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Targeting the PI3K/Akt/mTOR signaling pathway as a new therapeutic strategy for personalized treatments in acute lymphoblastic leukemia

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1. Introduction

1.1 The acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is an acute form of blood cancer, characterized by the uncontrolled clonal proliferation of cancerous, immature lymphoid cells, known as lymphoblasts (Fig. 1). In patients with ALL, lymphoblasts are overproduced and continuously multiply, causing damage and death by inhibiting the production of normal cells -such as red and white blood cells and platelets- in the bone marrow and by spreading (infiltrating) to other organs [1].

In T-cell acute lymphoblastic leukemia (T-ALL), the malignant cells are derived in the thymus from T-cell progenitor cells and express immature T-cell immunophenotypic markers [2, 3]. T-cell neoplastic transformation is a complex process in which multiple lesions, involving both oncogenes and tumor suppressor genes, cooperate to alter the normal signaling pathways that regulate proliferation, differentiation, and survival of developing T-cells [4-7]. T-ALL comprises about 15% of pediatric and 25% of adult ALLs. T-ALL was associated with a very bad outcome, however the introduction of intensified polychemotherapy protocols has improved the prognosis of this disorder and current therapies can achieve 5-year relapse-free survival rates of about 75% in pediatric patients and 40-50% in adults [8, 9].

B precursor cell acute lymphoblastic leukemia (B-pre ALL) is a form of leukemia in which too many B-cell lymphoblasts (immature white blood cells) are found in the blood and bone marrow. It is the most common pediatric malignancy and comprises 85% of childhood ALL [10]. New therapeutic protocols have improved pediatric patient survival rate to approximately 80% at 5 years, however some cases still relapse and are tried by long-term side effects of therapy [11-13]. The overall prognosis of children with relapsed disease remains poor with less than 40% survival at 5 years [14]. B-pre ALL is a heterogeneous disorder including several subtypes with specific cellular and molecular features, that are related to clinical outcome [15]. The Philadelphia (Ph) chromosome is the most common cytogenetic anomaly associated with B-pre ALL. The Ph chromosome results from a reciprocal translocation (t) between chromosomes 9 and 22 (t[9,22][q34;q11]) [16], and results in a fusion gene on chromosome 22, i.e. the breakpoint cluster region-Abelson leukemia (Bcr-Abl) viral proto-oncogene. Bcr-Abl fusion proteins

are constitutively active non-receptor tyrosine kinases that alter a myriad of intracellular signaling networks, thus contributing to leukemic cell proliferation and survival.

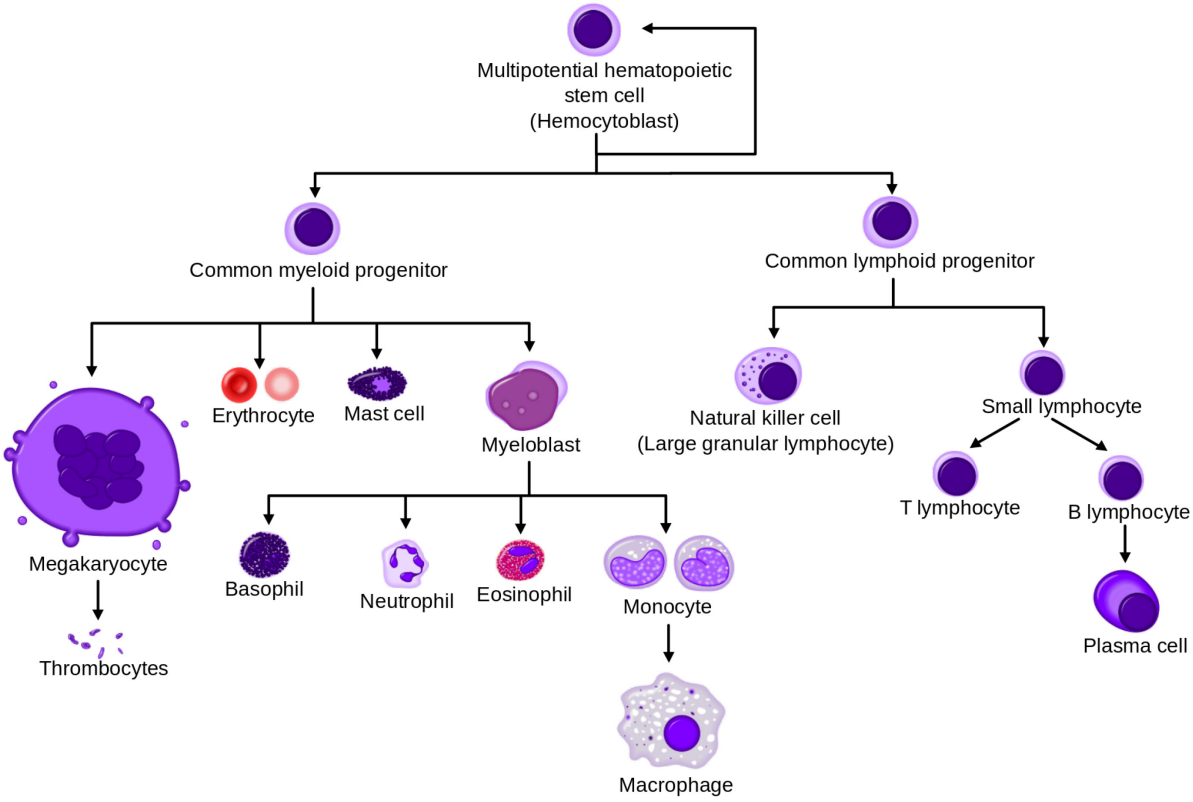


Figure 1: Scheme of myeloid and lymphoid cells maturation.

1.2 The PI3K/Akt/mTOR signal transduction pathway

1.2.a The PI3K family

The phosphoatidylinositol-3-kinase (PI3K) family consists of a number of serine/threonine and lipid kinases, including those that phosphorylate the 3'-OH of phosphatidylinositols. These enzymes consists of at least nine genes in mammalian systems, corresponding to various isoforms that are grouped into three classes, each one with distinct substrate specificity and lipid products: I, II, and III [17]. In mammalian cells, class I PI3Ks are the best understood PI3Ks and the most widely implicated in human malignancies [18].

Class I PI3Ks are further divided into two subgroups: A and B. Class IA PI3Ks contain one of three catalytic subunits (p110α, p110β, p110δ) that form heterodimers with one of the five adaptor (or regulatory) isoforms (p85α, p85β, p55α, p55γ, p50α). In general, class IA PI3Ks are activated downstream of both tyrosine kinase receptors (TKRs) and G protein-coupled receptors (GPCRs). The single class IB PI3K comprises a p110γ catalytic subunit which binds one of two related regulatory subunits, p101, and p87. Class IB PI3Ks mainly act downstream of GPCRs, however they can be stimulated also by TKRs [19]. Only class I PI3Ks have the ability to use phosphatidylinositol-4,5-bisphosphate (PtdIns 4,5P2) to generate the second messenger, phosphatidylinositol- 3,4,5-trisphosphate (PtdIns 3,4,5P3). Once activated by a variety of growth factors and cytokines, class I PI3Ks initiate a cascade of events that promote cancer cell proliferation, survival, and metabolism.

Class II PI3Ks can also be activated by tyrosine-kinase receptors (RTKs), cytokine receptors and integrins; the specific functions in response to these activators are not understood [20].

Class III PI3Ks are heterodimeric enzymes of catalytic (Vps34, 100 kDa) and adaptor (p150) subunits, and use only phosphatidylinositol as a substrate. Class III PI3Ks are implicated in the regulation of mammalian target of rapamycin (mTOR) activity in response to aminoacid availability and the regulation of autophagy in response to cellular stress, indicating the importance of class III PI3K in controlling cell growth and survival [17].

1.2.b Akt

Akt, a 60-kDa serine/threonine kinase, is a key effector of PI3K in carcinogenesis. Akt is a member of the AGC protein kinase family and is the cellular homolog of the *v-Akt* oncogene. The Akt family includes three highly conserved isoforms: Akt1/α, Akt2/β, and Akt3/γ [21] plus a fourth isoform defined Akt-γ1, have been identified in humans (Fig. 2). They are codified by different genes with 80% sequence homology. Akt-1 is the predominant isoform in the major part of tissues, Akt-2 is present in insulin sensitive tissues and Akt-3 has not been completely localized, but it is absent in central nervous system. Akt is constituted by three distinct modules: the pleckstrin homology (PH) domain in the amino-terminal region able to bind phospho-lipids; the central kinase domain which contains an highly conserved activation loop, called T-loop, with threonine residue important for the enzyme activation; a regulative carboxyl-terminal extension of about 40

amino acids containing the hydrophobic F-X-X-F/Y-S/T-Y/F motif. The recruitment of inactive Akt from the cytosol to the plasma membrane, requires that the PH domain of Akt binds to PtdIns 3,4,5P3 or PtdIns 3,4P2 synthesized at the plasma membrane by PI3K. Akt is then phosphorylated at Thr 308 by phosphatidylinositol-dependent kinase 1 (PDK1), and at Ser 473 by mTOR complex 2, resulting in full activation of Akt kinase activity [22]. Threonine 308 is located within the T-loop and is phosphorylated by PDK1, producing a conformational change which promotes the second phosphorylation on serine 473, on the carboxyl-terminal hydrophobic extension of kinase domain. Akt activity is maximal when the kinase is phosphorylated on both residues, increasing substrates affinity and greatly powering the catalytic potential.

Activated Akt is able to translocate from cytoplasm into the nucleus, where signaling events appear independent from those on the plasmatic membrane [23]. Akt phosphorylates a plethora of targets [19, 24, 25] on R-X-R-X-X-S/T consensus motifs [26]. Intriguingly, most of the Akt effects depend on its ability to phosphorylate proteins involved in cell cycle progression, apoptosis, mRNA translation, glycolysis, and angiogenesis, thus unlocking most, if not all, of the critical processes involved in tumorigenesis [27]. The identification of more than 400 different proteins containing the consensus sequence for Akt phosphorylation makes presume that in the future other Akt substrates will be characterized [28]. Thus, the heterogeneity of proteins potentially phosphorylated by Akt supports the key role of this kinase in different fundamental cell processes.

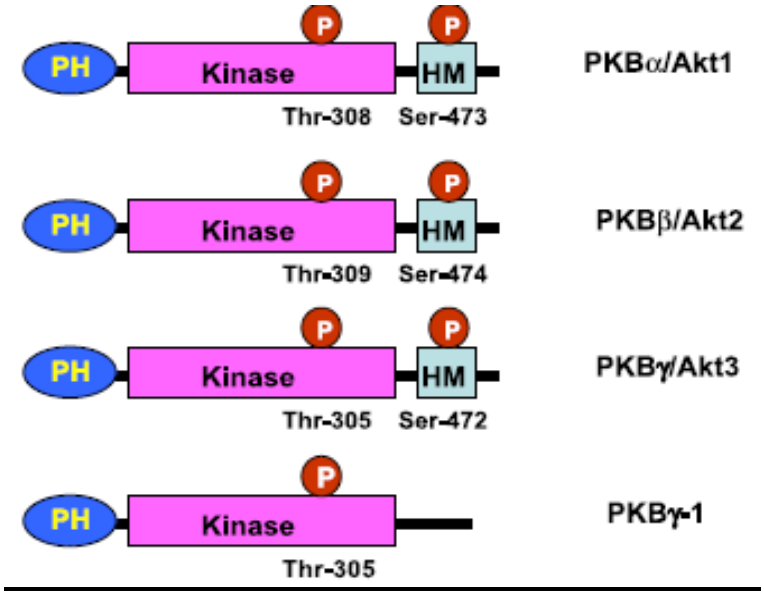


Figure 2. Scheme of the Akt isoforms structure.

1.2.c mTOR

mTOR is a 289-kDa serine/threonine kinase which belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family [29]. mTOR encompasses two functionally distinct multiprotein complexes, referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is a direct downstream effector of Akt, however its activity is controlled through other signaling networks that include the Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) 1/2 signaling network, and the liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) cascade [30, 31].

mTORC1 is characterized by the interactions between mTOR and the regulatory associated protein of mTOR (Raptor), which regulates mTOR activity and functions as a scaffold for recruiting mTORC1 substrates. mTORC1 is sensitive to rapamycin and its analogs (rapalogs) that include RAD001, CCI-779, and AP23753. Rapamycin/rapalogs are allosteric mTORC1 inhibitors and do not target the mTOR catalytic site [32, 33]. They associate with the FK506 binding protein 12 (FKBP-12, [34]), and, by doing so, they induce the disassembly of mTORC1, resulting in inhibition of its activity [33].

mTORC2 comprises the rapamycin-insensitive companion of mTOR (Rictor) and is generally described as being insensitive to rapamycin/rapalogs. However, long-term (>24 hours) treatment of about 20% of cancer cell lines (mainly of hematopoietic lineage) with rapamycin/ rapalogs resulted in mTORC2 activity inhibition [35, 36].

mTORC1 controls translation in response to growth factors/nutrients through the phosphorylation of p70S6 kinase (p70S6K) and 4E-BP1. p70S6K phosphorylates the 40S ribosomal protein, S6 (S6RP), leading to active translation of mRNAs [37]. Furthermore, p70S6K phosphorylates the eukaryotic initiation factor 4B (eIF4B) which is critically involved in translation [38, 39]. However, eIF4B is a downstream target also of MEK/ERK signaling [40]. Unphosphorylated 4E-BP1 interacts with the cap-binding protein, eukaryotic initiation factor 4E (eIF4E), and prevents the formation of the 4F translational initiation complex (eIF4F), by competing for the binding of eukaryotic initiation factor 4G (eIF4G) to eIF4E. 4E-BP1 phosphorylation by mTORC1 results in the release of the eIF4E, which then associates with eIF4G to stimulate translation initiation [41]. eIF4E is critical for translating 5'capped mRNAs, that include transcripts mainly encoding for proliferation and survival promoting proteins, such as c-Myc, cyclin-dependent kinase-2 (CDK-2), cyclin D1, signal activator and transducer of transcription-3 (STAT-3), B-cell

lymphoma (Bcl) -2, Bcl-xL, survivin, myeloid cell leukemia-1 (Mcl-1), ornithine decarboxylase [35, 41, 42].

