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Prístupy k inhibícii mTOR dráhy v akútnych leukemických líniách

Approaches to mTOR Pathway Inhibition in Acute Leukemic Cell Lines

Bachelor's thesis

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Podpis

Abstrakt

Cicavčí cieľ rapamycínu (mTOR, mammalian target of rapamycin) je serin/threoninová kináza, ktorá reguluje bunkový rast, proliferáciu, metabolizmus a prežitie. mTOR dráha v sebe integruje stimuly rastových faktorov, živín a energetického stavu bunky a vedie k aktivácii Akt, 4E-BP1 a S6K. Fosforylácia 4E-BP1 a S6K smeruje k zvýšenej syntéze proteínov ako aj zvýšenej tvorbe ribozómov a hraje dôležitú úlohu v postupe bunkového cyklu. Zvýšenú aktivitu mTOR dráhy je možné pozorovať v rôznych typoch rakovín, vrátane akútnej myeolidnej leukémie (AML, acute myeloid leukemia) a akútnej lymfoblastickej leukémie (ALL, acute lymphoblastic leukemia), ktoré sú obe charakteristické neprirodzenou proliferáciou bielych krviniek a nízkou mierou prežitia pacientov.

Sú používané tri rôzne prístupy na inhibíciu mTOR dráhy, pričom sa líšia mierou úspešnosti a štádiom výskumu. Rapamycin a jeho deriváty sú najčastejšími inhibítormi, ale keďže nie sú jednoznačne špecifické, neponúkajú žiadané výsledky. Dvojaké inhibitory cielené na mTOR ako aj PI3K dráhu dosiahli niekoľko úspechov v pôsobení na AML a ALL bunky. Avšak nová generácia inhibítorov ako napríklad PP-242 alebo Torin-1 vykazujú najsľubnejšie výsledky s ohľadom na inhibíciu mTOR dráhy a dlhodobý ústup akútnej leukémie.

Kľúčové slová: inhibícia mTOR, akútna lymfoblastická leukémia, akútna myeloidná leukémia, analógy rapamycínu, PI-103, NVP-BEZ235, PP-242, OSI-027

Abstract

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase regulating cell growth, proliferation, metabolism and survival. The mTOR pathway integrates stimuli from growth factors, nutrients, and cellular energy status and leads to downstream activation of Akt, 4E-BP1 and S6K. Phosphorylation of 4E-BP1 and S6K results in increased protein synthesis in addition to ribosome biogenesis and plays an important role in cell cycle progression. mTOR pathway is overactivated in numerous cancer types including the acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), which are both characterized by abnormal proliferation of white blood cells and low patient survival rate.

Three distinct approaches that differ in efficiencies and research stages have been used to inhibit the mTOR pathway. Rapamycin and its derivatives are the most common inhibitors, but since they are not entirely specific, they provide only limited desirable outcomes. Dual inhibitors targeting mTOR as well as PI3K pathway have had several successes in treating both AML and ALL. However, the new generation of inhibitors such as PP-242 and Torin-1 are providing the most hopeful prospects for mTOR inhibition and long-term remission of acute leukemia.

Keywords: mTOR inhibition, acute lymphoblastic leukemia, acute myeloid leukemia, rapamycin analogs, PI-103, NVP-BEZ235, PP-242, OSI-027

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Glossary

Cell lines

CEM-R	drug resistant CEM-S
CEM-S	T-ALL cells
HL-60	AML promyeloblasts
Jurkat	T-ALL cells
K562	CML blasts
Kasumi 1	AML cells
Kasumi 1	AML cells
KBM-3B	AML cells
KG-1	AML cells
LK63	pre-B cells
MOLT-4	T-ALL cells
Nalm6	B-ALL cells
NB4	APL cells carrying t(15;17)
REH	B-cell precursor ALL
U937	hystiocytic lymphoma cells

Others

4E-BP1	eIF4E binding protein
Akt	protein kinase B
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Bcr-Abl	break point cluster region-abelson
CD34+	expressing Hematopoietic progenitor cell antigen CD34
CML	chronic myeloid leukemia
CR	complete response or remission
DEPTOR	DEP domain-containing mTOR-interacting protein
eIF4E	eukaryotic translation initiation factor 4E
eIF4F	complex of eIF4G, eIF4E and eIF4A
FKB12	peptidyl-prolyl cis-trans isomerase
FRAP	FKBP12-rapamycin-associated protein
FRB	FKBP12-rapamycin binding domain
GI_{50}	concentration for 50% maximal inhibition of cell proliferation
GβL	G protein beta subunit-like
IC_{50}	half maximal inhibitory concentration
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor
IRS1	insulin receptor substrate 1
IRS2	insulin receptor substrate 2
MCL	mantle cell lymphoma [I might delete this altogether]
MEC	mitoxantrone, etoposide, and cytarabine
mLST8	mammalian lethal with SEC13 protein 8
mSin1	mammalian stress-activated protein kinase-interacting protein 1
mTOR	mammalian target of rapamycin

mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
mTORC3	mammalian target of rapamycin complex 3
NOD/SCID	nonobese diabetic/severe combined immunodeficiency
p53	cellular tumor antigen p53
PDK1	phosphoinositide dependent kinase 1
PI3K	phosphoinositide 3-kinase 1
PIK3CA	phosphatidylinositol 3-kinase catalytic subunit alpha
PIKK	PI3K-related kinase
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PKCα	protein kinase C alpha
PR	partial response or remission
PRAS40	proline-rich Akt substrate 40 kDa
Protor	proline-rich protein 5
PTEN	phosphatase and tensin homolog on chromosome 10
Rac	Ras-related C3 botulinum toxin substrate
RAFT1	rapamycin and FKBP12 target
RAPT1	rapamycin target 1
Raptor	regulatory associated protein of mTOR
Rictor	rapamycin-insensitive companion of mTOR
S6K	S6 kinase
S6RP	S6 ribosomal protein
SEP	sirolimus effector protein
UTR	untranslated region

1. Introduction

Billions of cells in our bodies grow and divide everyday. For each cell to be allowed to undergo division, it has to pass several checkpoints monitoring availability of nutrients, successful replication of chromosomes, and intactness of DNA. However, the control mechanisms are not perfect and when the extent of damage is too high to be repaired a healthy cell undergoes apoptosis. Nevertheless, a cancerous cell is able to trick the switch machinery into continuing the cell cycle.

