatment of ALL. Combination of the above-mentioned agents were used as chemotherapy resembling induction regimen and used alone or in combination with rapamycin for the combination therapy. Individual patient derived a TTL^{short} (S6) and a TTL^{long} (L6) leukemia were treated with chemotherapy alone (VDA) or in combination with rapamycin (VDA+R). In TTL^{short} leukemia, a superior post treatment leukemia free survival in response to combination therapy compared to chemotherapy alone was observed. On the other hand, combination therapy showed very short delay of post treatment leukemia growth in the TTL^{long} leukemia with a median survival of 64 and 67 days respectively for the mice treated with chemotherapy alone compared to combination of chemotherapy and rapamycin (Figure 33, Table 10).

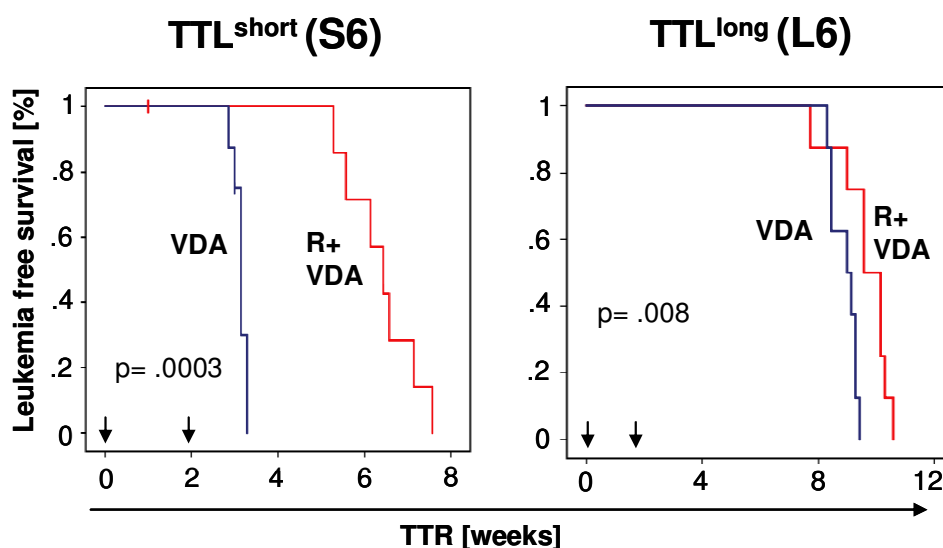


Figure 33: Rapamycin in combination with chemotherapy yield superior survival in TTL^{short} ALL

Superior leukemia free survival of recipient animals transplanted with TTL^{short} (S6) leukemia, but not TTL^{long} leukemia (L6) in response to combination of remission induction chemotherapy and rapamycin (vincristine, dexamethasone, asparaginase and rapamycin; VDA+R) compared to chemotherapy (VDA) alone. Arrows indicate treatment intervals (2 weeks); 8 animals per treatment group;

TTR, time to leukemia reoccurrence; Kaplan Meier analysis, log rank test; p, significance.

Modified from originally published article in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

Table 10: Probability of leukemia free survival of recipient animals engrafted with TTL^{short} or TTL^{long} ALL in response to combination of rapamycin and chemotherapy used in remission induction therapy.

LFS, leukemia free survival; SE, standard error; CI, confidence interval; p, significance (log rank test).

Leukemia	Treatment	Total	Censored	Events	Median LFS [weeks]	SE	CI	p
S6	VDA	8	1	7	3.14	.056	3.03-3.25	.0003
	VDA+R	8	1	7	6.42	.374	5.69-7.16	
L6	VDA	8	0	8	9.00	.50	8.01-9.99	.008
	VDA+R	8	0	8	9.57	.40	8.78-10.36	

Originally published in Oncotarget; Hasan MN, Queudeville M, Trentin L, Eckhoff SM, Bronzini I, Palmi C, Barth T, Cazzaniga G, te Kronnie G, Debatin KM, Meyer LH: Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia in a pre-clinical model. *Oncotarget* 6(3): 1382-1395 (2015).

