Therapeutic potential of targeting mTOR in T-cell acute lymphoblastic leukemia (Review)

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Abstract. T-cell acute lymphoblastic leukemia (T-ALL) is a heterogeneous neoplastic disorder of immature hematopoietic precursors committed to the T-cell lineage. T-ALL comprises about 15% of pediatric and 25% of adult ALL cases. Even if the prognosis of T-ALL has improved especially in the childhood due to the use of new intensified treatment protocols, the outcome of relapsed patients who are resistant to conventional chemotherapeutic drugs or who relapse is still poor. For this reason, there is a need for novel and less toxic targeted therapies against signaling pathways aberrantly activated in T-ALL, such as the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR). Small molecules designed to target key components of this signaling axis have proven their efficacy both *in vitro* and *in vivo* in pre-clinical settings of T-ALL. In particular, different classes of mTOR inhibitors have been disclosed by pharmaceutical companies, and they are currently being tested in clinical trials for treating T-ALL patients. One of the most promising approaches for the treatment of T-ALL seems to be the combination of mTOR inhibitors with traditional chemotherapeutic agents. This could lead to a lower drug dosage that may circumvent the systemic side effects of chemotherapeutics. In this review, we focus on the different classes of mTOR inhibitors that will possibly have an impact on the therapeutic arsenal we have at our disposal against T-ALL.

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Contents

1. Introduction

Acute lymphoblastic leukemia (ALL) is caused by the uncontrolled clonal proliferation of immature lymphoid cells which accumulate in the bone marrow (BM) and other body sites. The neoplastic lymphoblasts display an impaired differentiation program, are blocked at various maturation steps and are resistant to apoptotic stimuli and cell death. ALL accounts for approximately 20% of acute leukemias in the adult, however it is the most common malignant disease in the childhood (1). The clinical management of ALL is challenging, especially in the adults, even though current therapies can induce a complete remission in 65-90% of patients. Nevertheless, patients who are refractory to induction therapy or relapse after induction face a poor prognosis (2). ALL can be classified in two main subgroups, namely B-cell and T-cell ALL (B-ALL and T-ALL, respectively) (3).

T-ALL is an aggressive form of leukemia which arises in the thymus from T-cell progenitors expressing immature T-cell immunophenotypic markers (4,5). T-ALL accounts for 10-15% and 25% of pediatric and adult ALL, respectively. In the childhood, cure rate for T-ALL patients reaches 70-75%. In the adults, the cure rate remains low: 30-40% for adults below 60 years of age and 10% above this age (6,7). By immunophenotyping, it is possible to distinguish three subtypes of T-ALL, i.e., early, cortical and mature, which reflect different stages of healthy thymocyte differentiation. This classification is prognostically

relevant, as early and mature T-ALLs have a poorer outcome than cortical T-ALL (8).

Recent findings have documented that T-ALL is an extremely heterogeneous disease, characterized by chromosomal rearrangements causing aberrant expression of transcription factors (Myb; TAL/SCL; HOX) (9,10), altered expression of oncogenes (10), somatic gene mutations (11,12), multiple signal transduction pathway impairment (13-16) and microRNA dysregulation (17-19).

Activating mutations in Notch-1, the master regulator of T-cell development, are found in more than 60% of T-ALL patients, independently of the subtype (20). All of these alterations impact on T-ALL cell proliferation, differentiation, survival and drug-resistance (21).

In general, leukemia pathogenesis, treatment resistance and relapse are thought to be caused by leukemic stem cells or leukemia initiating cells (LICs) (22). LICs are characterized by asymmetric cell division and self-renewal capacity, unlimited repopulating potential and production of partially differentiated cells. Whereas the bulk of leukemic cells rapidly proliferate, LICs are mainly quiescent (23). This feature is associated with chemoresistance, as conventional chemotherapy strategies mainly target rapidly dividing cells (24).

The phenotype of T-ALL LICs is still under discussion. Cox *et al* (25) reported that either $CD34^+/CD4^-$ or $CD34^+/CD7^-$ cells were capable of serial engraftment in NOD/SCID mice (25). Afterwards, the leukemia initiating potential in xenografts of the $CD7^+/CD1a^-$ subset of primary T-ALL samples was found to be superior to other subsets (26). The importance of CD34 as a marker of LIC activity in T-ALL patients has nevertheless been documented by independent groups (27,28). However, it has been shown that also $CD34^-/CD7^+$ T-ALL cells displayed LIC proprieties, although at lower levels than $CD34^+$ cells (28). The above-outlined discrepancies could well reflect differences among distinct molecular T-ALL subtypes. Nevertheless, these studies have disclosed the complexity of LICs in human T-ALL.

Among the deregulated signaling pathways that have been identified in T-ALL, the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling network has been reported to be active in a high percentage (75-80%) of patients, where it portends a poorer prognosis (29).