Cells typically accumulate several of these mutations before they start exhibiting cancerous potential including, but not limited to, deregulated response to growth factors, alternate cell cycle control, decreased apoptosis regulation, abnormal activity of transcription factors, and corrupted DNA repair mechanisms. As a result, they usually begin to divide uncontrollably, grow in numbers and may even invade other tissues.

Acute leukemia is a quickly progressing disease of white blood cells, during which abnormal white blood cells grow rapidly and prevent healthy production of normal white blood cells, erythrocytes, and platelets. Two types of acute leukemia are recognized: acute myeloid leukemia (AML) and acute lymphoblastic or lymphocytic leukemia (ALL). In case of AML, deregulations are present in cells developing from myeloblasts (neutrophils, eosinophils, or basophils) and in case of ALL in cells developing from lymphoblasts (B lymphocytes or T lymphocytes). Combinational chemotherapy and additional stem cell transplantations have greatly increased survival rates of patients but they still remain low with less than 50% in both AML and ALL.

One of the prominent deregulations in both leukemias is the mammalian target of rapamycin (mTOR) pathway, which is constitutively activated in almost all acute leukemia samples (Tamburini *et al*., 2009). This provides a great potential target for molecular treatment of acute lymphoblastic and myeloid leukemia. First molecule used to target mTOR was rapamycin in 1991 (Heitman, Movva, & Hall, 1991). Since then it has entered into stage III clinical trials and new inhibitors have been identified and synthesized (Demetri *et al*., 2013).

In this thesis I aim to delineate the different approaches to mTOR pathway inhibition in acute leukemia cells, list the individual inhibitors that have been tested, and comment on the efficacy of most prominent ones.

2. The mTOR pathway

2.1 mTOR kinase

The mTOR, also known as FRAP (FKBP12-rapamycin-associated protein), SEP (sirolimus effector protein), RAPT 1 (rapamycin target 1) or RAFT1 (rapamycin and FKBP12 target) is a serine threonine kinase with molecular weight of 289 kDa and is a member of the PI3K-related kinase (PIKK) family (Sabatini, Erdjument-Bromage, Lui, Tempst, & Snyder, 1994).

The target of rapamycin (TOR) was first identified owing to the discovery of a metabolic product possessing antifungal activity and isolated from bacterium *Streptomyces hygroscopicus* (Brown *et al*., 1994; Paiva, 1988). The kinase was first described in *Saccharomyces cerevisae*, and later was also identified in mammals and across eukaryotes (Chiu, Katz, & Berlin, 1994; Helliwell *et al*., 1994; Oldham, 2000).

mTOR has been found in two distinct complexes mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2), both of which are well characterized (Dowling *et al*., 2010; García-Martínez and Alessi, 2008). Additionally, another complex, mTORC3 (mTOR complex 3) is thought to be present in the cytoplasm of cells as well (Klein Geltink, 2014).

2.1.1 mTORC1

Five proteins make up the mTORC1 complex, which is sensitive to rapamycin. Besides mTOR, there are Raptor (regulatory associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein 8) also known as GβL (G protein beta subunit-like), and two negative regulators PRAS40 (proline-rich Akt substrate 40 kDa) and DEPTOR (DEP domain-containing mTOR-interacting protein) (Kim *et al*., 2003; Peterson *et al*., 2009; L. Wang, Harris, Roth, & Lawrence, 2007). Importance of Raptor lies in its ability to regulate mTOR based on the protein's phosphorylation (Kim *et al.*, 2002, 2003). mLST8 plays a critical role in ensuring rapamycin-sensitive interaction between Raptor and mTOR (Kim *et al*., 2003). DEPTOR interacts with both mTOR complexes and inhibits their activity (Peterson *et al*., 2009).

mTORC1 is activated by nutrients, growth factors, and cellular energy status through PI3K/Akt pathway. It regulates protein synthesis through its effect on S6K (S6 kinase) and 4E-BP1 (eukaryotic translation initiation factor 4E binding protein). Hyper-phosphorylation of the mRNA translation repressor, 4E-BP1, results in its dissociation from eIF4E (eukaryotic translation initiation factor 4E), which is thereafter free to act in initiation of translation. However,

phosphorylation of 4E-BP1 by mTOR alone is not sufficient for the dissociation from eIF4E. It only serves as a priming phosphorylation event and another kinases might be needed for additional phosphorylation of 4E-BP1 at its carboxy-terminal residues. The data is inconsistent regarding the number and function of phosphorylation sites (residues) in the 4E-BP1 protein (Burnett, Barrow, Cohen, Snyder, & Sabatini, 1998; Gingras *et al*., 1999; Yip, Murata, Walz, Sabatini, & Kang, 2010).

eIF4E plays an essential role in assembly of the eIF4F (complex of eIF4G, eIF4E and eIF4A) complex, which is crucial for translation of mRNAs significant for cell growth, apoptosis prohibition, and cell transformation. These mRNAs are difficult to translate because they contain long 5' UTR (untranslated region) rich in Gs and Cs. When mTORC1 activity is increased, 4E-BP1 is phosphorylated and does not bind eIF4E which can in turn function in the eIF4F complex and recruit the 40S ribosomal subunit to these mRNAs and initiate their translation (reviewed in Hay & Sonenberg, 2004; reviewed in Martelli *et al*., 2011).

S6K regulates IRS1 (insulin receptor substrate 1) an IRS2 (insulin receptor substrate 1) by substrate phosphorylation but also by repressing its transcription. Constitutive activation of mTOR/S6K decreases the activity of IRS1/2, conferring resistance of PI3K activation by insulin. PI3K inhibition results in downstream inhibition of Akt, and subsequent reduced activity of mTORC1. Additionally, inhibition of mTORC1 pathway activates Akt also through a negative feedback loop (Breuleux *et al*., 2009; Harrington *et al*., 2004; Shah, Wang, & Hunter, 2004).

2.1.2 mTORC2

mTORC2 is a complex of mTOR, mLST8, mSin1 (mammalian stress-activated protein kinase-interacting protein 1), Rictor (rapamycin-insensitive companion of mTOR), and Protor (proline-rich protein 5) proteins (Frias *et al*., 2006; Kim *et al*., 2003; Pearce, *et al*., 2007; D. Sarbassov et al., 2004). It phosphorylates Akt on Ser473 residue and several other kinases (Zeng *et al*., 2007). Among essential components are Rictor, mSin1, and mLST8. mSin1 is important for proper internal bonds in the complex between proteins and for phosphorylation of Akt on Ser473 (Frias *et al*., 2006). mLST8 is a stable factor present in both mTOR complexes (Kim *et al*., 2003), while Rictor is a protein exclusively present in mTORC2 (Sarbassov *et al*., 2004). DEPTOR, as mentioned before, negatively regulates mTORC2 (Peterson *et al*., 2009).