3.7 Assessment of mTOR at diagnosis as a prognostic marker

In order to improve the treatment outcome of high-risk/early relapse patients, it is quite clear that new treatment strategies, ideally molecular targeted therapies are in dire need. Since the data presented in this study indicate association of hyperactivated mTOR pathway with high-risk ALL and its potential to be targeted with signal transduction inhibitors in combination with chemotherapy; now the challenge is to identify patients upfront at diagnosis who will benefit by mTOR targeted therapy. Two samples from primary ALL patients at diagnosis were investigated to assess the mTOR pathway activation using phospho flow cytometry analyzing S6 phosphorylation. One patient had an *MLL* and other an *ETV6-RUNX1* rearrangement. According to risk stratification by cytogenetics, *ETV6-RUNX1* is associated with good prognosis whereas *MLL* rearrangement is

associated with poor prognosis (Teitell and Pandolfi 2009). ALL cells of the patient with *MLL* rearrangement showed higher mTOR activity in contrast to low mTOR activity in the patient with *ETV6-RUNX1* rearrangement. Importantly, mTOR pathway activity could be repressed in the leukemic cells with *MLL* rearrangement but not in the cells with *ETV6-RUNX1* rearrangement (Figure 34). This result indicates that patients associated with hyperactivated mTOR signaling can be identified upfront at diagnosis providing options for treatment with compounds targeting mTOR signaling activity.

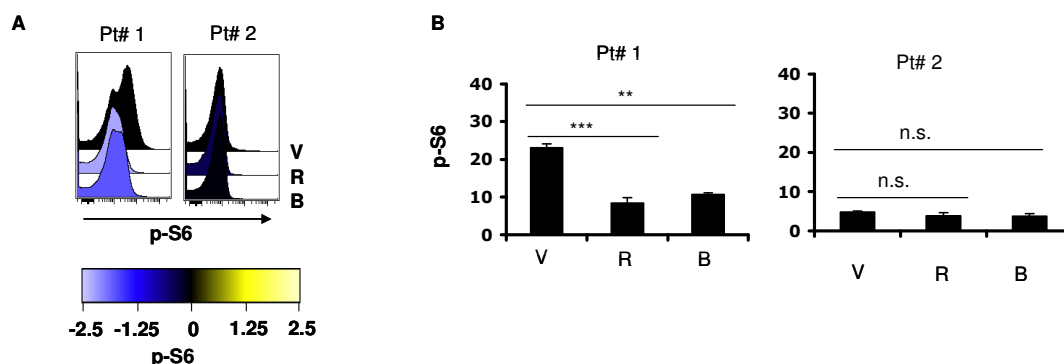


Figure 34: Higher mTOR signaling activity in high-risk ALL patient

(A, B) High mTOR signaling and significant reduction in signaling activity upon mTOR inhibition in patient 1 (*MLLr*) but not in patient 2 (*ETV6-Runx1*). V, vehicle; R, rapamycin; B, NVP-BEZ235; pt, patient. Histograms represent median fluorescence intensities (MFI), arcsinh-transformed data, reduced (negative, blue) phosphorylation according to the colorimetric scales. Columns represent mean values of triplicate measurement with corresponding standard deviations; Student's T test compared to vehicle; ** < .01, *** < .001; n.s., not significant.

4. Discussion

In children and adolescents the most frequent malignant disorder is acute lymphoblastic leukemia (ALL) with 80% cure rates, largely due to the improvement of multiagent chemotherapy regimen although 20% of patients encounter relapse (Conter, Arico et al. 2010, Pui and Evans 2006, Pui and Jeha 2007, Schrappe, Nachman et al. 2010, Schrappe, Reiter et al. 2000). Most of the conventional drugs used to treat ALL either target DNA, or inhibit nucleic acid synthesis, or interfere with the mitotic spindle formation and possess a narrow therapeutic index (Pui and Jeha 2007). On the other hand, the major cause of treatment failure is relapse for which a number of causes have been identified and inadequate therapy is the main cause (Pui and Evans 2006, Schrappe, Hunger et al. 2012). This dismal treatment outcome warrants for alternative strategies for the early identification of high-risk ALL as well as for therapies targeting aberrant signal transduction pathways. Previously, it has been identified that *in vivo* proliferation of patient leukemia cells transplanted onto NOD/SCID mice is indicative of poor patient outcome and characterized by a specific transcript profile involving survival pathways (Meyer, Eckhoff et al. 2011). The present study addresses activity of mTOR, a key survival pathway, in xenograft ALL cells on a functional level including preclinical evaluation of therapy targeting this key cellular pathway.