Over the last 10 years, mTOR has become an attractive therapeutic target in cancer patients, as several small molecule mTOR inhibitors have been developed and are being tested as monotherapy in clinical trials (30-32). Moreover, the use of targeted drugs combined with traditional anticancer agents could increase treatment efficacy, by lowering the required dosage of chemotherapeutic drugs and limiting their systemic side effects (33). In this review, we will describe the potential of several strategies for mTOR inhibition to improve the outcome of T-ALL patients.

2. The PI3K/Akt/mTOR pathway

mTOR is a 289-kDa serine/threonine (Ser/Thr) kinase which belongs to the phosphoinositide kinase-related family of protein kinases (PIKK) (34). The PIKK family includes ataxia telangiectasia mutated (ATM), ataxia telangiectasia- and RAD3-related (ATR), human suppressor of morphogenesis in genitalia-1 (hSMG-1) and the catalytic subunit of DNA-dependent protein kinase (DNA-PK) (35).

mTOR collects input from several signal transduction networks, such as the PI3K/Akt, the Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) and the AMP-activated protein kinase (AMPK) pathways, for regulating several physiological events. Indeed, mTOR is involved in cell cycle progression, cell survival, translation, metabolism, motility, autophagy and ageing (36). mTOR is the catalytic subunit of two distinct multi-protein complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), both of which are characterized by their different partner proteins and their substrate specificity (36) (Fig. 1).

mTORC1 is composed of the regulatory associated protein of mTOR (Raptor, a scaffolding protein), mammalian Lethal-with-Sec-Thirteen 8 (mLST8), proline-rich Akt substrate of 40-kDa (PRAS40), FK-506 binding protein 38 (FKBP38) and DEP-domain-containing mTOR interacting protein (Deptor). mTORC1 is sensitive to rapamycin and its derivatives (rapalogs) (37). Multiple exogenous stimuli regulate mTORC1 activity, including growth factors such as insulin and insulin-like growth factor-1 (IGF-1), stress signals, cellular energy status and amino acids (38).

mTORC1 activation is mainly regulated by PI3K/Akt signaling. Akt phosphorylates 200-kDa tuberous sclerosis 2 (TSC2 or hamartin). TSC2 is a GTPase-activating protein (GAP) that associates with TSC1 (tuberous sclerosis 1 or tuberin) for inactivating the small G protein Rheb (Ras homolog enriched in brain). Once TSC2 is phosphorylated by Akt, the GAP activity of the TSC1/TSC2 complex is repressed, allowing Rheb to accumulate in a GTP-bound state. As a consequence, Rheb-GTP upregulates the protein kinase activity of mTORC1 (39). Furthermore, Akt phosphorylates PRAS40 at Thr246. Phosphorylated PRAS40 dissociates from mTORC1 in response to growth factors, as well as glucose and nutrients, and thereby releases the inhibitory function of PRAS40 on mTORC1 (40).

mTORC1 positively regulates cell growth and proliferation by promoting many anabolic processes and by limiting catabolic processes such as autophagy (41) (Fig. 1). Regarding protein translation, mTORC1 phosphorylates components of the protein synthesis machinery, such as p70 S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). In turn, p70S6K phosphorylates the 40S ribosomal protein S6 (S6RP), leading to active translation of mRNA involved in ribosome biogenesis (42), while 4E-BP1 interacts with the eukaryotic initiation factor 4E (eIF4E), which critically regulates cap-dependent mRNA translation (43). Once phosphorylated by mTORC1, 4E-BP1 releases eIF4E, which then associates with eIF4G to stimulate translation initiation (44,45).

In addition to its role in protein translation, activation of mTORC1 triggers metabolic changes that are critically important in carcinogenesis, such as mitochondrial biogenesis and oxidative metabolism, aerobic glycolysis and *de novo* lipogenesis (41). mTORC1 controls mitochondrial biogenesis and oxidative metabolism by regulating the interactions between the transcription factor yin-yang 1 (YY1) and the peroxisomal proliferator-activated receptor γ (PPARγ)