Moreover, mTORC1 represses autophagy, a lysosome-dependent degradation pathway which allows cells to recycle damaged or superfluous cytoplasmic content, such as proteins, lipids, and organelles [43]. As a consequence, cells produce metabolic precursors for macromolecular biosynthesis or ATP generation. In cancer cells, autophagy fulfils a dual role, because it can have both tumor-suppressing and tumor-promoting functions. Indeed, the autophagic machinery prevents necrosis and inflammation, that can lead to genetic instability and tumorigenesis. However, autophagy might be important for tumor progression, by providing energy through its recycling mechanism during unfavorable metabolic circumstances, that are very common in tumors [44].

The mechanisms that control mTORC2 activity have only begun to be revealed [45], however mTORC2 activation by growth factors requires PI3K, as pharmacological inhibition of PI3K decreased mTORC2 activity *in vitro* [46]. mTORC2 phosphorylates Akt at Ser 473 which enhances subsequent Akt phosphorylation on Thr 308 by PDK1.

PI3K, Akt, and mTORC1/2 are linked to each other via regulatory feedback loops, that restrain their simultaneous hyperactivation [35]. A negative regulation of Akt activity by mTORC1 is dependent on p70S6K-mediated phosphorylation of insulin receptor substrate (IRS) -1 and -2 adapter proteins, downstream of the insulin receptor (IR) and/or insulin-like growth factor-1 receptor (IGF-1R) [47-49]. IRS-1 and IRS-2 are normally required to activate class IA PI3Ks after stimulation of IR/ IGF-1R tyrosine kinase activity. When mTORC1 is active, p70S6K phosphorylates the IRS-1 and -2 proteins on Ser residues, targeting them for proteasomal degradation [50, 51]. Therefore, inhibition of mTORC1 signaling by rapamycin/rapalogs blocks this negative feedback loop and activates Akt through PI3K. Recent findings have highlighted the existence of a rapamycin-sensitive, mTORC1/p70S6K-mediated phosphorylation of Rictor at Thr 1135. This phosphorylative event exerted a negative regulatory effect on the mTORC2-dependent phosphorylation of Akt *in vivo* [52]. Thus, both mTORC1 and mTORC2 could control Akt activation.

PI3K/Akt/mTOR signaling is negatively regulated by lipid and protein phosphatases. Phosphatase and tensin homolog (PTEN) is a lipid phosphatase which removes the 3'-phosphate from PtdIns 3,4,5P3, thereby antagonizing PI3K signaling [53, 54]. Two other lipid phosphatases, Src homology domain-containing inositol phosphatase (SHIP) 1 and 2, remove the 5-phosphate from PtdIns 3,4,5P3 to yield PtdIns 3,4P2 [55]. Protein phosphatase 2A (PP2A) downregulates Akt activity directly, by dephosphorylating it at

Thr 308 and several lines of evidence indicates that PP2A is a tumor suppressor [56]. Moreover, Ser 473 Akt is dephosphorylated by the two isoforms (1 and 2) of PH domain leucine-rich repeat protein phosphatase (PHLPP). Decreased PHLPP activity has been linked to specific cancer types [57, 58].

1.3 Downstream targets of PI3K/Akt/mTOR network

1.3.a GSK3-α/β

GSK3 is a serine (S) /threonine (T) kinase. GSK3 is a gene family comprised of two highly conserved members: GSK-3a and GSK-3b. The GSK3A gene encodes a 51 kDa protein while the GSK3B gene encodes a 47 kDa protein [59]. The larger GSK3-α protein has a glycine-rich extension at its amino terminus. GSK3-α and GSK3-β have 98% sequence identity in their highly conserved kinase domains but only 36% identity in their carboxyl termini [60].

GSK3-α and GSK3-β can be active in unstimulated cells. Both GSK3-α and GSK3-β are inactivated by diverse stimuli and signaling pathways. Inactivation of GSK3-α occurs when it is phosphorylated at Ser 21, while inactivation of GSK3-β occurs when it is phosphorylated at the corresponding residue, Ser 9.

GSK3 is believed to be an important regulatory enzyme in many diseases and disorders such as: cancer and aging (cancer stem cells, cellular senescence, control of stem cell pluripotency and differentiation), immune disorders, metabolic disorders (atherosclerosis, diabetes, and heart disease), neurological disorders (Alzheimer's, amyotrophic lateral sclerosis [ALS], bipolar disorder, mood disorders, Parkinson's, and schizophrenia), and other maladies. GSK3 may be a key therapeutic target for these and other diseases [61-66]. GSK3 has been implicated to play roles in cancers which are resistant to chemo-, radio-, and targeted therapy [67]. Targeting GSK3 may be a means to overcome the resistance of these cancers to certain chemotherapeutic drugs, radiation and small molecule inhibitors [68-70].

1.3.b FOXO

Forkhead box (Fox) proteins are an extensive family of transcription factors, which play a key role in the regulation of crucial biological processes, including cell proliferation, differentiation, metabolism, tissue homeostasis, senescence, survival, apoptosis, and DNA damage repair [71]. The unifying feature of Fox proteins is the "forkhead" box, a sequence of about 100 amino acids that enables binding to specific DNA sequences. The forkhead motif is also known as a "winged-helix" DNA binding domain (DBD) because of its distinct butterfly like appearance. Furthermore the deregulation of the PI3K/Akt signaling cascade has been implicated in the deregulation of almost all the aspects of cell physiology that promotes cell transformation including cell cycle progression, enhanced chemotherapeutic resistance, elevated cell metabolism, increased resistance to hypoxia and tumour metastasis [72, 73]. Many of these processes are controlled by the forkhead (FOXO) transcription family of proteins that bind to a conserved DNA motif (TTGTTTAC) driving transcription of crucial effecter proteins [74, 75]. The FOXO transcription factors are directly phosphorylated by Akt that promotes their export from the nucleus abolishing FOXO-dependent gene transcription, thus ensuring that FOXO activity is suppressed [76]. Given the importance of PI3K signaling in breast cancer and the overwhelming degree of validation for PI3K as a therapeutic target, it is not surprising that the pharmacological inhibition of PI3Ks are considered to be among the most promising strategies in drug development for cancer therapy [77]. Consequently a variety of small molecules with different mechanisms of action (including pan-PI3K, dual PI3K/mTOR, and isoform specific PI3K inhibitors) have been developed and entered a range of clinical trials [17].

1.3.c S6K

mTORC1 controls the hydrophobic motif of p70 ribosomal S6 kinase [32, 78]. Two isoforms of S6K1 are produced from the same transcript by alternative initiation of translational start sites: the shorter form of S6K1, which is largely localized in the cytoplasm, is termed p70S6K. A second isoform, p85S6 kinase, is derived from the same gene and is identical to p70S6 kinase except for 23 extra residues at the amino terminus, which encode a nuclear localizing signal [79]. The functional significance of the differential subcellular localization of the two S6K1 isoforms has not been established,

although it is tempting to speculate that the nuclear form is involved in phosphorylation of the nuclear pool of the free, chromatin-bound form of S6 [80].

p70S6K is probably one of the best characterized downstream effectors of mTORC1 [81]. Ribosomal S6 kinase p70 (p70S6K, S6K) is a member of the AGC family of serine/threonine protein kinases. It is a major substrate of mTOR and is a crucial effector of mTOR signaling [82].

The p70 ribosomal protein S6 kinase 1 (S6K1) plays a key role in cell growth and proliferation by regulating insulin sensitivity, metabolism, protein synthesis, and cell cycle. Thus, deregulation of S6K contributes to the progression of type 2 diabetes, obesity, aging, and cancer and will contribute to the ongoing efforts to develop novel drugs that provide effective treatments to combat diseases that are characterized by deregulation of the S6K signaling pathway.

The activity of S6K is regulated by a wide range of extracellular signals including growth factors, hormones, nutrients (glucose and amino acids), and stress. Work from many research groups has revealed the complexity of S6K1 activation via sequential phosphorylation at multiple sites [83]. The best characterized sites are Thr 229 in the activation loop and Thr 389 in a conserved hydrophobic motif [82]. It is known that PDK1 and mTOR can phosphorylate Thr 229 and Thr 389, respectively. The current model for S6K activation under nutrient and energy sufficient conditions is that PI3-kinase and/or Ras signaling converge to suppress the negative regulator of mTORC1 signaling, the tuberous sclerosis complex (TSC1/2). Inhibition of TSC GAP function results in Rheb-G protein and mTORC1 activation. mTORC1 then phosphorylates Thr 389, creating a docking site for PDK1, which is then able to phosphorylate the activation loop Thr 229 [38, 84]. More recently, it has been found that Ser 371, which resides within a turn motif, is essential for Thr 389 phosphorylation and S6K1 activity [82, 84]. However, it remains unclear how S371 phosphorylation is regulated. One report suggested that this site is also regulated by mTOR [85], but did not fully explain how it contributes to the mechanism of S6K1 activation. For example, rapamycin, an mTOR inhibitor, slightly inhibits Ser 371 phosphorylation, whereas it completely inhibits Thr 389 phosphorylation. Serum starvation and insulin treatment also do not substantially affect Ser 371 phosphorylation, whereas Thr 389 phosphorylation is significantly affected by these factors. These examples demonstrate that regulation of these two sites is very different, although it appears that mTOR is involved in regulating both sites through an unknown mechanism.

1.3.d 4EBP1

Protein synthesis is controlled primarily at the step of mRNA translation initiation [86]. A critical event in this process is the association of the eukaryotic translation initiation factor 4E (eIF4E) with the mRNA 5′ m7GpppN (where N is any nucleotide) cap structure. eIF4E binding to the cap structure is controlled by the eIF4Ebinding proteins (4E-BPs). Binding of 4E-BPs to eIF4E causes inhibition of cap-dependent translation initiation and is relieved by 4E-BP phosphorylation through the mechanistic target of rapamycin (mTOR) signaling [87].

Protein translation is a fundamentally important process that plays an essential role in maintaining normal homeostasis in cells. Numerous studies have demonstrated that mammalian target of rapamycin (mTOR) plays a critical role in controlling the translation initiation step in protein synthesis [38]. The mTORC1 complex is responsible for controlling protein translation downstream of growth factors, nutrients, and stress signals. Serving as one the major substrates of mTOR, 4E-BP1 directly regulates the rate of translation by affecting the assembly of the translation initiation complex [88]. During the translation initiation step in mammalian cells, the cap structure of the mRNA is recognized by the eIF4F complex, which is comprised of eIF4A, eIF4G, and eIF4E proteins. The hypophosphorylated form of 4E-BP1 binds to the cap-binding protein eIF4E and prevents it from interacting with the scaffolding protein eIF4G, thus suppressing cap-dependent translation. Activation of mTOR leads to phosphorylation of 4E-BP1 and disruption of the binding between 4E-BP1 and eIF4E. As a result, 4E-BP1 is released from the cap structure, which allows the association of eIF4G with eIF4E to form the initiation complex and protein translation to proceed [86, 89, 90]. Given its role in controlling protein translation, mTOR-mediated phosphorylation of 4E-BP1 has been studied extensively [38, 87, 91]. Specifically, two sets of phosphorylation sites have been identified in 4E-BP1 upon mTOR activation, in which mTOR is responsible for directly phosphorylating Thr 37 and Thr 46 and priming for additional phosphorylation at Ser 65 and Thr 70. Furthermore, the phosphorylation status of 4E-BP1 has been identified as a biomarker to indicate the efficacy of anticancer treatments because a complete dephosphorylation of 4E-BP1 is required to effectively inhibit cancer cell growth in vitro and in vivo [92].

1.3.e PTEN-mediated inhibition of the pathway

The PI3K/Akt activity is negatively modulated by the Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN) and SH2 Inositol 5-Phosphatase (SHIP) inhibitors. PTEN is a 3'-phosphatase which terminates the PI3K signaling in cells and was found to be inactivated in several human cancers, thus resulting in PI3K/Akt signaling constitutively activated. In particular, PTEN is a dual lipid and protein phosphatase. Its primary target is PIP3 [93], the direct product of PI3K. Since PTEN dephosphorylate PIP3, it acts as negative regulator of the PI3K/Akt pathway [17]. Loss of PTEN function, either in murine embryonic stem cells or in human cancer cell lines, results in accumulation of PIP3 mimicking the effect of PI3K activation and triggering the activation of its downstream effectors. PDK1 contains a C-terminal pleckstrin homology domain, which binds the membrane- bound PIP3 triggering PDK1 activation. Activated PDK1 phosphorylates Akt at Thr 308 activating its serine–threonine kinase activity (100-fold over the basal). Once phosphorylated in Thr 308, further activation of Akt occurs by PDK2 (the complex rictor–mTOR or DNA-PK) phosphorylation at Ser 473. It is known that Akt activation stimulates cell cycle progression, survival, metabolism and other crucial events through phosphorylation of many physiological substrates [94-97]. Activation of Akt results in the suppression of apoptosis induced by a number of stimuli including growth factor withdrawal, detachment of extracellular matrix, UV irradiation, cell cycle discordance and activation of FAS signaling [95, 96, 98]. Hyperactivated Akt has been also shown to promote cell proliferation, possibly through down-regulation of the cyclin-dependent kinase inhibitor p27 as well as up-regulation and stabilization of cyclin D1 [99]. Different genetic approaches have been used to directly assess the role of Akt in PTEN null-induced phenotype. Deleting Akt reversed the cell survival phenotype in PTEN-null cells and reversed its growth advantage [100]. Similarly, inactivation of Akt by dominant-negative mutants inhibits the survival advantage provided by activated class I PI3K [101]. These and other results point out the essential role of PTEN in modulating and turning off the PI3K/Akt network [102-106].

1.4 Role of the PI3K/Akt/mTOR network in acute leukemia

Since acute leukemias can still have an extremely poor outcome, at present great interest surrounds the development of novel and less toxic therapeutic strategies that may target aberrantly activated signaling networks involved in proliferation, survival, and drug resistance of leukemic cells [107].