In contrast to mTORC1, mTORC2 is stimulated only by growth factors and the pathway of this stimulation contains PDK1–AKT (phosphoinositide dependent kinase 1 – protein kinase B

pathway) kinases. The complex phosphorylates PKCα (protein kinase C alpha), Akt and regulates activities of Rac (Ras-related C3 botulinum toxin substrate) and Rho GTPases with functions in cell survival, migration and cytoskeleton regulation (Gan, Wang, Su, & Wu, 2011; He *et al*., 2013; Jacinto *et al*., 2004; Sarbassov, Guertin, Ali, & Sabatini, 2005).

There has been a long debate on whether mTORC2 is sensitive to rapamycin or not. The one research that majority of the articles cite in favor of the mTORC2 rapamycin sensitivity is from 2006 and describes rapamycin's inhibitory effect on mTORC2 complex after pro-longed treatment (Sarbassov *et al*., 2006). However, it has been shown also elsewhere that rapamycin negatively affects mTORC2 signaling by inhibiting its assembly – in 8 AML samples only half of the usual amount of the complex was formed (Zeng *et al*., 2007).

2.1.3 mTORC3

Experiments with ATP-site inhibitors of mTOR such as Torin-1 provoked ideas about additional presence of mTOR protein in the cells, besides being part of the mTORC1/2 complexes. The reasoning behind it was that Torin-1 had greater inhibitory effect than rapamycin but was similar to the effect of lacking Raptor in cells. Moreover, Rictor knock-down did not affect Torin-1 action. Later, in 2014, Klein Geltink declared discovery of mTORC3 complex, that is rapamycin-insensitive and is highly activated in many human cancers. They also filed a patent for detection and modulation of the new complex. Unfortunately, there is an extremely limited amount of data concerning this complex and thus it will not be further discussed (Gerard C. Grosveld, Frank C. HARWOOD, 2012; Klein Geltink, 2014).

2.2 mTOR pathway deregulation in acute leukemia

For better understanding of the deregulations in the mTOR pathway and its implications, it is beneficial to put the pathway into context of the PI3K/Akt/mTOR signaling network (refer to Figure 1). Akt is a prominent effector of the PI3K (phosphoinositide 3-kinase 1) pathway and mTOR is activated by PI3K/Akt network (Silva et al., 2008). At the same time mTOR inhibition may activate Akt (Breuleux *et al*., 2009; Wan, Harkavy, Shen, Grohar, & Helman, 2007).

Q. Xu and colleagues determined that not only S6 kinase and 4E-BP1 are phosphorylated in AML blasts but also the PI3K pathway is constitutively activated thus displaying activation on multiple levels of the PI3K/Akt/mTOR signaling pathway. As the main reason for deregulated

PI3K they suggested the function of PTEN (phosphatase and tensin homolog on chromosome 10) tumor suppressor (Xu, Simpson, Scialla, Bagg, & Carroll, 2003). This is in accord with the findings that PTEN is a potent negative regulator of PI3K/Akt pathway thanks to its phosphatase activity and counter effect on PI3K, which generates PIP_3 (phosphatidylinositol (3,4,5)-trisphosphate) from PIP_2 (phosphatidylinositol (4,5)-bisphosphate) (Carpenter *et al*., 1990; Huang *et al*., 2012; Maehama & Dixon, 1998). ALL cells also exhibit elevated activities of the PI3K/Akt pathway and mTOR signaling, which play an important role in cell viability, proliferation and growth in acute leukemia cell lines (Barata *et al*., 2004; Hasan *et al*., 2014).

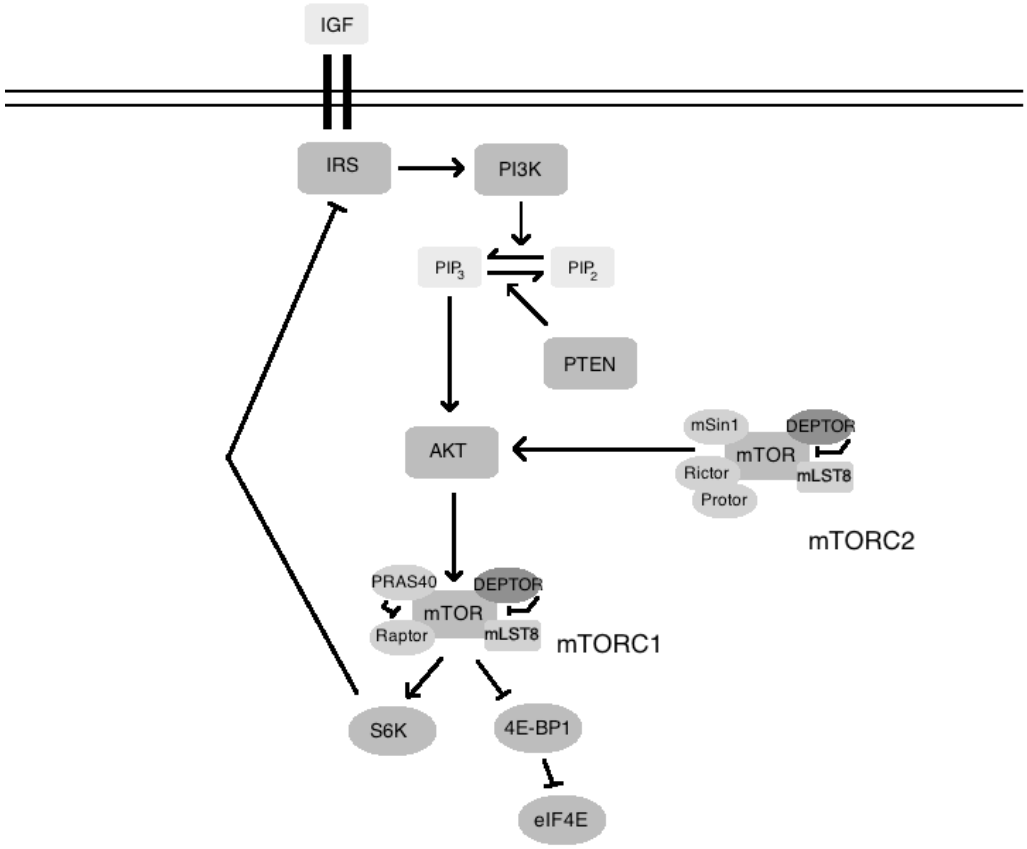

Figure 1: Diagram of simplified mTOR signaling network (see text for details) (adapted from review in Zhou & Huang, 2010).