4.1 NOD/SCID/huALL xenotransplant mouse model

Previously, a NOD/LtSz-scid/scid (NOD/SCID) xenotransplantation mouse model was established by our research group in order to study the molecular characteristics of ALL (Meyer, Eckhoff et al. 2011). Also in the present study, NOD/SCID xenotransplantation mouse model was used to address mTOR pathway activation and its potential as a molecular target for alternative treatment for TTL^{short}/high-risk ALL. Leukemia cells engrafted in our NOD/SCID model retained the intrinsic clinical and immunophenotypic characteristics (Meyer, Eckhoff et al. 2011), in line to the previous reports (Borgmann, Baldy et al. 2000, Lock, Liem et al. 2002, Nijmeijer, Mollevanger et al. 2001). Also the infiltration of leukemic blasts into different organs correlates with the proportion of leukemic cells present in the mouse peripheral blood (Lock, Liem et al. 2002, Nijmeijer,

Mollevanger et al. 2001). Unique to other studies, the xenotransplantation model used by our group described a short engraftment phenotype (time to leukemia short, TTL^{short}) and a long engraftment phenotype (time to leukemia short, TTL^{long}) upon transplantation of a series of primary ALL samples. Importantly, the TTL phenotype correlates with patient outcome. Patients with a TTL^{long} phenotype had superior survival compared to the patients with a TTL^{short} phenotype (Meyer, Eckhoff et al. 2011).

4.2 *In silico* prediction for the association of mTOR pathway with TTL phenotype

Earlier, a specific gene expression profile pointed differential regulation of molecules of the mTOR pathway between TTL phenotypes. In this study, gene set enrichment analysis (GSEA) identified enrichment of the TTL profile in mTOR annotated data sets further supporting involvement of the mTOR signaling in rapid leukemia engraftment in NOD/SCID mice and early patient relapse. Additionally, connectivity map analysis (CMAP) was performed to connect genes underlying rapid engraftment phenotype and potential drugs to revert the phenotype. CMAP analysis identified very strong similarity between the TTL signature with gene expression profile of mTOR pathway inhibitors further pointing to the association of mTOR pathway in driving rapid leukemia engraftment. Importantly, it points to the effectiveness of mTOR inhibition in TTL^{short}/high-risk ALL to revert the phenotype. In line to these findings, hyperactivated mTOR signaling activity was detected in xenograft ALL cells derived from patients associated with TTL^{short}/high-risk phenotype. Importantly, this activated pathway could be targeted by mTOR inhibitor rapamycin *ex vivo* and *in vivo*. Chemical genomic screening have been used to predict mTOR as a potential target in cancer such as breast cancer (Zhang, Cohen et al. 2014) and AML (Hassane, Sen et al. 2010). Additionally, this approach has been used to identify rapamycin as a potential agent to reverse the glucocorticoid resistance in ALL via down-regulating anti-apoptotic protein MCL1 (Wei, Twomey et al. 2006) further supporting the usefulness of chemical genomics approach in understating molecular mechanism of disease or a given phenotype.

4.3 mTOR pathway as a potential therapeutic target

4.3.1 mTOR pathway in cancer

mTOR signaling is a central pathway that controls cellular growth, proliferation and survival (Soliman 2013). mTOR is regulated by the AKT dependent PI3K pathway as well as by AKT independent mechanisms such as hypoxia, the Ras/MEK/ERK pathway, energy sensing and amino acids (Memmott and Dennis 2009). In addition to PI3K-AKT, Notch signaling positively regulates mTOR pathway in T-ALL (Chan, Weng et al. 2007). Phosphatase and tensin homolog (PTEN) is a lipid phosphatase that dephosphorylates PtdIns(3,4,5)P3 to PtdIns(4,5)P2 to negatively regulate the PI3K-AKT-mTOR pathway (Martelli, Chiarini et al. 2012). Mutational or functional loss of PTEN promotes the activation of PI3K-AKT signaling resulting in the development of T-ALL (Guo, Lasky et al. 2008, Hales, Taub et al. 2014, Palomero, Sulis et al. 2007, Silva, Yunes et al. 2008). In most AML patients PI3K-AKT-mTOR signaling is activated due to mutations of upstream receptor tyrosine kinases such as FLT3 and c-Kit (Vu and Fruman 2010). Also IGF-1/IGF-1R signaling and mutation of Ras family genes, N-RAS and K-RAS activate the PI3K-AKT signaling in AML (Park, Chapuis et al. 2010). Additionally, in Philadelphia-chromosome positive (Ph⁺) B-lineage ALL, activated PI3K/mTOR signaling is induced by BCR-ABL tyrosine kinase activity (Cilloni and Saglio 2012, Janes, Limon et al. 2010, Kharas, Janes et al. 2008, Redig, Vakana et al. 2011, Skorski, Kanakaraj et al. 1995).