One such pathway is represented by the PI3K/Akt/mTOR signaling network. Several lines of evidence, obtained in preclinical settings of acute leukemias, have documented how this network could be targeted by small molecule protein kinase inhibitors (SMIs) [108-112]. Indeed, the PI3K/Akt/mTOR pathway is probably the most easily druggable signaling network in human neoplasias, and an impressive array of inhibitors, targeting critical components of this cascade, have been designed by drug companies [113]. However, optimal therapeutic strategies have yet to be identified for a successful treatment of acute leukemias. Inhibition of critical signaling nodes such as PI3K or mTOR induced cell cycle arrest, apoptosis, and lowered drug-resistance of leukemic cells [108-112]. Several phase I/II clinical trials are now underway, in which PI3K or mTOR inhibitors are being tested in leukemic patients [114-116].

1.4.a PI3K/Akt/mTOR signaling in T-ALL

PI3K/Akt/mTOR signaling up-regulation is very common in T-ALL, being detectable in 70-85% of the patients [117], and portends a poorer prognosis [118]. Similarly to AML, multiple mechanisms could lead to PI3K/Akt/mTOR increased activity in T-ALL cells. Much attention has been devoted to PTEN, since the initial report by Ferrando and coworkers documenting that PTEN gene expression was inactivated in T-ALL cell lines and patients displaying Notch-1 activating mutations, through a repressive mechanism mediated by Hairy and Enhancer of Split homolog-1 (HES-1) [119-121]. In T-ALL cell lines, PTEN loss correlated with resistance to Notch inhibitors, raising concerns that patients with PTEN-negative disease could not respond to Notch inhibitor therapy [120]. However, it has been subsequently demonstrated that PTEN loss did not relieve primary T-ALL cells of their “addiction” to Notch-1 signaling [122]. It has been reported that PTEN down-regulation could be a consequence also of miR-19 overexpression, which resulted in lower expression of several genes controlling the PI3K/Akt/mTOR cascade, including

PTEN [123]. Furthermore, in a zebrafish model of T-ALL, c-Myc, which is typically overexpressed downstream of activated Notch-1 in T-ALL [124], caused PTEN mRNA down-regulation [125].

Nevertheless, in most T-ALL clinical samples PTEN is expressed, but is inactivated due to phosphorylation by casein kinase 2 (CK2) and/or oxidation by reactive oxygen species (ROS), which results in overactive PI3K/Akt/mTOR signaling [117].

Mutations in PI3K, Akt, PTEN, and SHIP1 have been described in T-ALL patients. However, their frequency is very low and their functional significance with regard to PI3K/Akt/mTOR activation, has not been thoroughly assessed [126, 127].

IGF-1/IGF-1R signaling plays an important role in the activation of the PI3K/Akt/mTOR cascade in T-ALL cells, as pharmacologic inhibition or genetic deletion of IGF-1R blocked T-ALL cell proliferation and survival [128]. 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 [128].

In T-ALL, cytokines produced by the thymic/bone marrow microenvironment could be involved in up-regulation of PI3K/Akt/mTOR signaling. These include interleukin (IL) -4 [129], and IL-7 [130, 131]. In particular, it has been recently reported that ROS produced by IL-7, are critical for activating PI3K/Akt/mTOR which then mediates proliferation and survival of T-ALL cells [132]. A source for IL-7 could be represented also by thymic epithelial cells [133]. However, increased signaling downstream of the IL-7 receptor (IL-7R) in T-ALL patients, could be a consequence of gain-of-function IL-7R mutations, which are detected in about 9% of T-ALL pediatric patients [134].

Another cytokine with the potential for activating PI3K/Akt/mTOR signaling is the CXC chemokine ligand 12 (CXCL12), referred to as SDF-1a (stromal cell-derived factor 1a), the ligand for the CXC chemokine receptor 4 (CXCR4) [153]. CXCL12 is produced by bone marrow stromal cells in T-ALL patients [135] and has been recently demonstrated to be involved in PI3K/Akt activation and drug-resistance in T-ALL cells [136].

It is not clear whether mTORC1 could be activated by signaling pathways other than PI3K/Akt in T-ALL cells. IL-7 activates MEK/ERK in T-ALL primary cells, however pharmacological inhibition of MEK/ERK did not have any negative effects on cell cycle progression and survival [130]. Thus, the pathophysiological relevance of MEK/ERK activation in T-ALL needs to be further investigated. In any case, MEK/ERK up-regulation is observed in about 38% of adult T-ALL patients [137].

1.4.b PI3K/Akt/mTOR signaling in B-pre ALL

In the Philadelphia chromosome, the breakpoint may occur within one of four sites on the Bcr gene to yield three proteins of different sizes: p190, p210, and p230 [138]. The p190 Bcr-Abl fusion protein occurs in about 90% of children and between 50% and 80% of adults with Ph+ B-pre ALL. The p210 Bcr-Abl gene constitutes the rest of the Ph+ B-pre ALL population, while p230 characterizes chronic myelogenous leukemia [15]. Until recently, Ph+ B-pre ALL patients treated with conventional chemotherapy carried a very poor prognosis irrespective of their age (approximately 10% survival at 5 years). However, the outcome for patients with Ph+ B-pre ALL has improved substantially with the introduction of the tyrosine kinase inhibitor (TKI) imatinib in combination with chemotherapy [139]. Second generation TKIs (dasatinib, nilotinib) have displayed a promising activity in Ph+ B-ALL cases that developed resistance to imatinib due to Bcr-Abl mutations, although there are Bcr-Abl mutations, such as T315I, that are resistant to these novel TKIs [15, 140].

In Ph+ B-pre ALL, the Bcr-Abl tyrosine kinase is upstream of the PI3K/Akt/mTOR pathway [141-145]. Bcr-Abl associates with a number of proteins (c-Cbl, Shc, GRB-2, and GAB-2) that bind the p85α subunit of PI3K [146], resulting in its activation [147]. Accordingly, the Bcr-Abl inhibitor imatinib down-regulated mTORC1 activity in Ph+ chronic myelogenous leukemia cells [148], while Ph+ B-pre ALL cell lines were hypersensitive to rapamycin [149].

PI3Ks play a key role in Bcr-Abl-dependent models of murine leukemogenesis. Indeed, it was possible to create mice that had *Pik3r1* (p85α/p55α/p50α) deleted specifically in the B-cell lineage and *Pik3r2* (p85β) deleted in all cells. As a consequence, there was decreased p190 Bcr-Abl-mediated *in vitro* colony transformation of both α- and α-/β- progenitor B-cells. Moreover, p190+/α-/β- B-cells displayed a severe loss of leukemogenic potential *in vivo* [150]. However, it was found that either genetic or pharmacological (wortmannin, LY294002) inhibition of PI3K only partially reduced mTORC1 activity, as assessed by phosphorylation of S6RP in these cells. The role of two other potential mTORC1-controlling pathways (MEK/ERK and amino acid sensing) has been investigate in order to explore the mechanism of PI3K/Akt-independent mTORC1 regulation. Basal ERK phosphorylation was consistently elevated in α-/β- leukemic colony forming cells (L-CFCs) and blocked by treatment with a MEK inhibitor [150]. Nevertheless, MEK inhibition did not affect mTORC1 activity, as judged by phosphorylation of 4E-BP1, while

p-S6RP levels were modestly reduced in both control and α-/β- L-CFCs, most likely due to stimulatory effects of ERK on p70S6K [151]. When the contribution of amino acid sensing by withdrawal of leucine from the culture media was assessed, mTORC1 activity was rapidly extinguished in α-/β- L-CFCs, as reported in other cell systems [152]. Amino acid sensing by mTORC1 was promoted by class III PI3K (hVPS34), an enzyme whose activity is sensitive to wortmannin [153]. This might explain the partial inhibition of mTORC1 signaling by wortmannin in α-/β- L-CFCs that lack class IA PI3Ks. Therefore, residual mTORC1 activity in α-/β- L-CFCs was MEK/ERK-independent and sustained by amino acid sensing and, perhaps, other pathways that remain to be defined [150].

However, there are some Bcr-Abl-independent mechanisms of PI3K activation that resulted in imatinib resistance [154], but they have not been analyzed thoroughly. Another reason for enhanced PI3K/Akt/mTOR signaling in Ph+ B-pre ALL is due to the fact PP2A is functionally inactivated during the blast crisis of chronic myelogenous leukemia through the inhibitory activity of SET protein, which is regulated by Bcr- Abl [155]. Reactivation of PP2A activity by FTY720 (fingolimod, a PP2A activator which has been approved as an immunomodulator for oral use in patients with multiple sclerosis [156]), led to leukemic cell growth suppression, enhanced apoptosis, impaired clonogenicity, and decreased *in vivo* leukemogenesis of imatinib- and dasatinib-sensitive and -resistant Ph+ B-pre ALL cells, as well as Ph+ B-pre ALL progenitors (CD34+/CD19+). Importantly, healthy CD34+ and CD34+/CD19+ bone marrow cells were unaffacted by FTY720. Moreover, pharmacologic doses of FTY720 suppressed *in vivo* Bcr-Abl-driven leukemogenesis (including leukemogenesis promoted by the T315I Bcr-Abl mutant which is resistant to imatinib and second generation TKIs) without exerting any toxicity in mice [157]. In Ph- B-pre ALL cases, the mechanisms for PI3K/Akt/mTOR up-regulation are unclear, however, they could be dependent on activation of signaling downstream of cytokine receptors, through interactions of leukemic cells with bone marrow stromal cells [158-162]. Interestingly, pediatric B-pre ALL patients with high expression of VLA-4 displayed an adverse outcome, which might be related to activation of PI3K/Akt/mTOR signaling [163]. Moreover, gain-of-function mutations in IL-7R have been identified in pediatric Ph- B-pre ALL cases [164], that could account for pathway activation. Very recently, it has been shown that ETV6/RUNX1 silencing abrogated PI3K/Akt/mTOR signaling in pediatric precursor B-ALL, however, no mechanistic explanation for this phenomenon was presented [165].

1.5 Therapeutic strategies acting on PI3K/Akt/mTOR network in leukemia

1.5.a Advances in targeting the PI3K/Akt/mTOR pathway

The PI3K/Akt/mTOR pathway is also involved in drug resistance, sensitivity to therapy and metastasis [30, 112, 115, 166-172]. PIK3CA mutations may act as driver mutations in certain cancers responsible for metastasis [173]. Novel PI3K-alpha inhibitors have been isolated and they inhibit metastasis [174]. Most PI3K inhibitors are cytostatic rather than cytotoxic and it has been questioned whether treatment with a single PI3K inhibitor will be effective [175].

There have been many recent advances in the development of inhibitors which target this pathway. One of the key developments is in dual PI3K/mTOR inhibitors.

Waldenstrom's macroglobulinemia proliferates, in part, in response to aberrant PI3K/Akt activity. The dual PI3K/Akt inhibitor NVP-BEZ235 suppresses the growth of the Waldenstrom's anemia cells as well as has effects on the tumor microenvironment [176].

The PI3K/Akt/mTOR signaling network is activated in acute leukemias of both myelogenous and lymphoid lineage, where it correlates with poor prognosis and enhanced drug-resistance. Treatment of AML and ALL with dual PI3K/mTOR inhibitors has been shown to be more effective than treatment with rapamycin which blocks mTORC1 but not mTORC2 [177]. The dual PI3K/mTOR inhibitors suppressed the rapamycin-resistant phosphorylation of eukaryotic initiation factor 4E-binding protein 1. The novel dual PI3K/mTOR inhibitor NVPBEZ235, an orally bioavailable imidazoquinoline derivative, has entered clinical trials. NVPBEZ235 was cytotoxic to a panel of T-ALL cell lines as determined by MTT assays. NVP-BEZ235 induced cell cycle arrest and apoptosis. A dose- and time-dependent dephosphorylation of Akt and mTORC1 downstream targets was observed after NVP-BEZ235 treatment.

1.5.b Akt Inhibitors

Akt inhibition may represent a potential therapeutic strategy in acute lymphoblastic leukemia. Many attempts to develop Akt inhibitors (Fig. 10) have been performed over the years. In many of the earlier attempts, the various Akt inhibitors either lacked specificity or

had deleterious side effects. Part of their deleterious side effects of many "Akt" inhibitors are probably related to the numerous critical functions that Akt plays in normal physiology. Namely some Akt inhibitors will alter the downstream effects of insulin on Glut-4 translocation and glucose transport.

MK-2206 [8-(4-(1-aminocyclobutyl)phenyl)-9-phenyl-[1,2,4]triazolo[3,4-f][1,6]naphthyridin-3(2H)-one] is an allosteric Akt inhibitor which inhibits both Thr 308 and Ser 473 phosphorylation (Fig. 3). It also inhibits the downstream effects of insulin on Glut-4 translocation and glucose transport [178]. MK-2206 decreased T-acute lymphocytic leukemia (T-ALL) cell viability by blocking the cells in the G_0/G_1 phase of the cell cycle and inducing apoptosis. MK-2206 also induced autophagy in the T-ALL cells. MK-2206 induced a concentration-dependent dephosphorylation of Akt and its downstream targets, GSK3-α/β and FOXO3A.

MK-2206 also was cytotoxic to primary T-ALL cells and induced apoptosis in a T-ALL patient cell subset (CD34+/CD4-/CD7-) which is enriched in LICs [179]. MK-2206 is in at least 43 clinical trials either as a single agent or in combination with other small molecule inhibitors or chemotherapeutic drugs with diverse types of cancer patients.

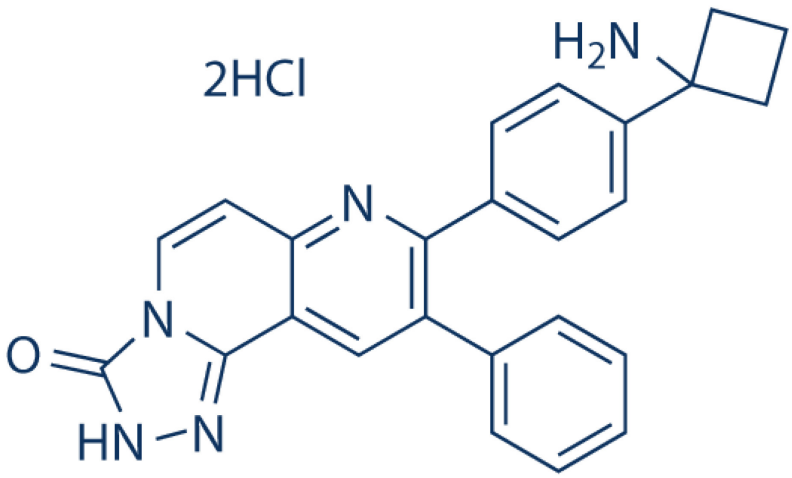


Figure 3: Chemical structure of MK-2206.