Overall, the mechanisms that may lead to increased mTOR pathway activity in acute leukemia include:

- Mutations or overproduction of growth factor receptors
- Overexpression of growth factors
- Tumor suppressor gene losses – loss of PTEN is the most common (Gutierrez *et al*., 2009).

- Point mutations in PI3K gene – constitutive activation of PI3K or Akt is the most frequent (Gutierrez *et al*., 2009).
- Mutation in mTOR or mTOR-linked pathways leading to gain of function phenotype – for example fusion protein Bcr-Abl (break point cluster region-abelson) production leading to increased protein synthesis (Kharas *et al*., 2008).

2.3 Note on Inhibition of the mTOR pathway in acute leukemia cells

Rapamycin was the first drug used to allosterically inhibit the mTOR pathway in acute leukemia cell lines. There were positive results from the beginning, but of a somewhat limited scope. After realizing the connections within and mutual regulation of PI3K/Akt/mTOR network that was discussed in previous subchapters, an effort was made to investigate dual inhibition of the pathways. The most recent approach has been to develop so called new generation inhibitors that target the active site of the mTOR kinase.

3. Rapamycin and its analogs

Mode of action of rapamycin is described as formation of a complex with FKBP12 (peptidyl-prolyl cis-trans isomerase), which interacts with the C-terminal region of TOR proteins and hence inhibits their activity (Brown *et al*., 1994).

Rapamycin and its three analogs are used to inhibit mTOR in acute leukemia. Temsirolimus, everolimus, and deferolimus where synthesized in order to improve pharmacokinetic properties and lessen immunosuppressive effects of the therapeutic agent (Jianxin Gu, Mark E. Ruppen, Panolil Raveendranath, Warren Chew, 2007; Moenius *et al*., 1999; Récher *et al*, 2009; Rivera *et al*., 2011).

3.1 Rapamycin (sirolimus)

Rapamycin inhibits cell growth of HL-60 cell line in concentration of 50 nM and 100 nM, increases the portion of cells in G_1 phase and decreases the portion of cells in S phase. When it was used with daunorubicin (antitumor drug used to treat acute leukemia) the effects were even more pronounced compared to daunorubicin treatment alone (Fukushima, Ueda, Uchida, & Nakamura, 1993; Liang, Xiong, Wang, & Chen, 2010).

Rapamycin is able to inhibit blast cell growth in high-risk pediatric B-ALL mouse xenograft studies and significantly ($P < 0.01$) prolong survival compared to control mice (Maude et al., 2012). Furthermore, in ALL cells with blocked mitochondria apoptosis rapamycin induces autophagy (Bonapace *et al*., 2010).

Sirolimus showed to be a potent inhibitory agent in AMl in clinical setting when 51 subjects with relapsed, refractory or high-risk AML were exposed to rapamycin plus MEC (mitoxantrone, etoposide, and cytarabine) chemotherapy treatment for 9 days. They received 12 mg of rapamycin on day 1 and additional 2 mg per day for 8 days. The peak blood concentrations were 22 ng/ml and did not differ between clinically responding and non-responding patients. 24 (47%) subjects responded with either complete or partial relapse and the median time to relapse or death was 261 days (Kasner *et al*., 2012).

A phase I study of sirolimus with MEC chemoterapy was preformed on 29 subjects. 2 of them did not finish the 7-day treatment due to toxicity, 4 subjects underwent complete remission (CR), and 2 subjects partial remission (PR). Three of the complete or partial remission subjects received the maximum tolerated dose, which was 12 mg on day 1 and 4 mg on each day from day

2 to day 7. However, S6 kinase in bone marrow blasts was inhibited in only one of the six CR/PR subjects. Only three of the complete remission subjects that underwent transplantation remained alive in a remission (Perl *et al*., 2009).

To support these treatments in a more quantitative way of mTOR inhibition, the group used an assay to determine S6RP (S6 ribosomal protein) phosphorylation in AML blast cells by intracellular flow cytometry. 8 out of 10 patients showed increased baseline phosphorylation of S6K. 6 subjects out of these 8 were inhibited *in vivo*. The other two subjects were resistant to rapamycin *in vivo* and when one of them was assessed for rapamycin sensitivity *in vitro*, results emerged indicating resistance to sirolimus (Perl, Kasner, Shank, Luger, & Carroll, 2012).

In a different phase I study, 13 patients with relapsed and refractory AML were treated daily for 5 days with 20 mg/m^2 of decitabine, which is a cytosine analog that inhibits DNA methylation (Yang *et al*., 2006). After decitabine pre-treatment, the patients received 2, 4, or 6 mg or rapamycin per day for 20 days. No significant non-hematological toxicities occurred except reversible mucositis in 4 patients. At the end, in 4 (31%) patients blast proportion declined, in 5 (38%) kept stable, and 4 (31%) patients had even higher blast count than before the treatment. Median survival of this group was 4 months with two patients undergoing stem cell transplantations. The limited positive results were probably due to patients' complex cytogenetics and heavy pretreatment before the phase I trial, as well as undergoing only 1 cycle of decitabine + rapamycin treatment. For comparison, in a different trial, AML patients with no previous therapy resulted in response rate (assessed by bone marrow biopsies) of 25% after 1-25 cycles of five-day-long daily administration of 20 mg/m^2 of decitabine (Cashen, Schiller, O'Donnell, & DiPersio, 2010). In 6 patients of the phase I study for which samples were evaluated by Western blotting, half of them had decreased expression of phosphorylated 4E-BP1, the other half's expression was increased (Liesveld *et al*., 2013).

When chemotherapy-refractory AML patients received doses of 2 mg of rapamycin every day in a 14-day long period, it led to concentrations of 10-20 ng/ml of blood. In only two out of five patients the treatment resulted in decreased leukocyte count and only in one of those patients, the reduction was long-lived. No negative immunosuppressive effects of rapamycin were observed in the patients (Boehm *et al*., 2009). In a different research, patients were administered 2 mg of rapamycin per day over 4 weeks. After the four weeks, 4 patients were labeled with partial response, since the number of marrow blasts reduced by more than 50%. They observed variable blood concentration of rapamycin among patients suggesting that rapamycin analogs might be

better suited for the treatment of AML (Récher *et al*., 2009).