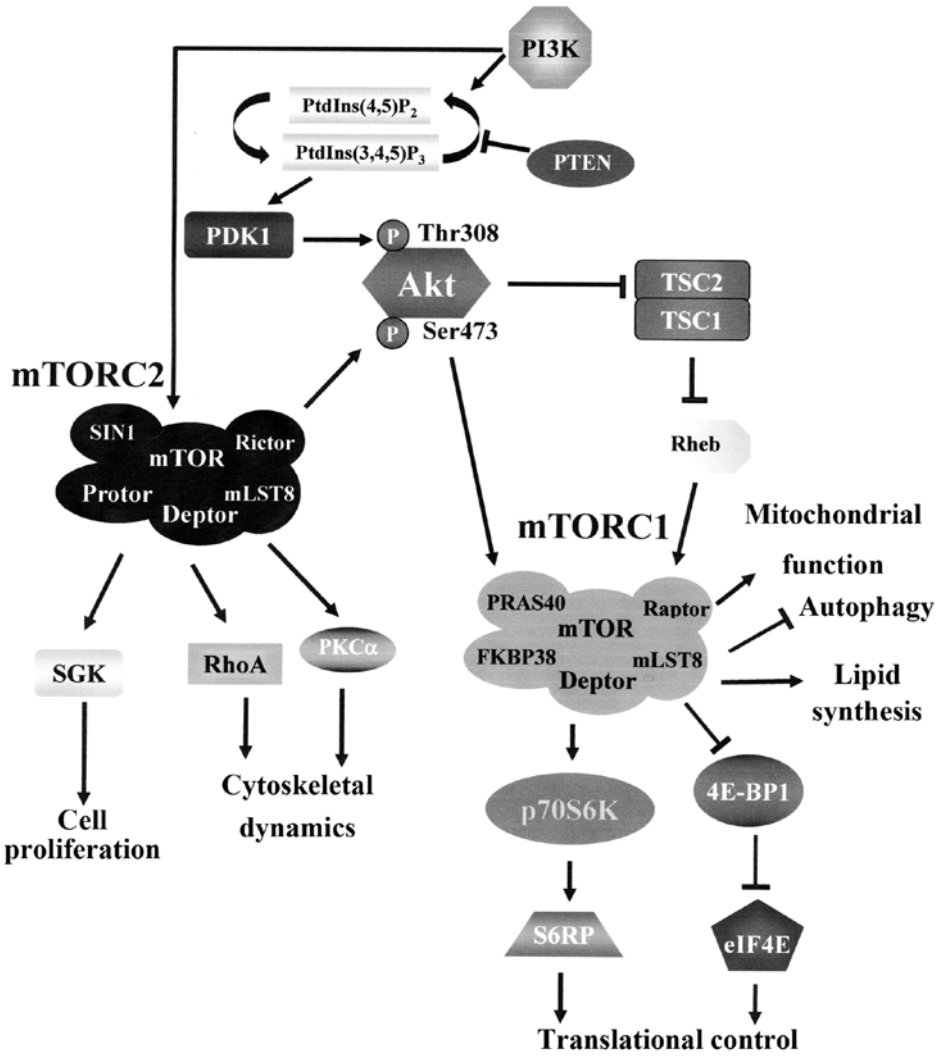

Figure 1. The PI3K/Akt/mTOR pathway. PI3K generates PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2. PtdIns(3,4,5)P_3 attracts to the plasma membrane PDK1 which phosphorylates Akt at Thr 308. Full Akt activation requires Ser 473 phosphorylation by mTORC2. Active Akt inhibits TSC2 activity through direct phosphorylation. TSC2 is a GTP-ase activating protein (GAP) that functions in association with TSC1 to inactivate the small G protein Rheb. Akt-driven TSC1/TSC2 complex inactivation allows Rheb to accumulate in a GTP-bound state. Rheb-GTP then upregulates mTORC1 activity. However, mTORC1 is controlled by Akt also through PRAS40 phosphorylation. The activation mechanisms of mTORC2 are not fully understood yet, but they require PI3K activity. Arrows indicate activating events, while perpendicular lines indicate inhibitory events. 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; Deptor, DEP-domain-containing mTOR interacting protein; eIF4E, eukaryotic initiation factor 4E; FKBP38, FK-506 binding protein 38; mLST8, mammalian lethal-with-sec-thirteen 8; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PKCα, protein kinase C α; PRAS40, proline-rich Akt substrate of 40-kDa; Protor, protein observed with Rictor; PtdIns(4,5)P_2, phosphoinositide (4,5) bisphosphate; PtdIns(3,4,5)P_3, phosphoinositide (3,4,5) trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome ten; p70S6K, p70S6 kinase; Raptor, regulatory associated protein of mTOR; Rheb, Ras homolog enriched in brain; Rictor, rapamycin insensitive companion of mTOR; S6RP, S6 ribosomal protein; SGK, serum- and glucocorticoid-stimulated kinase; SIN1, stress-activated protein kinase-interacting protein 1; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2.

coactivator 1 (PGC-1), thereby preventing the coactivation of YY1 (46).

As far as aerobic glycolysis is concerned, mTORC1 promotes it through induction of a transcriptional program affecting metabolic glycolytic gene targets of hypoxia-inducible factor 1α (HIF1α) (47,48).

Regarding lipid synthesis, mTORC1 activates the transcription factors sterol regulatory element binding protein 1 (SREBP1) and PPARγ which are necessary and sufficient for the differentiation of preadipocytes and lipid accumulation (41).

mTORC1 negatively regulates autophagy, a complex catabolic process that sustains cellular metabolism through recycling of cellular components during growth unfavorable conditions. Nevertheless, autophagy has also been associated with promoting cell survival during nutrient or hypoxic stress and may promote cancer cell survival (49). mTORC1 suppresses the kinase activity of unc-51-like kinase 1 (ULK1), thus preventing the ULK1/autophagy-related gene 13 (Atg13)/FIP200 complex formation (50) that plays an essential role at the early stages of autophagosome formation (51).