GSK690693 [4-(2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-((S)-piperidin-3-ylmethoxy)-1H-imidazo[4,5-c]pyridin-4-yl)-2-methylbut-3-yn-2-ol] is a pan Akt inhibitor developed

by GSK (Fig. 4). GSK690693 is an ATP-competitive inhibitor effective at the low-nanomolar range. Daily administration of GSK690693 resulted in significant antitumor activity in mice bearing various human tumor models including SKOV-3 ovarian, LNCaP prostate, and BT474 and HCC-1954 breast carcinoma. The authors also noted that GSK690693 resulted in acute and transient increases in blood glucose level [180]. The effects of GSK690693 were also examined in 112 cell lines representing different hematologic neoplasia. Over 50% of the cell lines were sensitive to the Akt inhibitor with an EC50 of less than1 μM. ALL, non-Hodgkin lymphomas, and Burkitt lymphomas exhibited 89%, 73%, and 67% sensitivity to GSK690693, respectively. Importantly GSK690693 did not inhibit the proliferation of normal human CD4+ peripheral T lymphocytes as well as mouse thymocytes.

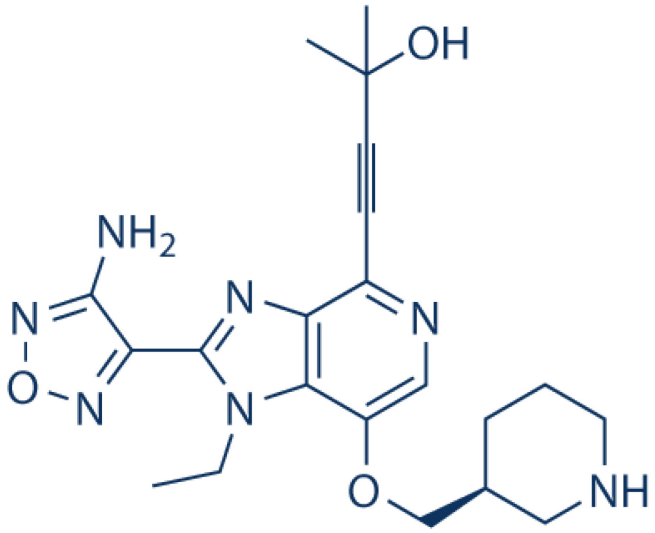


Figure 4: Chemical structure of GSK690693.

Alkylphospholipids and alkylphosphocholines (APCs) are promising antitumor agents, which target the plasma membrane and affect multiple signal transduction networks including Akt.

Perifosine [octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate] (KRX-0401) is a synthetic novel alkylphospholipid (Fig. 5) which inhibits the translocation of Akt to the cell membrane, blocking the growth of several different human cancers [181]. So, via its

interference with the turnover and synthesis of natural phospholipids, disrupts membrane-linked signaling pathways at several sites including lipid rafts, thereby inhibiting the PI3K/Akt survival network. The effects of perifosine have been examined on many different tumor types. Perifosine induces caspase-dependent apoptosis and downregulates P-glycoprotein expression in multidrugresistant T-ALL cells by a JNK-dependent mechanism [182]. Perifosine is or has been in at least 43 clinical trials to treat various cancer patients, with either blood cancers or solid tumors, either by itself, or in combination with other agents. It has advanced to phase III clinical trials for CRC and MM. In the USA it has orphan drug status for the treatment of MM and neuroblastoma.

Figure 5: Chemical structure of Perifosine.

1.5.c mTORC1 Inhibitors

Rapamycin (Rapamune, Pfizer) is a macrolide, produced by the microorganism *Streptomyces hygroscopius* and showed antifungal properties. Shortly after its discovery, immunosuppressive properties were detected, which later led to the establishment of rapamycin as an immunosuppressant. In the 1980s, rapamycin was also found to have anticancer activity although the exact mechanism of action remained unknown until many years later [183-185]. In the 1990s there was a dramatic change in this field due to studies on the mechanism of action of rapamycin and the identification of the drug target [186]. It was found that rapamycin inhibited cellular proliferation and cell cycle progression. It was approved by the FDA in 1999 to prevent rejection in organ transplant patients. Rapamycin/rapalogs (Fig. 10) act as allosteric mTORC1 inhibitors and do not directly affect the mTOR catalytic site [115, 177]. They associate with the FK506 binding protein

12 (FKBP-12) and by so doing, they induce disassembly of mTORC1, resulting in repression of its activity [33, 34].

The rapalogs have been examined in clinical trials with patients having various cancers including: brain, breast, HCC, leukemia, lymphoma, MM, NSCLC, pancreatic, prostate, and RCC [187, 188]. Furthermore rapamycins are being considered as anti-aging and anti-obestity drugs as well as to prevent diabetic neuropathy [189-192].

The rapalogs includes temsirolimus, and everolimus (RAD001), which are being evaluated in cancer clinical trials [193].

The rapalogs temsirolimus [Rapamycin, 42-[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate]] (CCI-779) is approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), for the treatment of renal cell carcinoma (RCC) and mantle-cell lymphoma. Temsirolimus (Fig. 6) has higher water solubility than rapamycin and is ester analogue of sirolimus that is rapidly converted to the parent compound after intravenous administration therefore administrated by intravenous injection [185, 194].

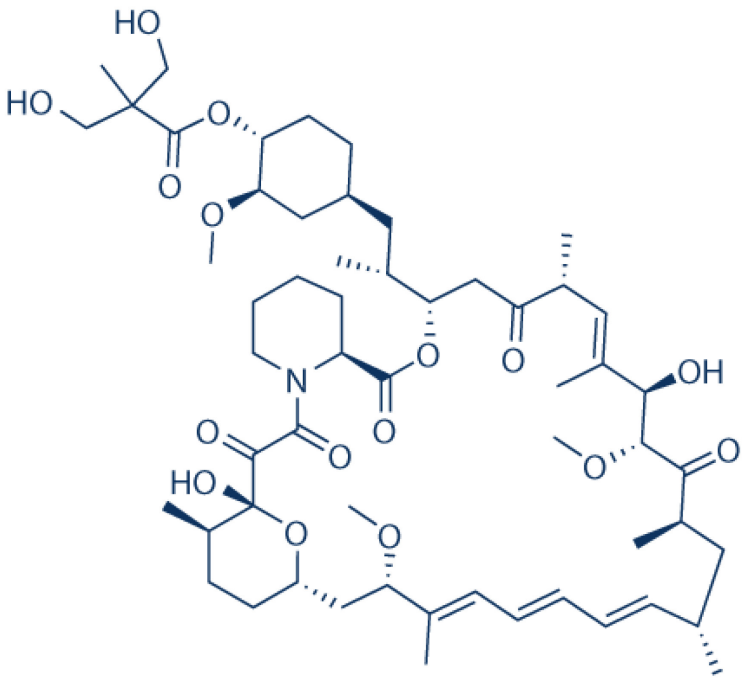


Figure 6: Chemical structure of CCI-779.

The rapalogs everolimus [23,27-Epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine, rapamycin derive] (RAD001) (Fig. 7) is the second novel rapamycin analog [183]

approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced renal cell carcinoma, subependymal giant cell astrocytoma (SEGA) associated with Tuberous Sclerosis (TS), and Progressive neuroendocrine tumors of pancreatic origin (PNET), as single agent therapy, and for the treatment of hormonereceptor positive breast cancer as combination therapy with exemestane [195].

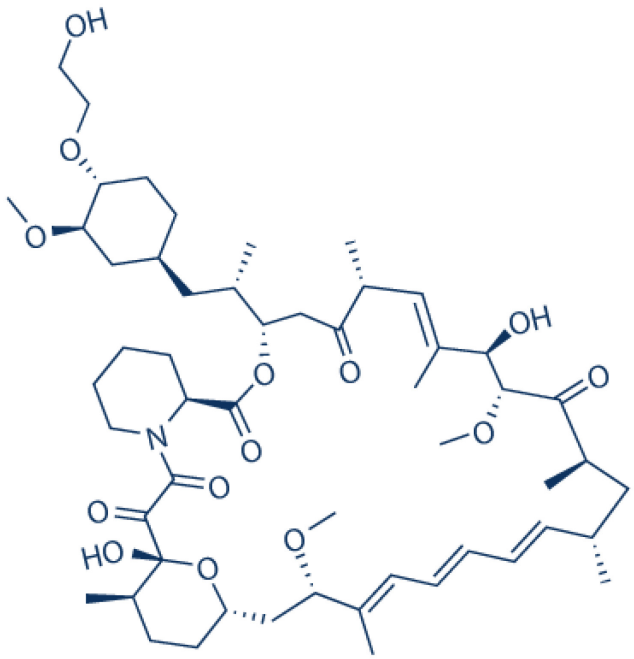


Figure 7: Chemical structure of RAD001.

A reason for the limited success of the mTOR inhibitor is that there is a feedback loop between mTORC1 and Akt in certain tumor cells. It seems that mTORC1 inhibition by rapalogs fails to repress a negative feedback loop that results in phosphorylation and activation of Akt [196]. These limitations have led to the development of the second generation of mTOR inhibitors.

1.5.d mTOR Inhibitors

The mTORC1/mTORC2 dual inhibitors (Fig. 10) are the second generation of mTOR inhibitors designed to compete with ATP in the catalytic site of mTOR (ATP-competitive kinase inhibitors). They inhibit all of the kinase-dependent functions of mTORC1 and mTORC2 and therefore, block the feedback activation of PI3K/Akt signaling, unlike

rapalogs that only target mTORC1 [196, 197]. This is the most important advantages of these mTOR inhibitors, i.e. the considerable decrease of Akt phosphorylation on mTORC2 blockade and in addition to a better inhibition on mTORC1 [198]. These types of inhibitors have been developed and several of them are being tested in clinical trials. Like rapalogs, they decrease protein translation, attenuate cell cycle progression, and inhibit angiogenesis in many cancer cell lines and also in human cancer. In fact they have been proven to be more potent than rapalogs [197].

Torin-2 [9-(6-aminopyridin-3-yl)-1-(3-(trifluoromethyl)phenyl)benzo[h][1,6]naphthyridin-2(1H)-one] is a second generation ATP-competitive mTOR inhibitor, with a superior pharmacokinetic profile to previous inhibitors (Fig. 8). It potently target mTORC1-dependent T389 phosphorylation on S6K. Torin-2 also exhibited potent biochemical and cellular activity against PIKK family kinases including ATM, ATR and DNA-PK, the inhibition of which sensitized cells to irradiation. Similar to the earlier generation compound Torin-1 and in contrast to other reported mTOR inhibitors, Torin-2 inhibited mTOR kinase and mTORC1 signaling activities in a sustained manner suggestive of a slow dissociation from the kinase [199].

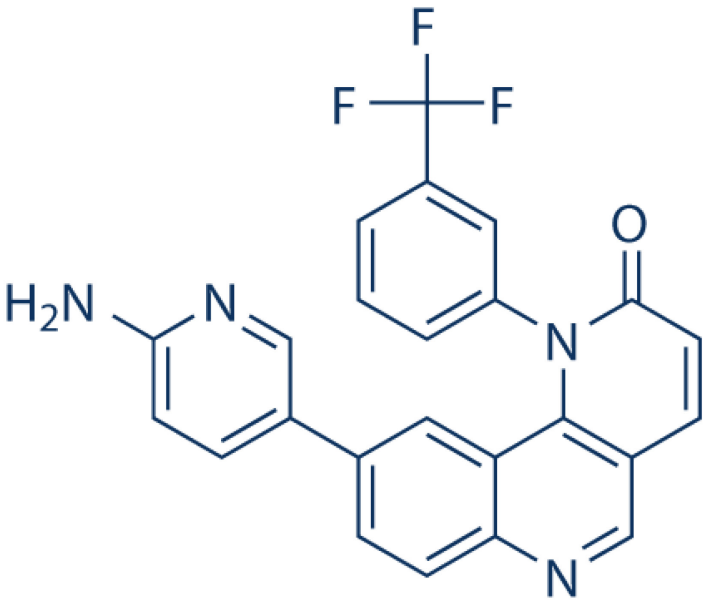


Figure 8: Chemical structure of Torin-2.

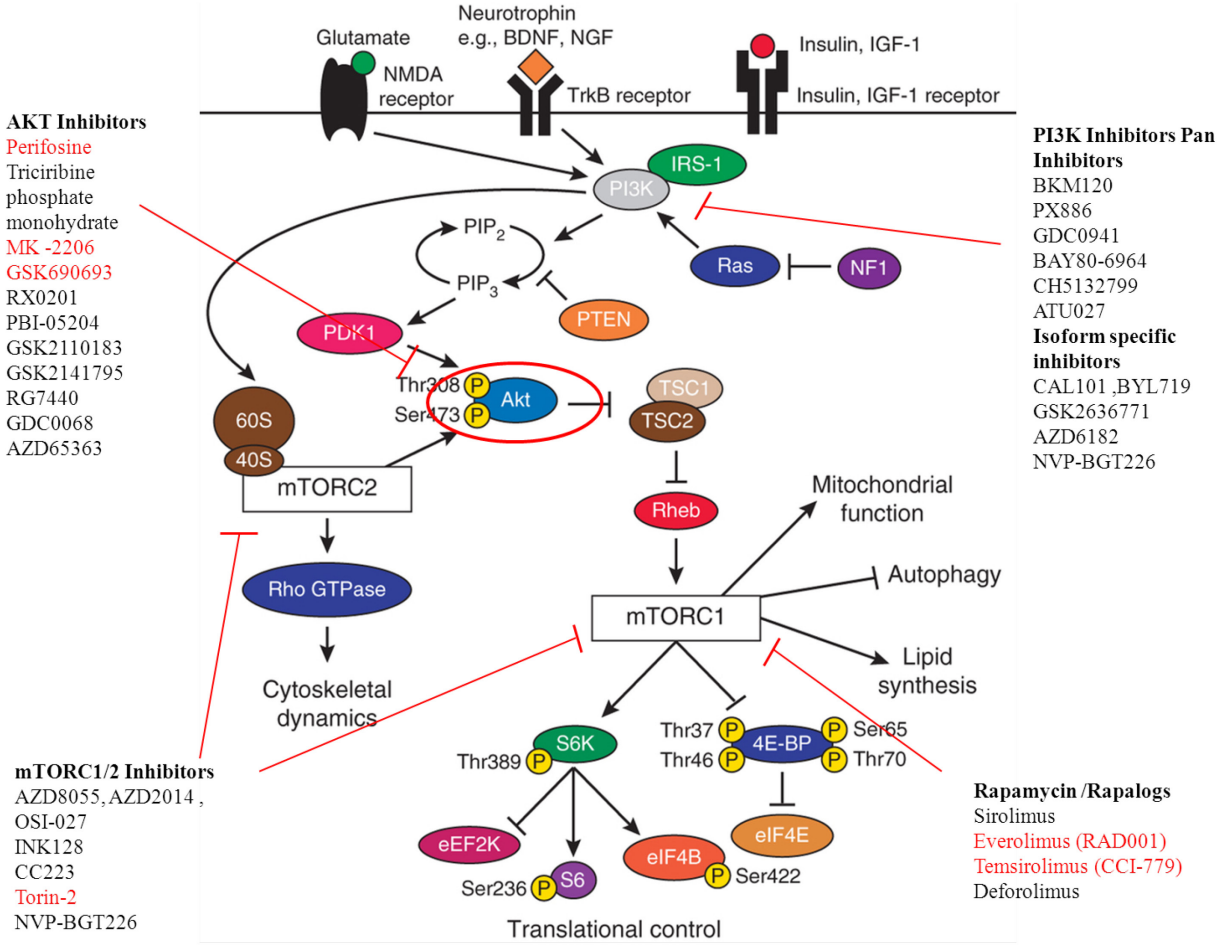


Figure 9: Drugs against the PI3K/Akt/mTOR network.