3.3 Everolimus (RAD001)

Mechanism of action of everolimus is very similar to rapamycin's but RAD001 was also shown to inhibit mTORC1 in absence of FKBP12, binding directly to FRB (FKBP12-rapamycin binding domain) (Shor *et al*., 2008).

Everolimus was shown to trigger an increase in Akt phosphorylation in 86% of 19 acute leukemia cell lines tested. Using a PI3K selective inhibitor IC87114 it was indicated that activating mTOR pathway is independent from activating the PI3K pathway, but not its downstream neighbor Akt. More importantly, it was observed that inhibition of both mTOR and PI3K/Akt pathway had additional benefit in causing cell anti-proliferation (Tamburini *et al*., 2008).

A change in already increased Akt phosphorylation after 24 hours of AML incubation with RAD001 was not observed, even though there were articles, which claimed that 24-hour rapamycin treatment inhibits mTORC2 and thus decreases Akt phosphorylation (Sarbassov *et al*., 2006; Tamburini *et al*., 2008). Furthermore, RAD001 failed to inhibit 4E-BP1 phosphorylation in AML cells or induce apoptosis. (Tamburini *et al*., 2009).

When NOD/SCID mice received engraftments of human ALL and began to be treated with RAD001 the day after injection, their median survival length increased to 42.0 days while control mice lived only for 21.3 days. RAD001 also decreased the number of ALL cells in bone marrow (Crazzolara *et al*., 2009).

24-hour treatment of 2 μm RAD001 resulted in significantly decreased levels of phosphorylated 4E-BP1 and S6RP in ALL cells from spleen. In one particular xenograft, the reduction was less pronounced than in other samples, which correlated with its decreased sensitivity to RAD001 treatment in previous experiments. It can be inferred from the results, that not all ALL cells respond to the treatment of mTOR activation in the same way (Crazzolara *et al*., 2009).

After RAD001 treatment was performed, bone marrow sections of the mice were analyzed by TEM. Even though the cells exhibited apoptosis only sporadically, a significant number of them contained cytoplasmic polyphagic vacuoles. The vacuoles contained different organelles in several stages of breakdown. Moreover, Nalm6 and REH cell lines treated with rapamycin

contained greater number and proportion of acidic vacuoles after the treatment. Caspase 3 was not detected in the ALL cell lines Nalm6 and REH treated *in vitro* with rapamycin. Together, these results indicate that apoptosis is not the mechanism of cell death induced by rapamycin, but autophagy is (Crazzolara *et al*., 2009).

Combination of RAD001 (everolimus) and standard chemotherapy (daunorubicin with cytarabine) in first relapse AML patients under age 65 showed low toxicity with less than 10% of patients experiencing gastrointestinal tract or lung problems. 70 mg dose of everolimus was administered twice in a seven-day interval and resulted in complete remission in 19 out of 28 (68%) patients. The dose resulted in strong inhibition of S6K phosphorylation, even though it was not the maximal tolerated one (Park *et al*., 2013).

Response to RAD001 of 27 people with relapsed or refractory hematologic malignancies, 9 of which suffered from AML, was assessed in phase I/II study. Even though the response was very limited, the study proved in *in vivo* settings that a rapamycin analogue can decrease the activity of Akt in hematopoietic cells (Yee *et al*., 2006). The drug is currently in phase I/II study of treatment of relapsed or refractory ALL (M.D. Anderson Cancer Center, 2015).

3.2 Temsirolimus (CCI-779)

A 2006 investigation established suitability of CCI-779 for inhibition of ALL growth when it showed that CCI-779 treatment increased apoptosis in lymphoblast cells 2- to 5-times and decreased overall cell numbers by almost 30-time. Interestingly enough, a significant difference between doses of 1 ng/ml and 100 ng/ml of culture was not observed. Moreover, treatment did not exhibit many considerable toxicities in mouse xenograft models, yet significant decrease in platelet count was observed (Teachey *et al*., 2006).

Temsirolimus shows to be a very promising therapeutic agent among the rapamycin analogs. Namely, prolonged rapamycin treatment inhibits mTORC2 assembly in addition to phosphorylation of Akt and similar results were obtained from experiments with temsirolimus (Sarbassov *et al*., 2006), even though Akt activation after 24-hour treatment with RAD001 was not observed. Zeng and colleagues state it could have in fact been because CCI-779 activity is higher with longer incubation time, or because of its possible additional inhibitory effects, but also because of different culturing of the primary blast cells between the experiments (Tamburini *et al*., 2008; Zeng *et al*., 2007). Moreover, combined treatment of temsirolimus and clofarabine

(nucleoside analog) displayed cytotoxic effects in AML cell lines and primary cells from AML patients. Temsirolimus in synergy with clofarabine blocked cells in the G_0/G_1 phase of cell cycle, induced autophagy, and inhibited formation of the eIF4F complex (Chiarini *et al*., 2012).

3.4 Deforolimus (AP23573)

Phase I trial of deforolimus, also called ridaforolimus, in which patients were treated with the drug for 5 days every two weeks provided good results for the use in phase II trials. There were negative reactions amongst subjects, which consisted mostly of mouth sores and rash but disappeared overtime with administration of symptomatic treatment. Deforolimus was well tolerated and 76% of 29 patients experienced tumor shrinkage or stabilization (Mita *et al*., 2008).

Phase II clinical trial of deforolimus used the dosing schedule determined in phase I, which meant daily delivery of 12.5 mg of deforolimus intravenously for five days every two weeks. They were able to detect lower level of phosphorylation of 4E-BP1 but concerning clinical effects of deforolimus, none of the 22 patients with AML experienced partial or complete remission. Most favorable response was observed in patients with MCL, of whom 33% underwent partial remission. The authors suggested possible explanations which was negative effect of deforolimus as initial decreased Akt phosphorylation in these cells after treatment (Rizzieri *et al*., 2008).

Ridaforolimus is currently in trial III to treat metastatic sarcoma (Demetri *et al*., 2013).

Overall, rapamycin analogs have been widely studied and produced positive effects on blocking acute leukemia progression. Some are more successful in AML than ALL like rapamycin, but all of them decrease the mTOR pathway's downstream signalization, prolong survival of xenograft models and result in partial or complete response in acute leukemia patients. However, they do so only to a limited extent, failing to successfully affect all cell lines and patients tested. To elicit a complete response in around 50% of subjects, they require to be administred with other established chemotherapeutics putting into question the harmlessness in terms of toxicity of such treatments. Investigations of other inhibitory agents of mTOR, but also other established chemotherapeutics and their combinations could prove extremely beneficial for lowering toxicity and increasing positive response in acute leukemias.