mTORC2 comprises rapamycin-insensitive companion of mTOR (Rictor), mLST8, stress-activated protein kinase-interacting protein 1 (SIN1), protein observed with Rictor (Protor), and Deptor, and is generally described as being insensitive to rapamycin/rapalogs. Nevertheless, it has been demonstrated that long-term rapamycin treatment leads to dissociation of

mTORC2 with resulting inhibition of Akt feedback phosphorylation at Ser 473, in primary leukemic cells both *in vitro* and *in vivo* (52). mTORC2 is mainly activated by growth factors through PI3K/Akt, and controls several downstream AGC kinases such as Akt itself, serum- and glucocorticoid stimulated kinase (SGK) and protein kinases Cα (PKCα) (53-55) (Fig. 1). Therefore, mTORC2 regulates cell proliferation, but it is also involved in the spatial control of cell growth via cytoskeleton regulation, through actin fibers, paxillin, RhoA, Rac1 and PKCα (56).

The regulation of PI3K/Akt/mTOR axis is extremely complex, and this is due mainly to the existence of multiple feedback loops and direct activation mechanisms that place mTOR both upstream and downstream of several oncogenic pathways. Importantly, these regulation loops are relevant *in vivo* and influence therapeutic responses based on mTOR inhibition, contributing to the drug-resistance that can occur in mTOR-targeted therapies using rapamycin or rapalogs (45). When Akt activates mTORC1, a negative feedback circuit antagonizes the formation of mTORC2 and reduces Akt activity (57). Moreover, when activated, mTORC1 phosphorylates p70S6K, which in turn inhibits insulin receptor substrate 1 (IRS-1) by phosphorylating it at multiple sites (Ser 307 and Ser 636/639), inducing its degradation and altering its localization, all of which ultimately dampen PI3K/Akt/mTORC1 activation (58-61). mTORC1 is also capable of downregulating IRS-2 expression by enhancing its proteosomal degradation (62). Recent findings have also highlighted the existence of a rapamycin-sensitive, mTORC1/p70S6K-mediated phosphorylation of Rictor on Thr1135. This phosphorylative event exerts a negative regulatory effect on the mTORC2-dependent phosphorylation of Akt at Ser 473 *in vivo* (63).

PI3K/Akt/mTOR signaling is antagonized by phosphatases. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a potent repressor of this pathway that removes 3'-phosphate from phosphoinositide (3,4,5) trisphosphate [PtdIns(3,4,5)P_3] to yield PtdIns (4,5) bisphosphate [PtdIns(4,5)P_2] (64), thus counterbalancing the action of PI3K (Fig. 1). Loss of *PTEN*, due to inactivating mutations or silencing, has been reported in a wide range of sporadic human cancers, including leukemias, and it has been correlated to cellular proliferation, cancer susceptibility and tumor progression (65). *PTEN* plays an important role in T-ALL pathophysiology (see below).

The lipid phosphatases, Src homology domain-containing inositol phosphatase (SHIP) 1 and 2, remove 5'-phosphate from PtdIns(3,4,5)P_3 to yield PtdIns(3,4)P_2 (66), and play a fundamental role in the inhibition of proliferation and survival of hematopoietic cells (67). Mutations of SHIP1, that is predominantly expressed in hematopoietic cells, have been implicated in the development of different blood disorders, including T-ALL (68). Also protein phosphatases, such as protein phosphatase 2A (PP2A), impact on PI3K/Akt/mTOR signaling, as PP2A dephosphorylates Akt at Thr308 (69).

3. Disregulated mTOR activity and T-ALL development

It is established that *PTEN* deletion led to T-ALL development in mice (70) and that rapamycin treatment of preleukemic mice prevented LIC formation and halted T-ALL development (71). Both mTORC1 and mTORC2 have been implicated in T-ALL pathophysiology. Regarding mTORC1, it has been documented that loss of mTORC1 activity caused by *Raptor* deficiency, eradicated T-ALL in a murine model of disease, suggesting that mTORC1 played a key role in T-ALL LIC survival (72). However, rapamycin was not sufficient for T-ALL eradication. This could be due to the fact that rapamycin is an incomplete blocker of mTORC1 outputs (73). Therefore, dual PI3K/mTOR inhibitors or ATP-competitive mTORC1/mTORC2 inhibitors (see below) could be more effective agents against T-ALL, as they efficiently targeted rapamycin-resistant mTORC1 activity in T-ALL cells (74-76).

An important role for mTORC2 in T-ALL development is suggested by the findings of another group (77). It was documented that deletion of the mTORC2 component, *Rictor*, prevented leukemogenesis and hematopoietic stem cell (HSC) depletion after *PTEN* deletion in adult mice. These observations implicated an important role for mTORC2 activation in these processes. However, *Rictor* deletion (and hence mTORC2 function inactivation) had little effect on the physiology of healthy (i.e., non-*PTEN*-deleted) HSCs. Moreover, *PTEN* deletion from neonatal HSCs did not activate PI3K/Akt signaling or promote HSC proliferation/depletion or leukemogenesis. Therefore, it was concluded that PTEN is required in adult, but not neonatal, HSCs for inhibiting mTORC2 signaling downstream of PI3K/Akt (77). These findings could explain why B-ALL, where *PTEN* deletions are very uncommon (78), is a disease of the early childhood with a peak incidence at 2-5 years of age (79), whereas pediatric T-ALL, in which *PTEN* deletion/inactivation is quite frequently observed (80), displays an older mean age of presentation (approximately 9-10 years) (81).