1.5.e Resistance to Rapamycin/Rapalogs and mTOR inhibitors and the effectiveness to multiple targeting the PI3K/Akt/mTOR signaling pathway

The obvious goal of current inhibitor development is to improve the effectiveness of treatments of cancer patients with small molecule signal transduction inhibitors. However, this has proven to be difficult for multiple reasons: first, there is a distinct genetic susceptibility for the success of a signal transduction inhibitor in suppressing cellular growth and proliferation, second, many of the small molecule signal transduction inhibitors are cytostatic as opposed to being cytotoxic and therefore they will need to be combined

with a therapeutic modality that induces cell death, and third, more than one signal transduction pathway may be activated in the cancer cells.

Rapalogs have shown objective responses in only a subset of patients, and unfortunately the responses are frequently shortlived. Mechanisms of acquired resistance to rapalogs are unknown. These therapeutic failures have been attributed, in part, to KRAS or BRAF mutations. Since KRAS is frequently mutated in human cancer, many cancers will have constitutive mTOR activity, but may not be sensitive to rapamycin as they will have Raf/MEK/ERK pathway activation. Since rapalogs function by binding FKBP-12, mutations in FKBP12 or the FKB domain of mTOR can suppress binding affinity and lead to rapalog resistance [200-203]. Direct mTOR inhibitors will overcome this resistance. The presence of the IGF1R/PI3K-mediated feedback loop, which results in ERK activation, is another mechanism of resistance to rapamycin rapalogs [115, 171, 177, 204-207].

Resistance to rapamycin has been also associated with rapamycin-induced Akt activation, as a result of inhibition of the S6K/IRS-1 feedback loop. Rapamycin not only inhibits S6K phosphorylation but also induces Akt Ser 473 phosphorylation, hence activating Akt [208, 209]. Therefore, there is a growing interest in multi-component target therapies: the combined delivery of multiple drugs is an attempt to overcome drug resistances and to improve clinical outcome. Approaches to prevent Akt activation, such as the use of specific inhibitors, are being pursued [210]. However, an alternate approach is to target this pathway with mTOR kinase inhibitors that potently inhibit mTORC1 as well as mTORC2, thus inhibiting Akt Ser 473 phosphorylation, and thereby preventing or attenuating the feedback loop activation of Akt and potentially treating PI3K/Akt/mTOR dependent cancers more effectively [45]. Another approaches is targeting simultaneously both Akt and mTOR protein, in order to obtain a more complete inhibition of the pathway, without the feedback loop activation of Akt.

At last, it is important to use SMI (small molecule inhibitors) which are able to inhibits both Raf/MEK/ERK and PI3K/Akt/mTOR pathways, in order to prevent the rebound activation of a second pathway that carry out a cellular escape to overcome Akt inhibition.

All these approaches could lead to a synergistic effect in cancer inhibition and could represent a new promising therapeutic strategy for the treatment of acute lymphoblastic leukemia.

2. Aim of the study

The acute lymphoblastic leukemia is an aggressive malignant disorder characterized by the abnormal proliferation of cell progenitors. In this pathology chemotherapy resistance and refractory relapses occur, with a poorer prognostic outcome.

The PI3K/Akt/mTOR signaling pathway is a key regulatory cascade controlling cell growth, survival and drug resistance, and it is frequently up-regulated in ALL, where it plays important roles in the etiology, maintenance and progression of acute leukemia.

It is very plausible that the oncogenic signature of some acute leukemia cases embrace activation of this key pathway, and that those cases may benefit from tailor-made therapies involving the use of signaling-specific antagonists. Therefore, the analysis of the intracellular signaling profile of leukemia patients could not only serve to reveal novel molecular targets for treatment of this disease, but also to identify critical biomarkers for accurate and clinically relevant diagnosis and prognosis. Moreover, data suggest that inclusion of inhibitors of the PI3K/Akt/mTOR pathway into current leukemia therapeutic protocols may be of particular relevance.

Targeted therapy with small molecule inhibitors (SMIs) could represent a new therapeutic strategy that has been successful for the treatment of multiple tumors (e.g., gastrointestinal stromal tumors, chronic myelogenous leukemia). Hence, this research is aimed at investigating the PI3K/Akt/mTOR signaling pathway in acute lymphoblastic leukemia cells. In particular, the use of specific inhibitory compounds directed against key proteins of the pathway, such as Akt and mTOR proteins, has been analyzed. The approach has employed single compounds as well as combination of inhibitors in order to investigate the use of strategies targeting signaling cascade at specific levels. These pharmacological strategies allow to inhibit the PI3K/Akt/mTOR pathway and could represent new promising and innovative therapeutic treatments in acute lymphoblastic leukemia.

3. Materials and Methods

3.1 Materials

Torin-2, RAD001, CCI-779, MK-2206, Perifosine and GSK690693 were purchased from Selleck Chemicals (Houston, TX, USA). For cell viability determination, Cell Proliferation Kit I (MTT) was purchased from Roche Applied Science (Basel, Switzerland). For western blotting, primary Akt-1, Ser 473 p-Akt-1, Tyr 202/204 p-ERK 1/2, ERK 1/2 and FoxO3A primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) while all the other antibodies were from Cell Signaling Technology (Danvers, MA, USA), including the rabbit secondary antibody. The mouse secondary antibody, Bafilomycin A1, Chloroquine, Z-VAD-fmk, 3-Methyladenine (3- MA), 1,4-Diazabicyclo[2.2.2]octane (DABCO) and 4′, 6 diamidino-2-pheny-lindole (DAPI) were from Sigma Aldrich (Milan, Italy). Signals were detected with the ECL Plus reagent purchased from Perkin Elmer (Boston, MA, USA). Alpha-MEM, McCoy's 5A and RPMI-1640 mediums, fetal bovin serum (FBS), penicillin and streptomycin were from Lonza (Lonza Milano SRL, Milan, Italy). Annexin V/7-AAD detection kit was from Merck-Millipore (Darmstadt, Germany). The kits for magnetic labeling separation of CD4+ or CD34+ cells were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CD34-phycoerythrine, anti-CD4-PC5, anti-CD7- PC7 and anti-Ser 473 p-Akt-AlexaFluor488 were from Beckman Coulter (Miami, FL, USA). Interleukin (IL)-2, -4, -7, -9 and -15 were from Peprotech (Rocky Hill, NJ, USA). SignalSilence control small interfering RNA (siRNA) and Beclin-1 siRNA II were obtained from Cell Signaling Technology.

3.2 Cell culture and patient samples

All the cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The T-ALL cell lines, JURKAT, MOLT-4, CEM-S (drug-sensitive) and CEM-R (CEM VBL100, drug-resistant cells overexpressing 170-kDa P-glycoprotein) were grown in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS). PEER and BE-13 were grown in RPMI 1640 with 20% FBS. The B-pre ALL cell lines SEM, REH, NALM6 and BV-173 were grown in RPMI 1640 medium 10% FBS, RS4;11 cells were grown in Alpha-MEM medium 10% FBS, TOM-1 in RPMI 1640 medium with 20% FBS and SUP-B15 in McCoy's 5A

medium with 20% FBS. All the media were supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin. The cells were grown at a density of 0.5 to 2 x 10^6 cells/ml and were incubated at 37°C with 5% CO_2. Primary samples from adult B-pre ALL patients (CD10+/-, CD19+, HLA DR+ and cytoplasmic IgM+) were obtained with informed consent according to institutional guidelines. B-pre ALL patient lymphoblasts were cultured in triplicate in flat-bottomed 96-well plates at 37 1C with 5% CO_2 at a density of 2 x 10^6 cells/ml, using RPMI medium supplemented with 20% fetal bovine serum and 2mM l-glutamine.

3.3 MTT test

The Cell Proliferation Kit (MTT, Roche) is designed to be used for the nonradioactive, spectrophotometric quantification of cell proliferation and viability in cell populations using the 96-well-plate format. It can be used for the measurement of cell proliferation in response to treatment and for the analysis of cytotoxic and cytostatic compounds, such as anti-cancer drugs and other pharmaceutical compounds.

MTT (3-(4,5-dimethylthythiazol-2-yl)-2,5-diphenyltetrazolium bromide) is mostly cleaved to formazan by enzymes of the endoplasmic reticulum. This bioreduction occurs intracellularly in viable cells only, and is related to NAD(P)H production through glycolysis. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

The assay is based on the cleavage of a soluble tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) in the presence of an electron-coupling reagent. Cells (2 x 10^6 cells/ml), cultured in triplicate in flat-bottomed 96-well plates at 37°C with 5% CO_2, are treated with different drugs at scalar concentrations and with the control samples, then are incubated with 10 μl of MTT solution for approximately 4 hours. After incubation, a water-insoluble formazan dye is produced and must be solubilized in an additional step with 100 μl of solubilization buffer, overnight at 37°C. After solubilization, the formazan dye is quantified using a scanning multi-well spectrophotometer (ELISA reader) at the wavelength of 550-600 nm. The measured absorbance directly correlates to cells number.

3.4 Cell cycle

Cell cycle analysis was performed using the Muse™ Cell Analyzer (Merck Millipore, Milan, Italy) and/or propidium iodide (PI)/RNase A staining by flow cytometry according to standard techniques. In brief, after 24h of drug treatment, cells were harvested, centrifuged at 300 g for 5 min and washed once with 1X PBS. After fixing them with 70% ethanol at 20°C, cells were centrifuged at 300 g for 5 min and washed once with 1X PBS. Then 200 µl of Muse™ Cell Cycle reagent or 100 µl of propidium iodide (PI)/RNase A staining was added to each tube with an incubation of 30 min at room temperature in the dark. Samples were then analyzed with Muse™ Cell Analyzer or with EPICS XL flow cytometer Beckman Coulter (Miami, FL, USA) with the appropriate software (System II, Beckman Coulter). At least 15000 events/sample were acquired.

3.5 Western blot

For protein extraction 2×10^6 cells were washed twice in PBS and lysed with RIPA buffer (50 mM Tris HCl pH 7.4; 150 mM NaCl; 0.1% SDS and 1% NP40) including protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Samples were incubated for 30 min in ice. Cell extracts were sonicated and centrifuged at 13000 g for 10 min at 4°C. Total protein concentration of supernatants was determined using the BCA Protein Assay (Euroclone, Milan, Italy). Equal amounts of protein samples were loaded on a polyacrylamid gel for electrophoresis separation (8% or 15%) and transferred to a nitrocellulose transfer membrane. Membranes were blocked in TBS with 5% not-fat dry milk and 0.1% Tween-20 1 h at RT and, after 3 washes in TBS with 0.1% Tween-20, incubated at 4°C overnight with the primary polyclonal antibodies (1:1000 dilutions). After 3 washes in TBS with 0.1% Tween-20, samples were incubated 60 min at RT with secondary antibody and washed as previously described. Specific horseradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit) were used. Blots were incubated with mouse anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA) as a loading control. Signals were detected with ECL Plus reagent (Amersham Biosciences; Buckinghamshire, UK) and a ImageQuant LAS4000 detection system (GE Healthcare Europe GmbH, Freiburg, Germany).

3.6 DAPI staining

Cell nuclear morphology was evaluated by fluorescence microscopy following DAPI staining. Cells were treated with Torin-2 for 24 h. The cells were washed with PBS (pH 7.4), cytocentrifuged, fixed with 4% paraformaldehyde/PBS and stained for 3 min with 1 μg/mL DAPI. The cells were then washed with PBS, specimens were embedded in glycerol with antifading agent (DABCO) and examined under Zeiss Axiophot fluorescence microscope (Zeiss, Germany).

3.7 SiRNA experiments

Pre-B and T-ALL cells were transfected with a nucleofection device (Amaxa Inc., Cologne, Germany), using kit C and program X-001, according to the manufacturer's instructions. The day before transfection, cells were seeded at 5 x 10^6 cells/well in12-well tissue culture plate. Then, cells were centrifuged at 90 g for 10 minutes at RT and resuspended in 100 μl of Nucleofector® Solution at RT. Then, the 100 μl of cell suspension were combined with 2 μg DNA, 2 μg pmaxGFP® Vector or 30 nM – 300 nM siRNA (3- 30 pmol/sample). After the transfection, cells were analyzed by the appropriate Nucleofector® Program X-001 (X-01 for Nucleofector® I Device) and partly lysed for western blotting.

For silencing Beclin-1 protein, 100 nM of control (scrambled) or Beclin-1-specific siRNA were used.