Recently, it has been shown that a subtype of B-ALL, cytokine receptor-like factor 2 (*CRLF2*) rearranged ALL is associated with hyperactivated mTOR signaling (Maude, Tasian et al. 2012, Tasian, Doral et al. 2012). Being the ligand of *CRLF2*, thymic stromal-derived lymphopoietin (TSLP) binds to *CRLF2* and activates mTOR pathway (Brown, Hulitt et al. 2007). TSLP also plays important role in early B-cell proliferation and differentiation (Scheeren, Van Lent et al. 2010). About 50% *CRLF2* rearranged ALL also harbor *JAK2* mutation, the most common of which is *JAK2* (R683G) whereas almost all the *JAK2* mutated ALL are associated with *CRLF2* alterations (Tasian, Doral et al. 2012). All the samples used in this study did not carry any *BCR-ABL* gene fusion. Additionally, no alterations of *CRLF2* gene, high *CRLF2* transcript or TSLP receptor complex (TSLPR)

expression and *JAK2* mutation were detected. These findings indicate that hyperactivated mTOR pathway in all the samples used in this study is not due to BCR-ABL kinase activity or aberrant *CRLF2* and *JAK2* activity.

The Philadelphia chromosome-like ALL (Ph-like ALL) has been identified by gene expression profiling studies which have a kinase activating gene expression profile similar to that of BCR-ABL1 rearranged ALL (Den Boer, Van Slegtenhorst et al. 2009, Mullighan, Su et al. 2009). A recent study performed genomic profiling of a large number of BCP-ALL and described that kinase-activating alterations in ALL with Ph-like expression profile are associated with STAT5 activation (Roberts, Li et al. 2014). STAT5 is also activated in *CRLF2* rearranged and *JAK2* mutated leukemia (Tasian, Doral et al. 2012). Additionally, JAK2-STAT5 activity has been described to reduce the efficacy of mTOR inhibitors in breast cancer cells (Britschgi, Andraos et al. 2012). In this study, no difference in STAT5 activity was detected between TTL^{short} and TTL^{long} xenograft ALL indicating no cross-talk between STAT5 and mTOR signaling in all samples used in this study.

4.3.2 4EBP1 phosphorylation is insensitive to rapamycin

Despite being the downstream molecule of mTORC1, 4EBP1 phosphorylation in AML blasts is shown to be independent of mTORC1 activity (Tamburini, Green et al. 2009). Additionally, the phosphorylation of 4EBP1 is largely resistant to rapamycin although S6k1 phosphorylation is inhibited by rapamycin and rapalogs (Chiarini, Evangelisti et al. 2014, Wander, Hennessy et al. 2011). Both, mTORC1 and mTORC2 have been attributed for the phosphorylation of 4EBP1 as dual inhibitors of mTORC1 and mTORC2 reduced 4EBP1 phosphorylation but not the allosteric mTORC1 inhibitors in *BCR-ABL* or *TEL-ABL* (*ETV6-ABL*) positive ALL (Badura, Tesanovic et al. 2013). In line to these findings, in all the samples used in this study, p-4EBP1 was resistant to the allosteric mTORC1 inhibitor rapamycin but was sensitive to dual PI3K-mTOR inhibitor NVP-BEZ235 which also inhibits mTORC2 activity. However, only rapamycin was used for *in vivo* treatment experiments as no PI3K-AKT pathway activity was observed in any of the samples used in this study.