4. Causes for PI3K/Akt/mTOR pathway activation in T-ALL

PI3K/Akt/mTOR pathway aberrant activation is a common feature in T-ALL, being detectable in 70-85% of the patients (82) and is associated with a poorer outcome (80,83).

Mutations in PI3K, Akt and PTEN have been described in T-ALL patients. Collectively, they were found in about 50% of 44 T-ALL samples (84). However, while PI3K or Akt mutations are extremely rare (two and one case, respectively, in the above mentioned study), *PTEN* mutations occur more frequently in both adult and pediatric T-ALL (85,86). In adults, *PTEN* mutations have been identified in 10% of patients in a study in which 90 T-ALL cases were analyzed (87), whereas in children, *PTEN* was found mutated in 52 out of 301 (17.3%) patients (85). However, some *PTEN* mutations affected exon 7, and were predicted to truncate the C2 domain without disrupting the lipid phosphatase domain of PTEN (84). Therefore, these mutations should not impact on PI3K/Akt/mTOR signaling, even though this has never been documented.

Moreover, *PTEN* could be either deleted (84) or repressed due to several mechanisms. In T-ALLs displaying Notch-1 activation (50-60% of cases), *PTEN* could be repressed through the hairy enhancer of Split-1 (HES-1), a downstream target of Notch-1 signaling (88). Another Notch-1 target gene which negatively impacts on *PTEN* expression is c-Myc (89,90). Overexpression of miR-19 has also been documented in T-ALL patients and resulted in lower expression of several

genes controlling the PI3K/Akt/mTOR cascade, including *PTEN* (91).

However, in most pediatric T-ALL clinical samples, PTEN is expressed, but displays elevated phosphorylation at the C-terminal Ser/Thr cluster, due to phosphorylation by casein kinase 2 (CK2), and/or oxidation by reactive oxygen species (ROS). Phosphorylation and/or oxidation resulted in PTEN stabilization and functional inactivation, with ensuing overactivation of PI3K/Akt/mTOR signaling (92). Decreased activity of PP2A on Thr308 p-Akt could also account for PI3K/Akt/mTOR upregulation in *PTEN*-null T-ALL cells (93).

IGF-1/IGF-1R signaling plays an important role in the activation of the PI3K/Akt/mTOR cascade in T-ALL cells. Indeed, pharmacologic inhibition or genetic deletion of IGF-1R negatively affected T-ALL cell proliferation and survival (94). Interestingly, IGF-1R is a Notch-1 target gene and Notch-1 was required to maintain IGF-1R expression at high levels in T-ALL cells. Furthermore, a moderate decrease in IGF1-R signaling compromised T-ALL LIC activity (94).

Cytokines produced by the thymic/BM microenvironment, including interleukin (IL)-4 (95) and IL-7 (96), could be involved in upregulation of PI3K/Akt/mTOR signaling in T-ALL. An important source for IL-7 could be represented by thymic epithelial cells (97). In this connection, it has been recently reported that ROS, produced through IL-7 signaling, are critical for activating PI3K/Akt/mTOR which in turn mediates proliferation and survival of T-ALL cells (29). However, in T-ALL patients, increased signaling downstream of the IL-7 receptor α chain (IL-7Rα) could also be a consequence of gain-of-function IL-7Rα mutations, which were detected in about 9% of pediatric T-ALL patients (98).

CXC chemokine ligand 12 (CXCL12), referred to as SDF-1α (stromal cell-derived factor 1α), the ligand for the CXC chemokine receptor 4 (CXCR4), is another cytokine with the potential for activating PI3K/Akt/mTOR signaling (99). CXCL12 is produced by BM stromal cells in T-ALL patients (100) and it has been recently demonstrated to be involved in PI3K/Akt/mTOR activation and drug-resistance in T-ALL cells (101).

5. mTOR inhibitors

We will summarize the three main classes of mTOR inhibitors that have been tested in pre-clinical models and/or entered clinical trials for treatment of T-ALL: rapamycin/rapalogs that are allosteric mTORC1 inhibitors; dual PI3K/mTOR inhibitors that target both PI3K and mTORC1/mTORC2; ATP-competitive, 'active-site' mTORC1/mTORC2 inhibitors that target the catalytic site of mTOR (Fig. 2).