For the down-regulation of Bcl-XL, Bcl-XL si-Genome duplexes D-003458-01-0010 and D-003458-04-0010 from Dharmacon were used (Chicago, IL, USA). Scrambled siCONTROL Nontargeting siRNA no. 1 D-001210-01-20 from Dharmacon was used as negative, nonsilencing control.

3.8 PI/Annexin V assay

Apoptosis analysis was performed by staining with Annexin V/7-AAD, using the Muse™ Cell Analyzer (Merck Millipore) and with Annexin V-fluorescein isothiocyanate (FITC) (BD Biosciences, Heidelberg, Germany) and propidium iodide (PI) (Sigma Aldrich, St. Louis, USA) using a FC500 flow cytometer by Beckman Coulter with the appropriate software (CXP, Beckman Coulter) for analyses. In the first case, a 100 μl treated cell

suspension was labeled for 20 min in the dark with the same volume of the Muse™ Annexin-V & Dead Cell reagent (Merck Millipore). Subsequently, quantitative detection of Annexin-V/7-AAD positive cells was performed with the Muse™ Cell Analyzer. In the second method, 5×10^5 cells were harvested and washed twice (180 g, 5 min, 4°C) with cold PBS at indicated points in time. Each cell pellet was resuspended in 100 µl of binding buffer (1×) and 5 µl Annexin V FITC and 5 µl of PI were added. After an incubation time of 15 min in dark at room temperature, cells were centrifuged and resuspended in 100 µl of binding buffer, and then analyzed by flow cytometry. Unstained and single stained controls were included in each experiment.

3.9 Combined drug effect analysis

To characterize the interactions between RAD001 and MK-2206, Torin-2 and MK-2206 and Perifosine, MK-2206 and GSK690693 the combination effect and a potential synergy were evaluated from quantitative analysis of dose-effect relationship, described by Chou and Talalay [211]. In this method, for every combination of two or more agents tested, dose-response curves are generated for each agent individually, and these data are used to analyze the results obtained from the combination treatment within the same experiment.
For each drugs combination experiment, a combination index (CI) number was calculated using the formula:

$$CI = Ca/Cxa + Cb/Cxb;$$

where Cxa and Cxb are the concentrations of compound a and b alone, respectively, needed to achieve a given effect (x%) and Ca and Cb are the concentrations of drugs needed for the same effect (x%) when the drugs are combined.
For each combination experiment, a combination index (CI) number was calculated using the BiosoftCalcuSyn software (Biosoft, Cambridge, UK). This method of analysis generally defines CI values from 0.9 to 1.1 as additive, from 0.3 to 0.9 as synergistic and below 0.3 as strongly synergistic, whereas values over 1.1 are considered as antagonistic.

3.10 Statistical evaluation

The data are presented as mean values from three separate experiments ± s.d. Data were statistically analyzed by a Dunnet test after one-way analysis of variance (ANOVA) at a level of significance of $P<0.05$.

4. Results

4.1 Treatment with MK-2206 affects T-ALL cells

4.1.a MK-2206 displays cytotoxic pro-apoptotic effects on T-ALL cell lines and induces cell cycle arrest

To determine whether MK-2206 could affect viability of T-ALL cell lines, MOLT-4, CEM-R and CEM-S cells were incubated in the presence of increasing concentrations of MK-2206 for either 24 or 48h. Then, the rates of cell survival were analyzed by MTT assays. The experiments documented that all three cell lines were sensitive to MK-2206. After 24h of incubation with the drug, the IC50 was 2.6 μM for MOLT-4, 4.1 μM for CEM-R and 6.9 μM for CEM-S cells (data not shown). After 48h of treatment, the cytotoxic effect was slightly more evident, being the IC50 1.7 μM for MOLT-4, 3.3 μM for CEM-R and 5.1 μM for CEM-S cells (Figure 10a). To establish whether the decreased viability was related to apoptosis, extracts from MOLT-4 and CEM-R cells, treated for 4h with MK-2206 concentrations ranging from 1 to 10 μM, were analyzed by western blotting, which demonstrated cleavage of procaspase-8, procaspase-9, procaspase-3 and of poly(ADP-ribose)polymerase (Figure 10c).

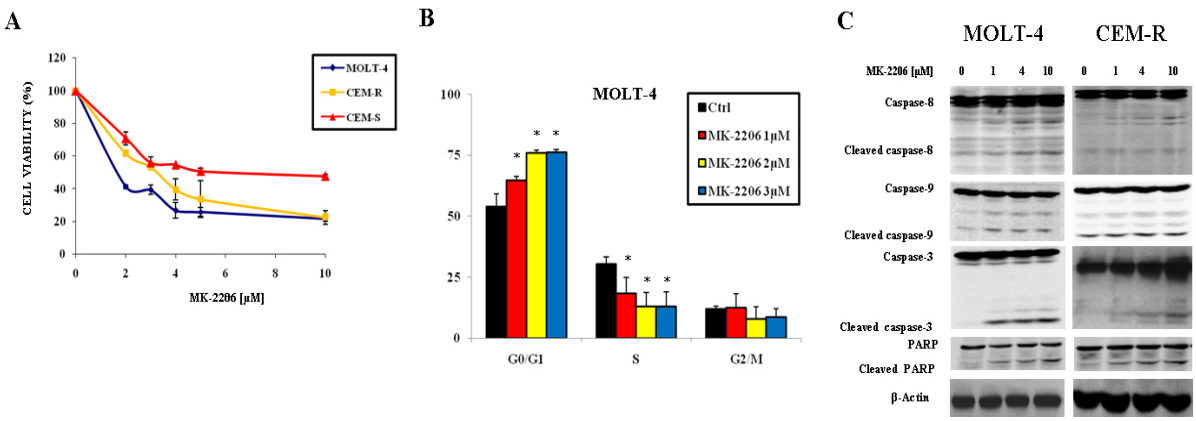


Figure 10. MK-2206 is cytotoxic to T-ALL cell lines and induces cell cycle arrest and apoptosis. **(a)** MTT assay of T-ALL cell lines treated with increased concentrations of MK-2206 for 48h. **(b)** MOLT-4 cells were treated with increasing concentrations of MK-2206 for 24h. Then cell cycle analysis was performed by flow cytometry. MK-2206 treatment resulted in an increase in cells in the G_0/G_1 phase and in a decrease in cells in S phase. CTRL, control (untreated) cells.

Asterisks indicate significant differences compared with CTRL. In **(a)** and **(b)** results are mean of three different experiments ± s.d. **(c)** Western blot analysis documenting caspase-8, -9, -3 and poly(ADP-ribose)polymerase cleavage in MOLT-4 and CEM-R cells, treated for 4h with MK-2206. Antibody to β-actin served as a loading control.

Given the importance of the PI3K/Akt/mTOR signaling pathway in the regulation of cell proliferation [212], the effects of MK-2206 on cell cycle progression were also investigated. MOLT-4 cells were treated with MK-2206 for 24h. Flow cytometric analysis of propidium iodide-stained cells documented a concentration dependent increase in cells in the G_0/G_1 phase of the cell cycle and a concomitant decrease in cells in both S and G_2/M phase (Figure 10b).
Overall, these findings demonstrated that MK-2206 potently reduced the growth of T-ALL cell lines and that this effect was due to apoptosis and G_0/G_1 cell cycle arrest.

4.1.b MK-2206 affects PI3K/Akt/mTOR signaling in T-ALL cell lines

As MK-2206 is an allosteric Akt inhibitor, it was analyzed whether treatment with this drug resulted in down-regulation of Akt phosphorylation. Upon 4h of incubation with MK-2206, a concentration-dependent decrease in both Thr 308 and Ser 473 p-Akt levels was detected in all the cell lines analyzed (Figure 11a). Total Akt levels were unaffected by MK-2206. Akt inhibition had functional consequences on the phosphorylation levels of two well-established Akt substrates, GSK3-α/β and FoxO3A. Both of these proteins displayed dephosphorylation at amino acidic residues (Ser 21/9 for GSK3-α/β and Thr 32 for FoxO3A) that are targeted by Akt. In contrast, expression of total GSK3-α/β and FoxO3A was unaffected by treatment with MK-2206.
MK-2206 affected also mTOR complex 1 (mTORC1) activity, as it dephosphorylated p70S6K on Thr 389 and 4E-BP1 on Thr 37/46. MK-2206 also diminished mTOR complex 2 (mTORC2) activity, as documented by a decrease in the levels of Ser 2481 p-mTOR, a readout for mTORC2 activity (Figure 11b).

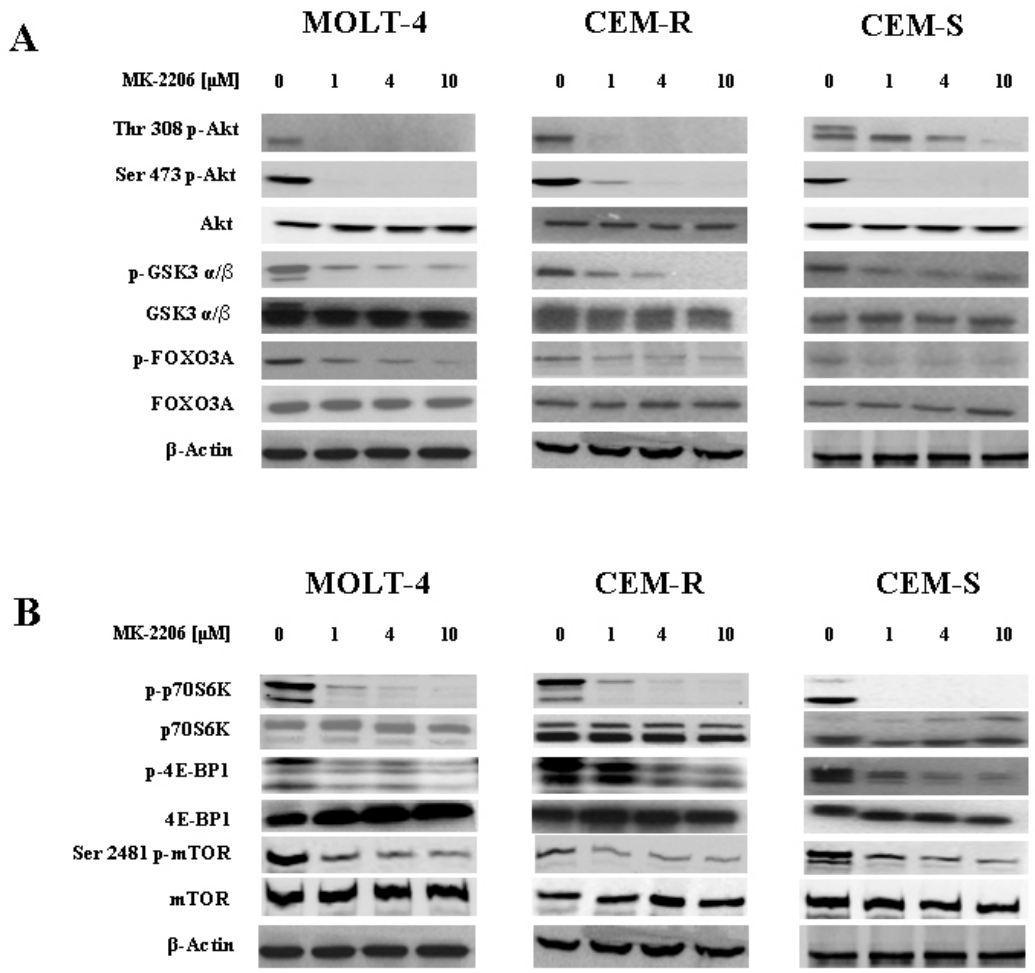


Figure 11. Effects of MK-2206 on the phosphorylation status of critical components of the PI3K/Akt/mTOR signaling pathway. **(a)** Western blot analysis for Akt, GSK3-α/β and FoxO3A. **(b)** Western blot analysis for mTOR and its downstream targets, p70S6K and 4E-BP1. In **(a)** and **(b)**, MK-2206 treatment was for 4h, and β-actin served as a loading control.

4.1.c MK-2206 induces autophagy

It has been reported that MK-2206 induces autophagy in human glioma cells and this protected tumor cells against apoptosis [213]. Microtubule-associated protein 1 light chain 3 (LC3A/B) is a structural component in the formation of autophagosomes and is widely used as an autophagic marker, as its lipidated form (LC3A/B-II) monitors the occurrence of autophagy [214]. Therefore, we investigated whether MK-2206-induced autophagy also in T-ALL cell lines. MK-2206 increased the amount of cleaved (14-kDa form) LC3A/B, a well-established autophagy marker (Figure 12a). Interestingly, the increased cleavage was detected by western blot in MOLT-4 and CEM-S, but not in CEM-R cells.

Beclin-1, an essential initiator of autophagy, interacts with BH3 domain proteins such as Bcl-2, Bcl-XL and Mcl-1 and these interactions could inhibit beclin-1-mediated induction of autophagy [215]. Bcl-XL levels were then down-regulated by siRNA in CEM-R cells and the cleavage of LC3A/B was investigated by western blot. In Figure 12b, we document that specific siRNA (but not scrambled siRNA) to Bcl-XL lowered the levels of Bcl-XL in CEM-R cells. Moreover, when cells treated with Bcl-XL-specific siRNA were treated with MK-2206, the cleavage of LC3A/B was much more evident than in cells treated with scrambled siRNA. Overall, these findings demonstrated that the levels of Bcl-XL are important for determining the induction of MK-2006-dependent autophagy in CEM-R cells.

We then inhibited autophagy using either Bafilomycin A1 or Chloroquine and measured cell viability by MTT assays. Both Bafilomycin A1 and Chloroquine, when used alone, displayed only limited cytotoxic effects against CEM-S cells. However, when they were combined with MK-2206, it was possible to detect an increased cytotoxicity in CEM-S cells (Figure 12c). Overall, these findings suggested that autophagy could protect T-ALL cells by the cytotoxic effects of an Akt inhibitor.