4. Dual inhibitors of mTOR and PI3K

Targeting the pathway PI3K/Akt/mTOR at different levels may lead to a highly effective treatment by disrupting the feedback loops. Treatment with dual PI3K/mTOR inhibitor may be sufficient to disrupt the following feedback loop in the PI3K/Akt pathway (Chiarini *et al*., 2010). Inhibitors used in this approach to mTOR pathway inhibition are PI-103, NVP-BEZ235, BGT226, LY294002, wortmannin, GNE477, XL765 and WJD008, of which the first four will be discussed in depth.

4.1 PI-103

PI-103 is a molecule synthesized in 2004 to target mTOR and class I of PI3K. It inhibits also some other kinases, but to a limited extent (Knight *et al*., 2004).

PI-103 inhibited Akt in concentration of 1μM and also inhibited both PI3K/Akt and mTORC1. It has strong anti proliferative effect on blast cells in basal as well as induced conditions, which supports the fact that PI3K and mTORC1 are needed for blast cell proliferation. When studying the response of primary AML bone marrow patient cells to treatment, Park and colleagues observed proapoptotic effects (Park *et al*., 2008).

When T-ALL cell lines Jurkat, MOLT-4, CEM-S, CEM-R were assessed for PI-103 sensitivity, survival rate of cells decreased dramatically after 48 hour treatment of PI-103 with the critical concentrations IC_{50} being between 0.25 and 0.40 μM PI-103. Wortmannin and LY294002 were less effective in negatively affecting cell growth than PI-103 with signs of toxicity starting at 25 uM. After 8 hours of treatment with 0.75 μM PI-103, 0.1 μm rapamycin, and 10 μM LY294002 the cells were assessed for aptoptosis using flow cytometry. More than 40% cells were apoptotic when treated with PI-103 compared to less than 10% in case of the other inhibitors. 0.75 μM PI-103 treatment resulted in significant decrease of Akt Ser473 phosphorylation at 8 hours. Total levels of Akt did not change in PI-103 treatment nor in rapamycin treatment. Downstream substrates of mTOR, S6K, 4E-BP1, and S6RP, were significantly dephosphorylated compared to controls (Chiarini *et al*., 2009).

Bone marrow and peripheral blood samples from patients with T-ALL were isolated and assessed for levels of Akt Ser473 and Ser37/46 phosphorylation, as well as PI-103 sensitivity. Samples treated with PI-103 showed decrease in phosphorylated Akt Ser473, increased levels of

caspase-3, and an extensive reduction in cell viability was observed after 96 hours of PI-103 treatment with IC_{50} ranging between 0.18 and 0.63 μM of PI-103 (Chiarini *et al*., 2009).

PI-103 is cytotoxic to T-ALL cell lines independently of p53 gene status, which is in contrast to the findings in AML cell lines and induces cell cycle arrest in G0/G1. It possesses apoptotic activity but, which is lower compared to the activity in AML cells. Even though PI-103 was more effective in inducing apoptosis in T-ALL cell lines than rapamycin, it was less effective in growth inhibition in Bcr-Abl positive B-ALL mouse model (Chiarini *et al*., 2009).

PI-103 eventually did not enter clinical trials because its rapid metabolization is a highly undesirable quality (Raynaud *et al*., 2007), but it did give a fine idea on implementing two points of inhibition of the PI3K/Akt/mTOR pathway (Chiarini *et al*., 2009).

4.2 NVP-BEZ235

NVP-BEZ235 is a dual PI3K/mTOR inhibitor which has strong cytotoxic effect on T-ALL cells. It is an imidazo-quinoline derivative structurally designed to inhibit four PI3K paralogs and mTOR. It inhibits PI3Kalpha and mTORC1 and mTORC2 by competing for the ATP binding site (Maira *et al*., 2008).

Compared to the PI-103 inhibitor, using flow cytometry to determine the effectivity of apoptosis, NVP-BEZ235 proved to be a more powerful agent. Also, the treatment led to an increase in cells in G_0/G_1 phase and decrease in cells in S and G_2/M phases. At the same time, T lymphocytes obtained from healthy donors and stimulated prior to experiments showed less sensitivity to NVP-BEZ235 than the tested T-ALL cell lines. Chiarini and colleagues confirmed that NVP-BEZ235 induces autophagy using microscopy of autophagy vesicles and western blot. Total levels of Akt did not change but Ser473 was dephosphorylated as well as several mTORC1 downstream substrates S6K, 4E-BP1, and S6RP. In contrast, rapamycin did not cause 4E-BP1 dephosphorylation (Chiarini *et al*., 2010).

NVP-BEZ235 has dose-dependent effects on Jurkat and MOLT-4 cell lines in terms of induction of apoptosis, arrest in G_2/M cell phase, cleavage of caspase 3, 8, and 9, decreased content of cyclin B1 protein, decreased phosphorylation of 4E-BP, SK6 and Akt (Pereira *et al*., 2014).

NVP-BEZ235 entered into phase I/II clinical trial in 2008 but in treatment of solid malignancies, but not yet treatment of leukemia (Novartis Pharmaceuticals, 2013).

4.3 BGT226

Dual inhibitors NVP-BEZ235 and BGT226 were used to treat ALL cell lines NALM6, REH and LK63 reaching IC_{50} between 13 and 26 nM compared to 1.5 - 10 μM of everolimus. When assessing the potential to induce cell death, BGT226 had the most pronounced effect, requiring only 0.6-1.3 μM to induce it. Everolimus had a slightly weaker effect with IC_{50} of 10 – 13 μM, while NVP-BEZ235 was the least efficient with IC_{50} between 30 μM and 128 μM. However, with longer time, much less ($P<0.001$) of NVP-BEZ235 and BGT226 was needed to achieve cell death induction, while increased assay time did not change the effect of everolimus (Wong, Welschinger, Hewson, Bradstock, & Bendall, 2014).

Thanks to the use of pan-caspase inhibitor Z-VAD the researchers were able to determine each of the inhibitor's mode of action concerning apoptosis. BGT226 apoptosis was not significantly reduced with use of Z-VAD in contrast to NVP-BEZ235 , whose apoptotic action was considerably alleviated with the use of caspase inhbitor. An interesting result surfaced when NOD/SCID mouse were engrafted with human ALL and treatmet was delivered when 1% ALL was detected in the peripheral blood. NVP-BEZ235 , BGT226, and rapamycin were used daily resulting in doubling the overall survival of mice, which was a significant improvement compared to control. However, the overall effect of dual kinase inhibitors was not notably better than rapamycin's. Even more interestingly, when individual xenografts were considered, each treatment resulted in significant improvement of survival regardless of the xenograft tested (Wong *et al*., 2014).