Rapamycin/rapalogs. Rapamycin (sirolimus), a natural compound discovered from the bacterium *Streptomyces hygroscopicus* in the Easter Island more than 30 years ago, is an allosteric mTORC1 inhibitor that at first interacts with the intracellular protein, FK506 binding protein 12 (FKBP12) (102). The rapamycin/FKBP12 complex results in the dissociation of Raptor from mTORC1 and loss of contact between mTORC1 and its substrates (103). Therefore, rapamycin does not directly target the mTOR catalytic site and does not affect mTORC2 activity, except in some cell types after prolonged exposure (52). Since no other cellular protein has been identified as rapamycin targets and a cofactor (i.e., FKBP12) is required, rapamycin is a very selective mTORC1 inhibitor. Rapamycin is now FDA-approved as an immunosuppressive agent in solid organ transplantation.

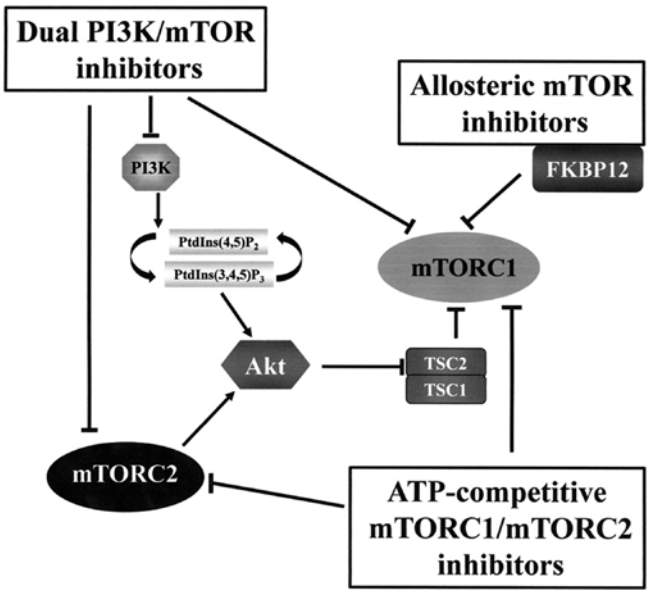

Figure 2. Targets of mTOR inhibitors. Allosteric mTOR inhibitors (rapamycin and rapalogs) associate with FKBP12 leading to dissociation of Raptor from mTORC1 complex and loss of contact between mTORC1 and its substrates. Dual PI3K/mTOR inhibitors target both PI3K and mTORC1/mTORC2. ATP-competitive mTORC1/mTORC2 inhibitors target the catalytic site of the enzyme, thus acting on both mTORC1 and mTORC2. FKBP12, FK506 binding protein 12; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; PI3K, phosphoinositide 3-kinase; PtdIns(4,5)P_2, phosphoinositide (4,5) bisphosphate; PtdIns(3,4,5)P_3, phosphoinositide (3,4,5) trisphosphate; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2.

Rapamycin derivatives (rapalogs) display an improved bioavailability when compared to rapamycin, and include CCI-779 (temsirolimus), RAD001 (everolimus) and AP23573 (ridaforolimus). The orally available RAD001 is more efficacious than rapamycin, as it has a higher affinity to FKBP12 (104).

Rapamycin has been tested *in vitro* in pre-clinical models of T-ALL, where it induced apoptosis and/or cell cycle arrest and synergized with chemotherapeutic drugs (doxorubicin, idarubicin, dexamethasone) (105-107). Interestingly, rapamycin synergized with the glycolysis inhibitor, 3-BrOP in T-ALL cell lines, where the combined treatment induced apoptosis (108). It was concluded that when ATP is depleted by glycolysis inhibition, blocking mTORC1 may further limit nutrient uptake, which resulted in additional cytotoxicity.

CCI-779 was able to block *in vitro* IL-7-induced proliferation, survival and cell cycle progression of primary T-ALL cells, and synergized with both doxorubicin and dexamethasone (109).

The Pediatric Preclinical Testing Program (PPTP) evaluated rapamycin against T-ALL cell lines and xenografts. Rapamycin induced regression in the two T-cell ALL xenografts studied during PPTP (110).

However, the efficacy of rapamycin/rapalogs as broad-based monotherapies for acute leukemia treatment has not

been as promising as initially expected. Indeed, several mechanisms emerged as barriers to anti-leukemic activity of this class of mTORC1 inhibitors which could explain the mostly disappointing results of clinical trials (111,112).