4.3.3 In BCP-ALL mTOR is not activated by PI3K-AKT signaling

In majority of cancers including breast cancer, prostate cancer, cancer of bladder, lung cancer, melanoma, renal cell carcinoma, ovarian cancer and glioblastoma (brain cancer) the mTOR signaling is activated by AKT activation (Guertin and Sabatini 2005). Activation of mTOR in hematological malignancies e.g. CML, AML and T-ALL is also triggered by AKT (Guertin and Sabatini 2005, Vu and Fruman 2010). However, in *CRLF2* wild type and the majority of Ph-negative BCP-ALL patient samples no AKT activation was observed (Morishita, Tsukahara et al. 2012, Tasian, Doral et al. 2012). In line with these results, no AKT activation was observed in any of the samples used in this study. Concomitantly, dual PI3K-mTOR inhibition by BEZ235 did not show greater repression of the signaling activity compared to mTOR inhibition alone by rapamycin in xenograft ALL cells *ex vivo*, regardless of the TTL phenotype. Additionally, in BCP-ALL cell lines no superior repression of cellular proliferation was observed by BEZ235 compared to rapamycin. In contrast, BEZ235 induced greater reduction of cellular proliferation compared to rapamycin in renal cell carcinoma which is characterized by activated AKT (Cho, Cohen et al. 2010). In line, AKT inhibition by specific AKT inhibitor (GSK690693) induced no superior survival of BCP-ALL xenografts, in contrast significant increase in survival in solid tumors e.g. osteosarcoma, glioblastoma were observed in response to AKT inhibition (Carol, Morton et al. 2010). Thus, unlike solid tumors and some other hematological malignancies, BCP-ALL samples used in this study are characterized by an inactivated AKT signaling.

It has been described that in malignancies, including acute myeloid leukemia with constitutive PI3K-AKT pathway activity, mTOR inhibition leads to feedback activation of the PI3K/AKT pathway (O'reilly, Rojo et al. 2006, Tamburini, Chapuis et al. 2008). In line with absent constitutive PI3K-AKT activity in our xenograft samples, no feedback PI3K-AKT activation was observed in response to mTOR inhibition. Additionally, no PI3K-AKT activation as well as feedback activation in response to mTOR inhibition was observed in BCP-ALL cell lines in contrast to constitutively active PI3K-AKT pathway in T-ALL cell line.

4.4 mTOR inhibition decreases cellular proliferation but does not induce apoptosis and autophagy

Inhibition of mTOR signaling by rapamycin or rapalogs decreases cellular proliferation in BCP-ALL cell lines (Brown, Fang et al. 2003, Neri, Cani et al. 2014). Concomitantly, in this study, decreased cellular proliferation was observed upon *in vitro* mTOR inhibition using two BCP-ALL cell lines. More importantly, decreased proliferation was detected upon *in vivo* mTOR inhibition in line with previous findings showing reduced *in vivo* growth of leukemic cells upon mTOR inhibition (Crazzolara, Cisterne et al. 2009, Maude, Tasian et al. 2012, Teachey, Obzut et al. 2006). Despite the reduced proliferation upon mTOR inhibition, no cell death was observed in BCP-ALL cell lines, xenograft ALL cells *ex vivo* as well as upon *in vivo* treatment. However, divergent findings of apoptosis induction by mTOR inhibition have been reported. No apoptosis was detected in primary AML cells upon *ex vivo* treatment with rapamycin (Ryningen, Reikvam et al. 2012). Additionally, *in vitro* mTOR inhibition by rapamycin did not induce apoptosis in T-ALL cell lines (Guo, Zhou et al. 2013). In contrast, apoptosis was detected in about half of 15 pediatric primary BCP-ALL samples upon rapamycin treatment (Avellino, Romano et al. 2005). In line to these findings, no apoptosis induction was observed in lymphoma cell lines (Mallya, Fitch et al. 2014) and BCP-ALL cell lines (Crazzolara, Cisterne et al. 2009) in response to mTOR signaling inhibition.

Alike apoptosis, no autophagy was detected upon *in vitro*, *ex vivo* and *in vivo* mTOR signaling inhibition. Conflicting findings have been reported about autophagy induction in response to mTOR inhibition as well as the role of autophagy in cell death. Appearance of double or multiple vesicles engulf bulk cytoplasm or cytoplasmic organelles resulting in autophagic cell death (Gozuacik and Kimchi 2004). However, other evidences indicate that autophagy is primarily a pro-survival rather than a cell death mechanism at least in cells with intact apoptotic machinery (Levine and Yuan 2005). In BCP-ALL cell lines, mTOR inhibition has been reported to induce autophagy (Crazzolara, Cisterne et al. 2009, Neri, Cani et al. 2014). In contrast, in AML cells autophagy has been reported to promote cell survival with inhibition of autophagy resulting in more potent anti-leukemic effect of mTOR inhibition (Altman, Szilard et al. 2014). Thus,

the absence of autophagy upon mTOR inhibition in BCP-ALL cells might induce more potent anti-leukemic effect when treated with mTOR inhibitors.