Treatment of both dual inhibitors significantly inhibited phosphorylation of 4E-BP1 *in vivo* at 2 hours as well as at 24 hours. S6RP phosphorylation did not exhibit significant any changes at 24 hours. Detection of Ser473 phosphorylation of Akt showed a decrease at 24 hours but a correlation between Akt phosphorylation and survival of mice could not be established from the data. Decreased phosphorylation of Akt by NVP-BEZ235 and BGT226 did not directly result in *in vitro* cell death or better survival of the mice. As a possible reason stating, that Akt might not be equally important in all ALL cell lines for proliferation and survival (Wong *et al*., 2014).

4.4 LY294002

LY294002 is a direct inhibitor of PI3K family kinases, to which mTOR belongs as well. It effectively inhibits both 4EBP1 and S6K, but at the same time also inhibits several other lipid and protein kinases (Gharbi *et al*., 2007; X. Wang, Beugnet, Murakami, Yamanaka, & Proud, 2005).

To test the effect of LY294002 inhibitor on AML cell lines, HL-60, NB4, U937, and K562 cell lines were grown for 24, 48, 72, or 96 hours with LY294002, rapamycin or combination of the two inhibitors. Two concentrations 5 μM and 10 μM of LY294002 and two concentrations, 5 nM and 20 nM, of rapamycin were used totaling to 6 different combinations with one additional control. Rapamycin inhibited growth of leukemia cells only minimally, with significant results ($P<0.05$) in respect to controls in most HL-60 cells. LY294002 was more successful in growth inhibition, but the combination of inhibitors showed significant reduction in viable cell numbers in all four cell lines. Proportion of cells in sub-G1 phase of cell cycle was similar to the cell growth results, making the combination of inhibitors most successful treatments while LY294002 induced greater proportion of cells to G0/G1 phase compared to rapamycin (Mise, Dembitz, Banfic, & Visnjic, 2011).

LY294002 suppressed Akt activation in bone marrow blast cells from AML patients while RAD001 increased Akt activation on average by 86%. mTORC1 inhibits insulin/IGF-1 signaling by negatively regulating it on transcriptional as well as protein level (Tremblay, Gagnon, Veilleux, Sorisky, & Marette, 2005). When the interaction between IGF-1 and IGF-1R was blocked the phosphorylation of Akt induced by RAD001 returned to normal levels. Independently LY294002 and RAD001 inhibited blast cell proliferation to the same extent but when used together, gave a significant additional anti-proliferative effect (Tamburini *et al*., 2008).

Altogether, dual inhibitor approach to mTOR inhibition, targeting not only mTOR but also additional PI3K kinases, proved more successful than use of rapamycin alone in acute leukemia cell lines. It resulted in greater proportion of cells arrested in G_0/G_1 phase, more apoptotic cells, further decrease of Akt phosphorylation, and greater inhibition of 4E-BP1 phosphorylation. Unfortunately, not many studies in clinical phase or with xenografts models were performed, to thouroughly compare the effectivity of dual inhibitor approach to treatments with rapamycin analogs.

5. New generation inhibitors

Among new generation of inhibitors targeting the active site of mTOR are PP-242, PP-30, Torin-1, OSI-027, Ku-0063794, AZD8055, AZI-2014, WAY-600, WYE-687 and WYE-354. Again, the first four will be discusses in greater extent.

5.1 PP2-42 and PP-30

ATP-competitive mTOR kinase domain inhibitors PP-242 and PP-30 which selectively inhibit mTORC1 and mTORC2 were discovered in 2008. Their IC_{50} are only 8 nM and 80 nM respectively. The agents block phosphorylation of S473 residue of Akt and disable its complete activation. And inhibit phosphorylation of Akt, and S6K (Apsel *et al*., 2008).

A screen of over 600 human cancer cell lines was revealed markers of sensitivity to PP-242. Mutations in RAS gene indicate the most significant resistance and mutations in PIK3CA gene are evidence of the most significant sensitivity to PP-242 (Ducker *et al*., 2014).

They are both more powerful inhibitors of proliferation than rapamycin. It is based on the facts that PP-242 inhibits mTORC1 more effectively than rapamycin and inhibits cap dependent translation in a way rapamycin does not (Feldman *et al*., 2009). Additionally PP-242 had greater anti-proliferative effects than rapamycin, even in cells lacking the mTORC2 complex. Moreover, apamycin and PP-242 fully inhibit S6K phosphorylation but rapamycin inhibits phosphorylation of 4E-BP only partially (Feldman *et al*., 2009).

PP-242 is very selective for mTOR inhibition and it inhibits both of the mTOR complexes. At concentration of 800 nM (100-times its IC_{50}) PP-242 inhibited only four kinases by more than 75%. Consequently, it is more selective for PI3K/Akt/mTOR signaling inhibition than other inhibitors of Akt/mTOR such as PI-103 (Janes *et al*., 2010).

PP-242 is a better inducer of apoptosis than temsirolimus in AML cells isolated from patients at concentrations as low as 0.1 μM. It downregulated the expression of 4E-BP1 and Akt as well as suppressed the phosphorylation of 4E-BP1, S6K, and Akt. It did so in both floating leukemia cells as well as CD34+ AML cells. In contrast to rapamycin, PP-242 induced death of leukemic blast cells. Using a mouse model of AML leukemia and flow cytometry with antibodies against phosphorylated Akt Ser473 and phosphorylated S6K Ser235/236, it was shown that PP-242 effectively inactivates S6K and Akt after 2 hours of drug administration. It also substantially

decreased leukemia burdens in mice 15 days from leukemia-cell inoculation compared to rapamycin (Zeng *et al*., 2012).