The rapamycin/rapalog modest effects on leukemic cells, could be due to several reasons. Firstly, these drugs have only a poor pro-apoptotic activity, being mainly cytostatic. Secondly, they do not target all the mTORC1 outputs. In particular, phosphorylation of 4E-BP1 is usually resistant to rapamycin/rapalogs (73,113,114). This is a very critical issue, as 4E-BP1 controls the cap-dependent translation of mRNAs coding for critical factors which regulates cell survival and proliferation in cancer cells. These include c-Myc, cyclin-dependent kinase-2 (CDK-2), cyclin D1, signal activator and transducer of transcription-3 (STAT-3), B-cell lymphoma 2 (Bcl-2), Bcl-xL, survivin, myeloid cell leukemia-1 (Mcl-1), ornithine decarboxylase (45,82). Thirdly, upregulation of PIM protein kinase activity has been shown to contribute to resistance to rapamycin (115). Indeed, PIM1 protein kinase phosphorylates PRAS40 at the same amino acidic residue (Thr246) as Akt and, by doing so, activates mTORC1 (116). Moreover, PIM2 protein kinase phosphorylated 4E-BP1 at Ser 65 residue and this phosphorylative event was documented to be essential for oncogenic protein translation independent of mTORC1 activity in acute myelogenous leukemia cells (113). Interestingly, a small molecule inhibitor of PIM protein kinases (SMI-4a) was cytotoxic to T-ALL cell lines through the induction of a G_1 phase cell cycle arrest, and apoptosis (117). SMI-4a treatment reduced mTORC1, but not mTORC2, activity. However, it upregulated MEK/ERK signaling, possibly due to mTORC1/p70S6K inhibition (117).

In this connection, the disappointing performances of rapamycin/rapalogs have been also ascribed to the feedback loops that lead to re-activation of either PI3K/Akt and/or MEK/ERK signaling upon mTORC1 inhibition (118-121). However, it should be pointed out that the existence of these feedback loops has never been documented in T-ALL cells treated with rapamycin/rapalogs.

In agreement with pre-clinical studies, clinical trials with rapalogs combined with chemotherapy have provided more encouraging clinical results (122,123). Phase I/II clinical trials are ongoing in which CCI-779 is being tested in combination with intensive re-induction therapy (dexamethasone, mitoxantrone, vincristine and PEG-asparaginase) in children with relapsed T-ALL (ClinicalTrials.gov: NCT01403415). Also RAD001 has entered phase I/II clinical trials for T-ALL, in combination with standard chemotherapy regimens (ClinicalTrials.gov: NCT00968253; NCT01523977; NCT01403415).

Dual PI3K/mTOR inhibitors. PI3K and mTOR belong to the PIKK family of kinases, and share high sequence homology in their catalytic domains. Dual PI3K/mTOR inhibitors are ATP-competitive inhibitors that target the active sites of both the holoenzymes. The first compound of this class to be disclosed was the morpholinoquinazoline derivative, PI-103 (124). Dual PI3K/mTOR inhibitors downregulate signaling both upstream and downstream of Akt, thus avoiding the issue of Akt re-activation which follows mTORC1 inhibition. These compounds are more powerful apoptotic inducers than rapamycin/rapalogs and inhibit rapamycin-resistant mTORC1 outputs (125,126). They also target mTORC2 activity (127). PI-103 was cytotoxic to T-ALL cell lines and patient samples, where it inhibited 4E-BP1 phosphorylation, as well as oncogenic protein translation, more efficiently than rapamycin (74,75). Interestingly, Shepherd and coworkers have documented that PI-103 treatment of T-ALL cell lines with activating Notch-1 mutations, caused a compensatory upregulation of Notch-1 signaling, as demonstrated by increased levels of c-Myc (128). PI-103 and a γ-secretase inhibitor (compound E, which targets Notch-1 signaling) synergized in inducing T-ALL cell death, thus providing a rational basis for the use of drug combinations that target both the signaling networks (128). Although PI-103 displayed low toxicity and was well tolerated in mouse xenografts (124), it did not enter clinical trials, mainly because of its rapid *in vivo* metabolism (129).

NVP-BEZ235 is an orally bioavailable imidazoquinoline dual PI3K/mTOR inhibitor (130) that has entered a phase I clinical trial for relapsed/refractory ALL patients (ClinicalTrials.gov:NCT01756118). NVP-BEZ235 inhibited the proliferation and induced apoptosis in T-ALL cell lines and primary lymphoblasts (114). The drug synergized with several chemotherapeutic agents (cyclophosphamide, Ara-C, dexamethasone) currently used for treating T-ALL patients (114,131). In this connection, it is very important to emphasize that NVP-BEZ235 also inhibited DNA-PK and ATM/ATR kinases, that are key players of DNA damage response (DDR) (132). Chemotherapeutic drugs, such as Ara-C and doxorubicin, induce DDR and activate ATR (17,133). Therefore, abrogation of DNA repair by NVP-BEZ235 could potentiate the effects of traditional chemotherapeutic drugs.

NVP-BGT226 is another dual PI3K/mTOR inhibitor which has been tested *in vitro* against T-ALL cell lines and primary lymphoblasts (134). NVP-BGT226 was more powerful in inducing apoptosis than NVP-BEZ235. Nevertheless, a phase I clinical study of NVP-BTG226 in patients with advanced solid tumors, revealed that the drug displayed only a limited antineoplastic activity and inconsistent target inhibition. This was probably due to the fact that efficacious plasma concentrations were not achieved at the maximum safety dose (135).

The main limit of dual PI3K/mTOR inhibitors is that these drugs, by inhibiting PIKK family of kinases, could also result in more toxic side effects than rapamycin/rapalogs (136), even if they seem to be well tolerated when administered orally (137,138).