4.5 *In vivo* mTOR inhibition

In this study, a significant delay of post-treatment leukemia reoccurrence upon mTOR inhibition was observed in recipients engrafted with TTL^{short}/high-risk ALL consistent with reduced cellular proliferation upon mTOR signaling inhibition *in vitro*. Similarly, mTOR inhibitors led to post-treatment delay of leukemia growth in xenografted B-lineage ALL samples (Crazzolara, Cisterne et al. 2009, Houghton, Morton et al. 2008, Maude, Tasian et al. 2012, Teachey, Obzut et al. 2006) although incidences of no effect or less effect of mTOR inhibition were observed (Crazzolara, Cisterne et al. 2009, Houghton, Morton et al. 2008, Teachey, Obzut et al. 2006). However, no survival benefit or delay in post-treatment leukemia reoccurrence was observed in recipients engrafted with TTL^{long} ALL. This explains the low or no effectivity of *in vivo* mTOR inhibition as reported by earlier reports in contrast to the current study.

In a clinical setting, the major questions to treat acute leukemia are to define the risk-adapted alternative therapy and to determine whether current chemotherapy is sufficient for the treatment (Schiller 2013). It is unlikely to treat leukemia with a single agent targeting aberrant signaling activity. To efficiently establish rapamycin in a clinical setting for the treatment of high-risk ALL, it would be necessary to combine it with multiagent chemotherapy. In the current study, a clear post treatment delay of leukemia reoccurrence was observed in TTL^{short}/high-risk ALL in response to combination treatment compared to chemotherapy alone. Likewise, *in vivo* mTOR inhibition with temsirolimus in combination with cytostatic drug methotrexate was synergistic for ALL (Teachey, Sheen et al. 2008). Also, everolimus in combination with vincristine increased overall survival of mice engrafted with ALL over the single agents (Crazzolara, Cisterne et al. 2009). Additionally, positive interaction between mTOR inhibitors and different chemotherapeutic agents has been reported for solid tumors (Houghton, Morton et al. 2008, Mondesire, Jian et al. 2004, Stromberg, Dimberg et al. 2004). However, in TTL^{long} ALL, combination treatment did not exert superior effect over

the chemotherapy alone. Therefore, mTOR inhibitors in combination with chemotherapy may provide an excellent strategy for the treatment of TTL^{short}/high-risk ALL.

4.6 Assessment of mTOR pathway activation at diagnosis

Since TTL^{short}/high-risk ALL is associated with hyperactivated mTOR signaling, it is necessary to identify patients at diagnosis who will benefit of mTOR directed therapy. Study consisting appropriate sample size is necessary to address this issue. However, in order to get preliminary insight, mTOR pathway activation of primary cells derived from bone marrow aspirates of two BCP-ALL patient's employing phospho flow cytometry was investigated. Higher constitutive mTOR activity was detected in cells carrying *MLL* rearrangement in contrast cells with *ETV6-RUNX1* gene fusion showed low mTOR activity. Moreover, upon pathway inhibition, signaling activity was repressed in cells carrying *MLL* rearrangement in contrast no repression of signaling activity in cells carrying E/R gene fusion. In recent years few reports demonstrated the successful use of phospho flow cytometry to assess mTOR pathway (Tasian, Doral et al. 2012), PI3K-AKT pathway and JAK2-STAT5 pathway activation (Gomes, Soares et al. 2014, Tasian, Doral et al. 2012) of BCP-ALL patient primary material. Kotecha and colleagues successfully used phospho flow cytometry to monitor the disease status of juvenile myelomonocytic leukemia (JMML) patients at diagnosis, remission, relapse and transformation and detected the abnormal signaling profile (Kotecha, Flores et al. 2008). Phospho flow cytometry was also used to identify JMML patients by p-STAT5 profiling, although it was not used to stratify the patients into different risk-groups (Hasegawa, Bugarin et al. 2013). The phospho flow cytometry technique was successfully used to discover the disrupted relationship between pSYK/pPLC γ 2 in chronic lymphocytic leukemia (CLL) indicating potential of phospho flow cytometry to further classify subtypes of CLL (Palomba, Piersanti et al. 2014). Thus, evaluation of mTOR signaling activity including pretreatment evaluation of different inhibitors at diagnosis by phospho flow cytometry technique is a feasible strategy for early identification of patients with high-risk for relapse. Therefore, phospho flow cytometry technique can be implemented into current routine immunophenotypic diagnostic work-up.