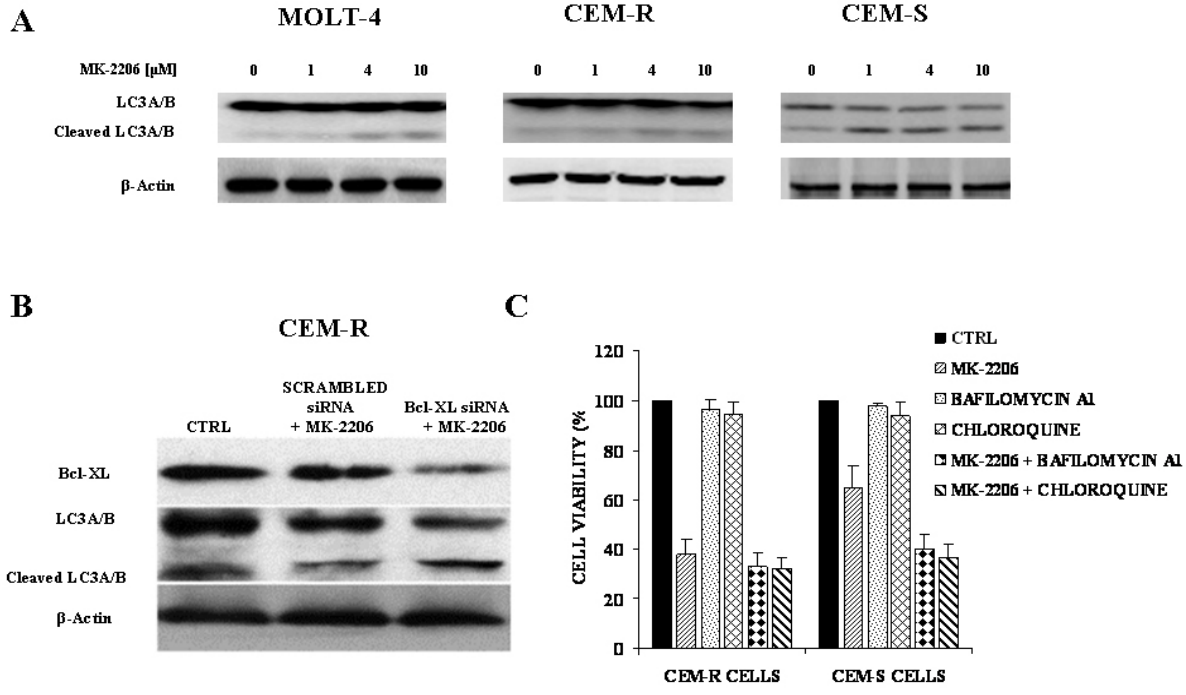


Figure 12. MK-2206 induces autophagy and siRNA down-regulation increases MK-2206-dependent cleavage of LC3A/B. **(a)** Western Blot analysis for LC3A/B in MOLT-4, CEM-R and CEM-S cells. Cells were treated with MK-2206 for 4h. **(b)** The levels of Bcl-XL were down-regulated by a 48-h-long treatment with siRNA, then cells were incubated for 4h with 4 μM MK-2206 and western blot analysis was performed. In **(a)** and **(b)**, β-actin served as a

loading control. **(c)** MTT assays documenting the effects of Bafilomycin A1 (4 μM) or Chloroquine (25 μM) on viability of CEM-S cells treated for 24h with MK-2206 (4 μM). Results are mean of three different experiments ± s.d. Asterisks indicate significant differences compared with CTRL (untreated cells).

4.1.d MK-2206 synergizes with Doxorubicin

We examined whether MK-2206 could synergize with the anthracycline antibiotic, Doxorubicin. Doxorubicin is frequently included in established protocols to treat T-ALL patients [216]. Two different strategies were explored to determine whether synergy occurred. In the first protocol, T-ALL cell lines were incubated with both MK-2206 and Doxorubicin in combination for 48h. In the second protocol, one drug was added before the other, the first drug was administered for 48h (throughout the entire experiment) while the second drug was added only for the last 24h of treatment. In all the experiments, the drugs were used at a fixed ratio (Doxorubicin: MK-2206, 1:30 for MOLT-4 cells and 1:1 for CEM-R cells). MTT assays were then performed. In MOLT-4 cells, synergistic effects were observed when MK-2206 was added with Doxorubicin together at low concentrations (10–30 nM of Doxorubicin) after 48h of treatment. Also in MOLT-4 cells, both of the sequential treatments resulted in synergistic results, that is, synergy was observed when MK-2206 was added first and Doxorubicin was added second, or when Doxorubicin was added first and MK-2206 was added second (Figure 13a).

In contrast, different results were observed with the drug-resistant CEM-R cell line. Namely, if the two drugs were administered together for 48h, synergism was observed at concentrations of Doxorubicin ranging from 2.5 to 7 μM. In the sequential exposure experiments, synergy was dependent upon which drug was added first. When MK-2206 was added after Doxorubicin treatment, synergism was detected at all the concentrations tested. In contrast, when the reverse sequence was performed, where cells were exposed to MK-2206 for 48h followed by post-treatment with Doxorubicin for 24h, antagonism was frequently observed (Figure 13b). Thus, in the drug-resistant CEM-R cells, addition of Doxorubicin for the entire period was required to detect synergy with the Akt inhibitor MK-2206.

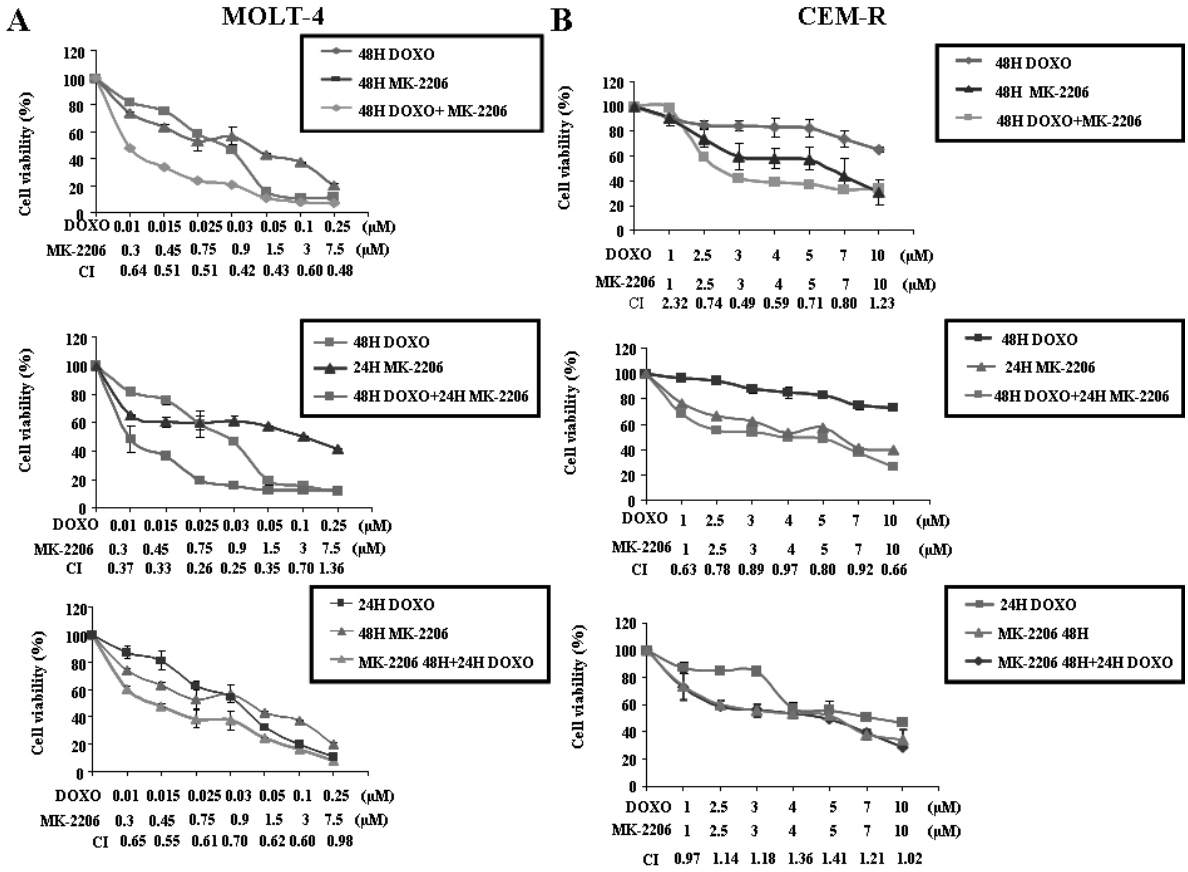


Figure 13. Synergistic effects of the MK-2206 plus Doxorubicin combination in MOLT-4 and CEM-R cells. (**a**) MOLT-4 cells were treated for 48h with MK-2206 or Doxorubicin, either alone, in combination or in sequential exposure (relative concentration ratio, MK-2206:Doxorubicin, 30:1). (**b**) CEM-R cells were treated for 48h with MK-2206 and Doxorubicin, alone, in combination or in sequential exposure (relative concentration ratio, MK-2206:Doxorubicin, 1:1). Viability was then analyzed by MTT assays. Results are mean of three different experiments ± s.d. Combination index (CI) value for each data point was calculated with the appropriate software for dose effect analysis.

4.2 Treatment with RAD001 affects B-pre ALL cells

4.2.a The PI3K/Akt/mTOR pathway activation status and RAD001 effects in B-pre ALL cell lines

By western blotting, the PI3K/Akt/mTOR pathway activation status in B-pre ALL cell lines was first evaluated. Ser 473 p-Akt and Ser 2481 p-mTOR (indicative of mTORC2 activity) [217] were maximally phosphorylated in SEM cells and, to a lower extent, in RS4;11 cells, whereas REH cells displayed very low levels of Ser 473 p-Akt phosphorylation. Also Ser 2448 p-mTOR levels (a readout for mTORC1 activity) [217]

were higher in SEM cells than in either RS4;11 or REH cells (Figure 14a). Phosphatase and tensin homolog PTEN, a negative regulator of the PI3K/Akt/mTOR pathway [218], was much more abundantly expressed in REH cells.

We next examined the RAD001 cytotoxic effects on B-pre ALL cell lines. Cell lines were treated with increasing concentrations of RAD001 for 48h and then analyzed by MTT assays. Cell viability decreased in a dose-dependent manner. Under these conditions, the RAD001 IC_{50} for cell lines ranged between 6.3 and 10.4 μM. It is noteworthy that SEM cells were the most sensitive to RAD001 when compared with the other cell lines (Figure 14b). The cytotoxic effects of RAD001 on SEM, REH and NALM6 cells were also studied by flow cytometry analysis of Annexin V-stained samples. Overall, the results were similar to those obtained with MTT assays, displaying a concentration-dependent increase of apoptotic cells (Figure 14c).

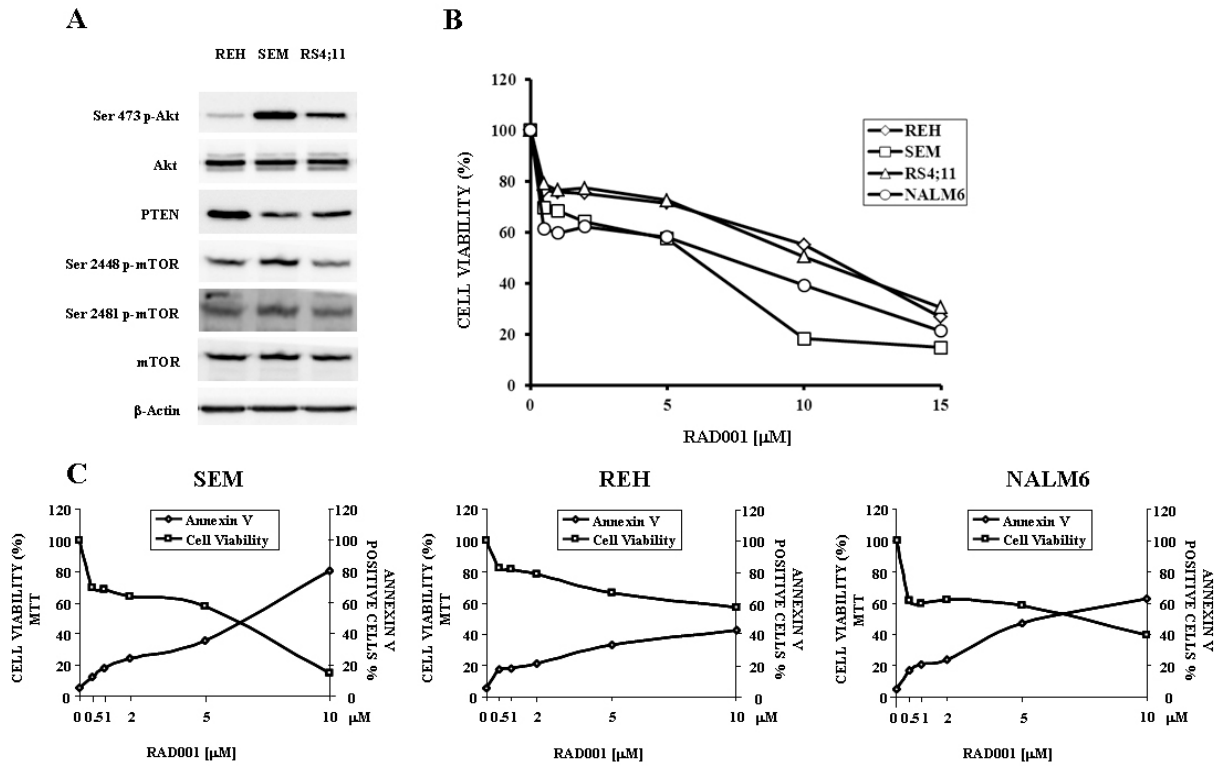


Figure 14. PI3K/Akt/mTOR signaling activation and RAD001 sensitivity of B-pre ALL cell lines. **(a)** Western blot analysis for phosphorylation levels of critical components of the PI3K/Akt/mTOR signaling network. Protein (50 mg) was blotted to each lane. An antibody to β-actin documented equal lane loading. **(b)** Cell viability of B-pre ALL cell lines treated with increasing concentrations of RAD001 for 48h. **(c)** Assessment of cell viability and apoptosis induction by RAD001 in SEM, REH and NALM6 cells. In **b** and **c**, one representative of three different experiments that yielded

similar results is shown. Viability was assessed by MTT assays, whereas apoptosis was assessed by Annexin V/7-AAD staining.