ATP-competitive mTORC1/mTORC2 inhibitors. Due to the limited success of rapalogs in the treatment of leukemia, a new generation of mTOR inhibitors, which target the ATP-binding site of mTOR and inhibit the catalytic activity of both mTORC1 and mTORC2, were developed. Acting on both mTOR complexes, these compounds display stronger effects on cell growth, proliferation and survival than rapalogs, and they offer a more efficient alternative to rapalogs in the treatment of T-ALL. Their use also minimize the re-activation feedback loops of Akt seen with rapamycin/rapalogs (139). This class of inhibitors displayed, in pre-clinical evaluations, more potent anti-leukemic effects when compared with rapamycin/rapalogs. In particular, they strongly suppressed both mTORC1-dependent phosphorylation of p70S6K and

4E-BP1 (140,141) and mTORC2-dependent phosphorylation of Akt at Ser 473, without affecting PI3K (136,142).

PP-242 was one of the first mTORC1/mTORC2 ATP-competitive inhibitors to be disclosed (143). PP-242 displayed cytotoxic activity against T-ALL and was a potent repressor of cap-dependent mRNA translation in T-ALL cells, most likely via inhibition of the rapamycin-resistant phosphorylation of 4E-BP1 (75). PP-242 has not been developed into the clinic, however its derivative, MLN0128 (formerly INK128), has entered phase I/II clinical trials for cancer patients, including hematological malignancies (e.g., ClinicalTrials.gov: NCT01058707; NCT01351350). MLN0128 displayed potent anti-leukemic activity in pre-clinical models of B-ALL (144).

Other mTORC1/mTORC2 ATP-competitive inhibitors which have been successfully tested *in vitro* against T-ALL cells include AZD-8055 and OSI-027 (75,76). Both of these drugs are being evaluated in clinical trials for individuals with lymphomas (ClinicalTrials.gov: NCT01194193; NCT00698243).

6. Conclusion

mTOR is activated in most T-ALL cell lines and primary samples, due to several mechanisms, which include *PTEN* gene deletion/suppression or PTEN protein phosphorylation/oxidation. mTOR activation confers a poorer prognosis to T-ALL patients. Both mTORC1 and mTORC2 play an important role in the pathophysiology of T-ALL, as they are involved in the proliferation/survival of T-ALL LICs.

Three main classes of mTOR inhibitors have been tested both *in vitro* and *in vivo* in pre-clinical settings of T-ALL: allosteric mTORC1 inhibitors (rapamycin/rapalogs), dual PI3K/mTOR inhibitors and ATP-competitive mTORC1/mTORC2 inhibitors. Some of these are now being tested, alone or in combination with chemotherapeutic drugs, in T-ALL patients. Therefore, in the future also T-ALL could be added to the growing list of disorders where mTOR inhibition is beneficial to patient outcome.

7. Perspectives

A growing body of evidence has documented that mTOR is a key node of the PI3K/Akt/mTOR signaling pathway, which is by far one of the most commonly upregulated signal transduction cascades in human cancer (32). The literature reviewed in this article suggests that there is a strong rationale for targeting mTOR in T-ALL, including the fact that both mTORC1 and mTORC2 are important for T-ALL LIC survival (72). These findings suggest that mTOR inhibition, by targeting LICs, has the potential for eradicating T-ALL.

Could it be possible to specifically target mTOR signaling in T-ALL LICs, without affecting the functions of healthy HSCs? Indeed, evidence suggests that mTOR is important for the biology of normal HSCs (145). However, preliminary findings have indicated that there are subtle differences in how HSCs and LICs utilize the same signaling pathways. This has been demonstrated in murine LICs treated with rapamycin (146), where the drug did not affect HSCs, while it was cytotoxic to LICs. Some of the side effects of rapamycin/rapalogs (anemia, leukopenia, thrombocytopenia) seem to indicate that this class of drugs does indeed affect normal hematopoiesis. However, these side effects are usually quite mild (147). The side effects of dual PI3K/mTOR inhibitors and of ATP-competitive mTORC1/mTORC2 inhibitors on healthy HSCs are at present not well known, although the only hematological toxicity which emerged from a phase I study of BGT-226 was anemia (148).

A major challenge in the clinical use of mTOR inhibitors remains the identification of patients who will likely respond to the treatment. For example, it has been recently documented that B-lymphoma cell lines which did not express 4E-BP1, were resistant to ATP-competitive mTORC1/mTORC2 inhibitors (149).

Additional work is therefore required to identify and confirm predictive biomarkers of constitutive/acquired resistance and sensitivity to each drug in large scale clinical trials using homogeneous patient populations (32). Future studies could also benefit from a more thorough analysis of the entire PI3K/Akt/mTOR pathway and of its cross-talk with other signal transduction networks aberrantly activated in T-ALL, including the Notch-1 pathway (85,86). All of these studies could provide the rationale for developing personalized pharmacological treatments, based on mTOR inhibitors, with or without chemotherapeutics or other targeted agents, aimed at T-ALL eradication.

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