In addition to the reports discussed here, clinical trials are currently ongoing to evaluate the efficacy of mTOR inhibition by different substances to treat leukemia. The clinicaltrials.gov database currently enlists 8 clinical trials employing different mTOR inhibitors to treat leukemia. However, rational strategy of using mTOR inhibitors alone or in combination with chemotherapy is necessary. The preclinical data of mTOR inhibition alone or in combination with chemotherapy point to its efficacy only in high-risk/TTL^{short} ALL, not in TTL^{long}/low-risk ALL providing rational of using mTOR inhibitors in treating leukemia. Patients associated with TTL^{short} phenotype encountered poor treatment outcome are not associated with classical high-risk criteria (Meyer, Eckhoff et al. 2011) which emphasize the importance of upfront identification of patients who will benefit from mTOR directed therapy. The current study provides an excellent strategy for early identification of ALL patients high-risk for relapse and thereby providing opportunity for novel therapeutic strategy targeting aberrant signaling activity.

5. Summary

In order to improve the dismal treatment outcome of acute lymphoblastic leukemia (ALL) patients with high-risk for relapse, novel treatment strategies targeting aberrant signaling that promotes leukemogenesis are necessary. A previous study of our group showed that a short engraftment phenotype is indicative for high-risk for relapse or poor treatment outcome. In this study, the involvement of mTOR pathway in rapid leukemia engraftment was investigated functionally. In TTL^{short}/high-risk ALL, hyperactivation of the mTOR pathway with effective repression of signaling activity upon mTOR inhibition was detected. But, unlike to other cancer types, mTOR pathway in BCP-ALL is not activated by upstream PI3K-AKT pathway with no superior reduction in signaling activity upon treatment with dual PI3K-mTOR inhibitor BEZ235 compared to only mTOR inhibition by rapamycin. Additionally, reduced *in vitro* and *in vivo* cellular proliferation upon mTOR inhibition was observed although no apoptosis or autophagy detected.

Rapamycin was used for *in vivo* mTOR pathway inhibition using a series of individual patient-derived xenograft with both TTL^{short} and TTL^{long} phenotypes, to confirm the efficacy of mTOR inhibition as a potential therapeutic strategy. In line to the *ex vivo* data, *in vivo* rapamycin administration resulted in superior survival of mice engrafted with TTL^{short} ALL, but not with TTL^{long} leukemia. The anti-proliferative activity of rapamycin without any apoptotic activity provides the rationale of combining rapamycin with chemotherapy to enhance the anti-leukemic effect. Combination of rapamycin with chemotherapy prolonged the survival of mice engrafted with TTL^{short} leukemia in contrast mice engrafted with TTL^{long} leukemia showed no superior survival upon combination treatment.

It is of utmost important to identify patients associated with TTL^{short} phenotype or high-risk for relapse upfront who will benefit of the mTOR directed therapy. Investigation of primary ALL cells from pediatric leukemia patients at diagnosis indicated the possibility to identify patients upfront for the association of the mTOR pathway activation. However, a study employing larger cohort of patients is necessary to validate initial results of the current study which will provide strong

basis of using phospho flow cytometry to identify high-risk patients upfront by assessing mTOR activity.

Taken together, ALL patients with high-risk for relapse are characterized by hyperactivated mTOR signaling which can be successfully targeted by signal transduction inhibitors; more efficiently in combination with remission induction multi-agent chemotherapy. This preclinical evidence of effectiveness of mTOR targeted therapy may provide opportunity for novel therapeutic options for the treatment of high-risk ALL patients.

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Declaration

I hereby declare that I wrote the present dissertation with the topic:

“Targeting of hyperactivated mTOR signaling in high-risk acute lymphoblastic leukemia as a novel treatment strategy”

independently and used no other aids other than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current rules of the University of Ulm for assuring good scientific practice.

Ulm

Md. Nabiul Hasan