Rapamycin treatment of B-ALL cells in mice resulted in decreased growth, but only up to 60% (with GI_{50} = 6.5 nM), while PP-242 decreased proliferation by more than 90% (with GI_{50} = 12 nM). PI-103 and NVP-BEZ235 completely suppressed growth as well. Overall, rapamycin's effect on cells was cytostatic while the effect of the mTORC1/2 inhibitors resulted in cell death or cell cycle arrest. Rapamycin treatment increased PIP_3 content in cells while PP-242 did not have an effect on the production of it. The leukemic burden of B-ALL mice was significantly reduced after treatment of PP-242 but not after rapamycin. The reason of different bioavailability was excluded and since the phosphorylation of S6K was inhibited and phosphorylation of Akt at Ser473 decreased, mTORC2 inhibition appeared as a potential reason. Combining PP-242 and rapamycin with tyrosine kinase inhibitors (dasatinib) led to decrease in leukemic burden in case of PP-242 but did not stop its increase in case of rapamycin (Janes *et al*., 2010).

5.3 Torin-1

Torin-1 inhibits mTORC1/2 with IC_{50} between 2 and 10 nM while inhibiting other kinases at much higher concentration, for example IC_{50} for PI3K is 1.8 uM. Torin-1 has no effect on the stability of the complexes in contrast to rapamycin and is able to completely inhibit mTOR, which results in induction of apoptosis. It inhibits proliferation more completely and reduces cell size more extensively than previous inhibitors. By diminishing the activity of mTORC2 through deleting one of its proteins, Rictor, the mTOR activity connected with Torin-1 could be assessed. Surprisingly, even after deleting Rictor, Torin-1 treatment led to extensive cell proliferation stop and size reduction. The effects could not be attributed to a different mTOR complex, or at least the one that does not contain Raptor protein, since deletion of Raptor led to very similar effects as did Torin-1 treatment (Holtmann *et al*., 2013; Thoreen *et al*., 2009).

5.4 OSI-027

OSI-027 is a potent inhibitor of mTORC1 and mTORC2 with a selectivity greater than 100-fold compared to other PI3K-related kinases and even different family kinases. Akt phosphorylation, which is a marker of mTORC2/Akt activity was perfectly inhibited by OSI-27 as opposed to rapamycin's effect. Moreover, rapamycin not only decreased phosphorylation of Akt in

a limited number of cases, it even showed an increase in the Akt phosphorylation in more than half of the cell lines tested (Bhagwat *et al*., 2011).

Evangelisti and colleagues observed inconsistent apoptotic effects of active-site mTOR inibitors on cells, suggesting that there might be mechanisms in malignant cells that block the induction of apoptosis. They specifically showed that OSI-027 and AZI-2014 induce autophagy in Kasumi 1 and Kasumi 3 cell lines as well as in isolated leukemic blasts from AML patients (Altman *et al*., 2014). However, in a study of ATP-site mTOR inhibitors in T-ALL, they induced not only autophagy and cell cycle arrest in G_0/G_1, but also a considerable level of apoptosis suggesting that the efficacy of ATP-competitive inhibitors is dependent on the particular cell transformations (Evangelisti *et al*., 2011).

OSI-27 decreases S6K phosphorylation and inhibits the mTORC1 complex without disturbing protein interactions within the complex in AML cell lines. It also suppressed cap-dependent translation through intensifying binding of 4E-BP1 with eIF4E and through inhibiting the formation of eIF4E with eIF4G complexes. Rapamycin also increased formation of the complex between 4E-BP1 and eIF4E, but it was unable to disrupt already established interaction of eIF4E with eIF4G. The used doses of both inhibitors were maximal corresponded to 20 nM and 10 μM for rapamycin and OSI-27 respectively. Furthermore, the effects of OSI-27 on HL-60, KG-1, KBM-3B, and U937 followed a dose-dependent manner with achieved response in all AML cell lines unlike rapamycin, in case of which proliferation failed to cease even after increasing rapamycin concentration (Altman *et al*., 2011).

The OSI-027 treatment in ALL cell lines showed similar effects, reducing the levels of phosphorylation of S6K, 4E-BP1 and Akt. Five-day-long 5 μM OSI-027 treatment significantly decreased viable cell mass in ALL cells and the IC_{50} was established to be 100 nM. OSI-027 induced both autophagy and apoptosis, utilizing caspase 9 and the mitochondrial apoptotic pathway and was proved not to affect the mTORC1/2 complexes through a yet unknown upstream signaling approach (Gupta *et al*., 2012).

Another beneficial properties of OSI-027 were determined as part of investigation of the results of its coupled activity with low doses of AraC cytotoxic chemotherapeutic. Their combined effect was more pronounced than when they were tested individually (Altman *et al*., 2011). Pharmacokinetic properties of OSI-027 showed to be favorable when tested in mouse xenograft models, since plasma levels of the inhibitor showed linear dependency on the administered dose of 20 to 200 mg/kg (Bhagwat *et al*., 2011).

So far, OSI-027 was used in clinical phase I study only to treat patients with advanced solid tumors and lymphoma showing good pharmacological activity and low toxicity (Tan *et al*., 2010). Further studies will need to be performed to assess its potential for treatment in leukemia patients.

Overall, use of active site inhibitors has a great potential in inhibiting mTOR in acute leukemia cell lines – the half maximal inhibitory concentrations are in nanomolar range, inhibitors plasma levels are stable, proliferation rate is significantly decreased, and phosphorylation of 4E-BP1, S6K and Akt inhibited. Additionally, the extent of desired effects of the inhibitors on cell growth and proliferation is dependent on the type of leukemia and cell line tested, suggesting that more patient-specific treatments of leukemia could prove beneficial. Moreover, majority of the new generation inhibitors still need to be tested in clinical settings to provide more information about their effectivity and toxicity.

6. Conclusion

Targeting mTOR pathway and decreasing its activity in AML and ALL has proven as a rational approach to treating acute leukemia. Using rapamycin derivates for the treatment results in cytostatic effects and a decrease of the abnormal proliferation of white blood cells. Targeting mTOR and PI3K pathway at the same time with dual inhibitors prevents undesirable activation of Akt pathway through a negative feedback loop, induces autophagy and also inhibits abnormal proliferation in bone marrow. New generation of inhibitors has proven to have the greatest potential in successful inhibition of mTOR pathway even though additional research will be essential.

Right now it looks like rapamycin derivatives will be used for mTOR inhibition in acute leukemia treatment since number of them are already in clinical trial phases. However, with active site inhibitors showing such promising results, we may witness a rapid rise of new generation of mTOR inhibitors and their use in clinical setting.

Additional knowledge of the PI3K/Akt/mTOR signaling network, such as the mTORC2 or mTORC3 upstream and downstream effectors, will allow us to better understand but most importantly inhibit unwanted mTOR activation in leukemia cells and thus provide a better basis for the treatment of AML and ALL.

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