4.2.b RAD001 modulates PI3K/Akt/mTOR signaling in B-pre ALL cell lines

We studied the effects of RAD001 on the phosphorylation levels of critical components of the PI3K/Akt/mTOR cascade. REH, SEM and RS4;11 cells were treated with increasing concentrations of RAD001 for 4h and then analyzed by western blotting (Figure 15a). RAD001 not only decreased the phosphorylation levels of mTOR mainly on the Ser 2448 residue (a readout for mTORC1 activity) but also on the Ser 2481 phosphorylation site (a marker for mTORC2 activity). The total amount of mTOR was unchanged. mTORC1 inhibition had functional effects on two well-known mTORC1 substrates, p70S6K and 4EBP1. p70S6K was completely dephosphorylated already at 2 μM concentration of RAD001, whereas 4EBP1 was dephosphorylated starting at a higher concentration. Total levels of these two proteins were instead unaffected by RAD001.

RAD001 also decreased the levels of Ser 473 p-Akt, p-GSK3-α/β on Ser 21/9 and p-Forkhead box O3A (FoxO3A) on Ser 318/321 in SEM cells. Interestingly, FoxO3A transcription factor was fully dephosphorylated already at 2 μM RAD001 only in SEM cell line (Figure 15b).

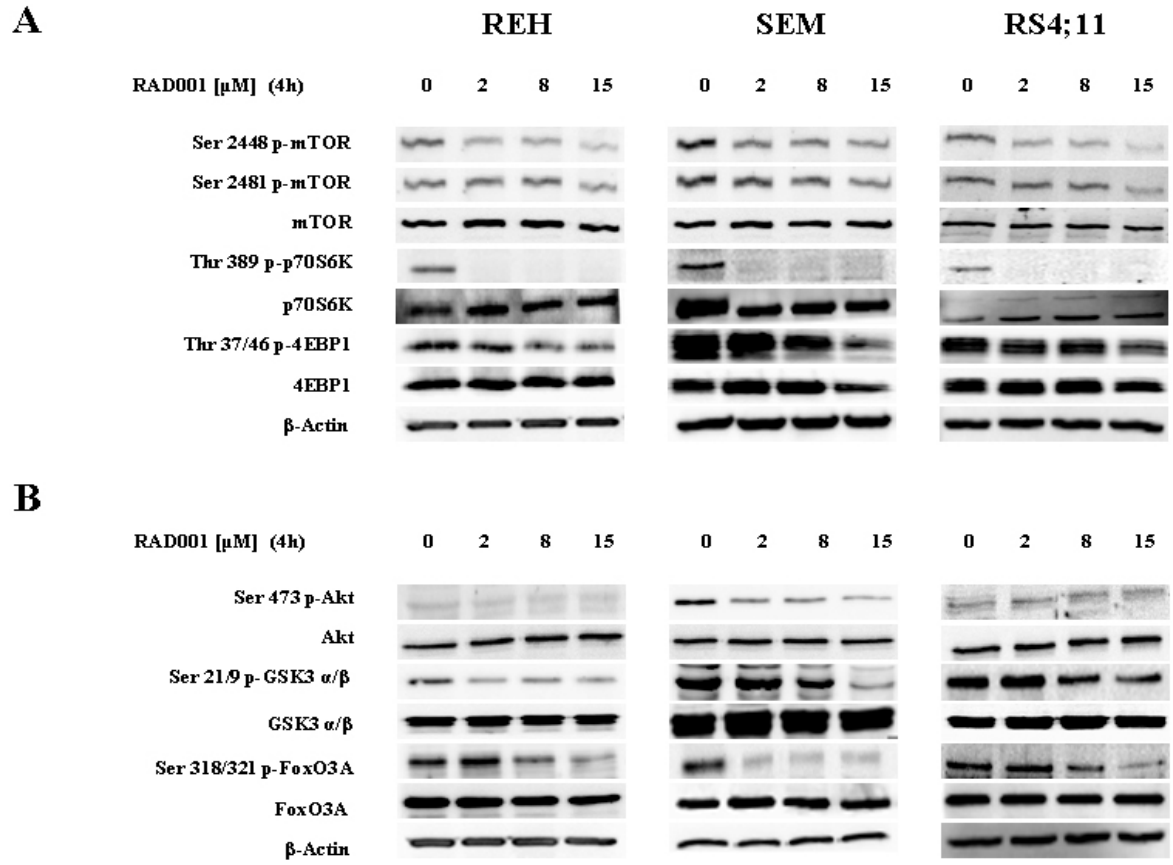


Figure 15. Effects of RAD001 on the phosphorylation levels of key components of the PI3K/Akt/mTOR pathway after 4h of treatment with increasing concentrations of the drug. **(a)** Western blot analysis for mTOR and its downstream targets, p70S6K and 4EBP1. **(b)** Western blot analysis for Akt, GSK3-α/β and FoxO3A. In **a** and **b**, 50mg of protein was blotted to each lane. β-actin served as a loading control.

4.2.c RAD001 induces cell cycle arrest, apoptosis and autophagy

To assess the effects of RAD001 on cell cycle, we performed flow cytometric analysis of PI-stained samples in REH and SEM cells cultured for 24h in the presence of increasing concentrations of RAD001. RAD001 increased dose dependently the percentage of cells in the G_0/G_1 phase of the cell cycle with a concomitant decrease of cells in S and G_2/M phases (Figure 16a).

Apoptosis and autophagy, two central mechanisms for programmed cell death [219], have important roles in the killing of malignant cells. To elucidate whether RAD001 cytotoxic effects were related to apoptosis and/or autophagy, we analyzed REH, SEM and RS4;11 cell lysates by western blotting. The cells were treated for 4h with increasing concentrations of RAD001. Procaspase-9 and poly-(ADP-ribose)polymerase (PARP) were

cleaved in a dose-dependent manner in all the three cell lines, but the apoptotic effect of RAD001 was more evident in SEM cells (Figure 16b).

Autophagy can be a form of programmed cell death, but is also involved in protective mechanisms against apoptosis [220]. To evaluate whether or not the treatment with RAD001 could lead to autophagy, we detected the expression of LC3A/B-I (non lipidated) and LC3A/B-II (lipidated) by western blotting. The expression levels of LC3A/B-II gradually increased in the three cell lines in a dose-dependent manner, but were more evident in SEM cells (Figure 16c).

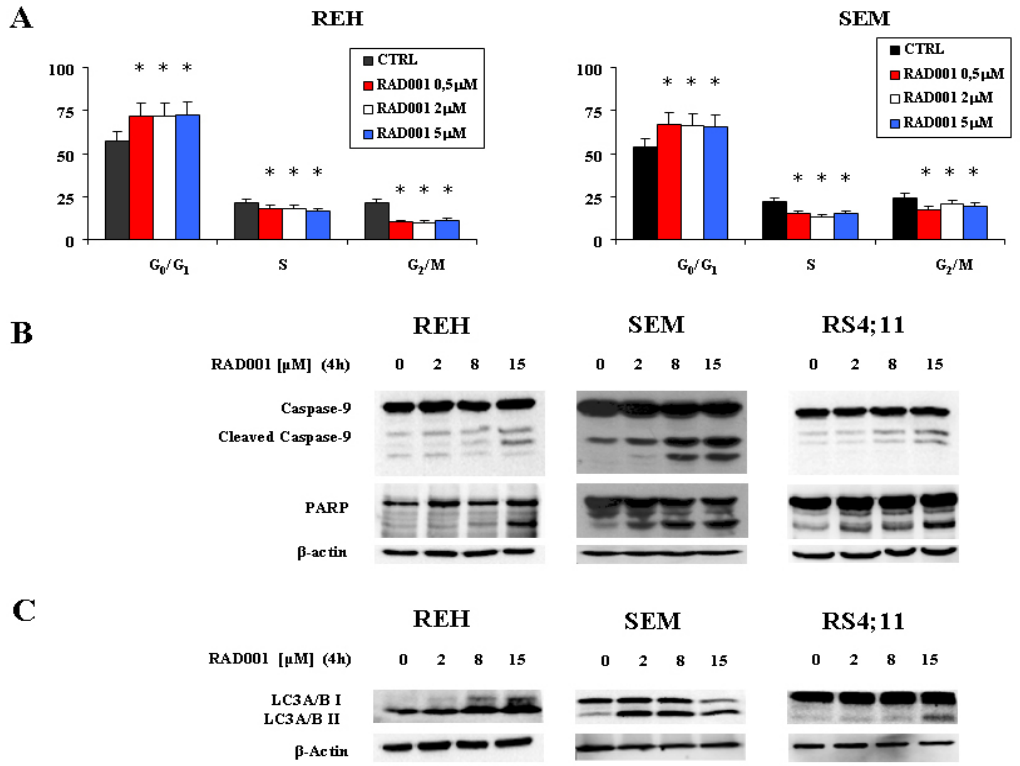


Figure 16. RAD001-induced cell cycle arrest, apoptosis and autophagy in B-pre ALL cell lines. **(a)** Cell cycle was analyzed by flow cytometry after PI staining. The results are the mean ± s.d. of three different experiments. Asterisks (*) indicate significant differences ($P<0.05$) in comparison with control. **(b)** Western blot analysis for caspase-9 and PARP cleavage in B-pre ALL cell lines. **(c)** Western blot analysis demonstrating increased expression of the fast-migrating (lipidated) form of LC3A/B in cell lines treated with RAD001. In **b** and **c** an antibody to β-actin documented equal lane loading.

Another protein critically involved in autophagy is Beclin-1, which is responsible for autophagosome formation, and its deletion has been reported to increase the incidence of

spontaneous tumorigenesis, abnormal proliferation of mammary epithelial cells and germinal center B lymphocytes [220].

Therefore, we down-regulated Beclin-1 expression by transfecting SEM cells with Beclin-1 siRNA (Figure 17a) and measured cell viability by MTT assay after cell exposure to increasing concentrations of RAD001. Analysis of the data showed that cells with silenced Beclin-1 were more resistant to RAD001 than the control (untreated) cells and the cells treated with scrambled siRNA (Figure 17b). Similar results were obtained with REH cells (Figures 17c–d).

These results indicated that autophagy is a critical determinant of the cytotoxic effects induced in B-pre ALL cells by RAD001.

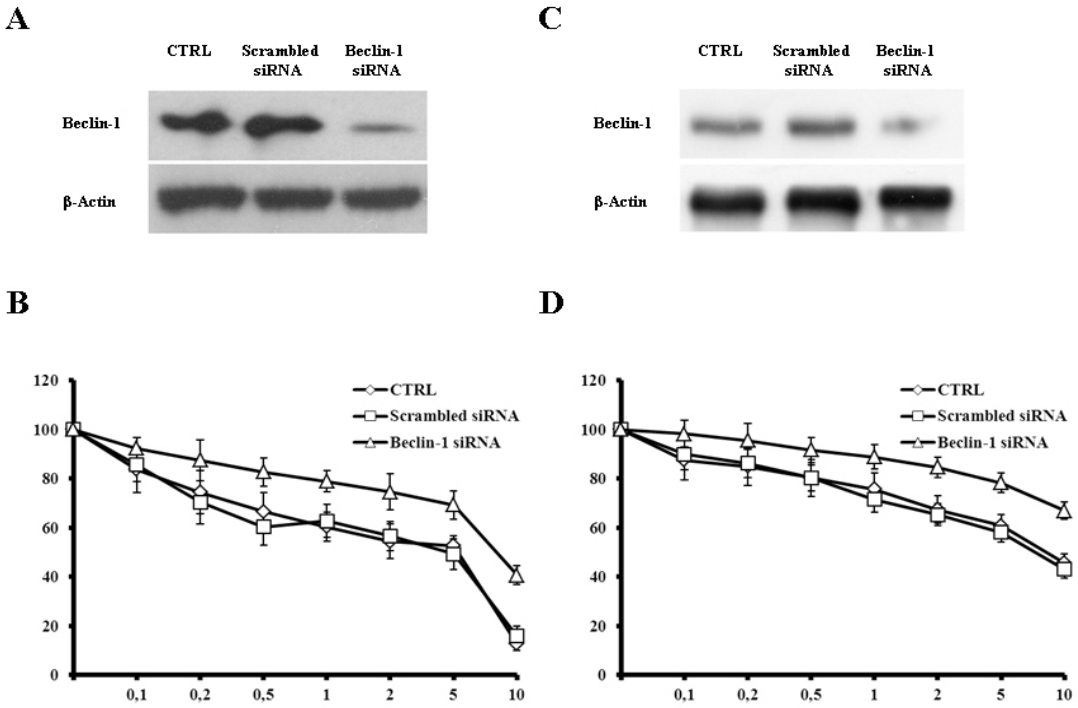


Figure 17. Beclin-1 silencing resulted in decreased RAD001 cytotoxicity in SEM and REH cells. **(a, c)** Western blot analysis for Beclin-1 in SEM and REH cells treated for 48h with either scrambled (control) siRNA or Beclin-1-specific siRNA. β-actin served as the loading control. **(b, d)** SEM and REH cell viability, analyzed by MTT assays, after silencing of Beclin-1. Transfected cells, that are, cells with decreased levels of Beclin-1, displayed a lower sensitivity to RAD001. The results are the mean ± s.d. of three different experiments. CTRL, untreated cells.

4.3 Two hit pathway with RAD001 and MK-2206 leads to synergistic effects

4.3.a Dual targeting of mTOR and Akt results in synergistic inhibition of proliferation in B-pre ALL cell lines

For therapeutic targeting of the PI3K/Akt/mTOR pathway, the combined inhibition at different points of the cascade often leads to more effective results than the use of a drug that acts on a single or dual targets. However, most of the studies in this field have been performed in solid tumor models [221-224].

Therefore, we decided to evaluate whether the simultaneous administration of RAD001 and MK-2206 could lead to synergistic cytotoxic effects in B-pre ALL cell lines. More than one strategy was used to determine whether the two inhibitors could synergize. In the first condition, cells were treated with the two drugs simultaneously for 48h. In the second condition, the first drug was administered during the entire experiment (48h), whereas the second drug was added only for the last 24h of the treatment. Then, MTT assays were performed. As shown in Figure 18a, in SEM cells, all the conditions were tested, and the strongest synergy was observed when RAD001 was added first and MK-2206 was added second. In REH, RS4;11 and NALM6 cells, RAD001 was combined with MK-2206 for 48h. A synergistic inhibition of cell viability was observed for all cell lines (Figures 18b-c-d).

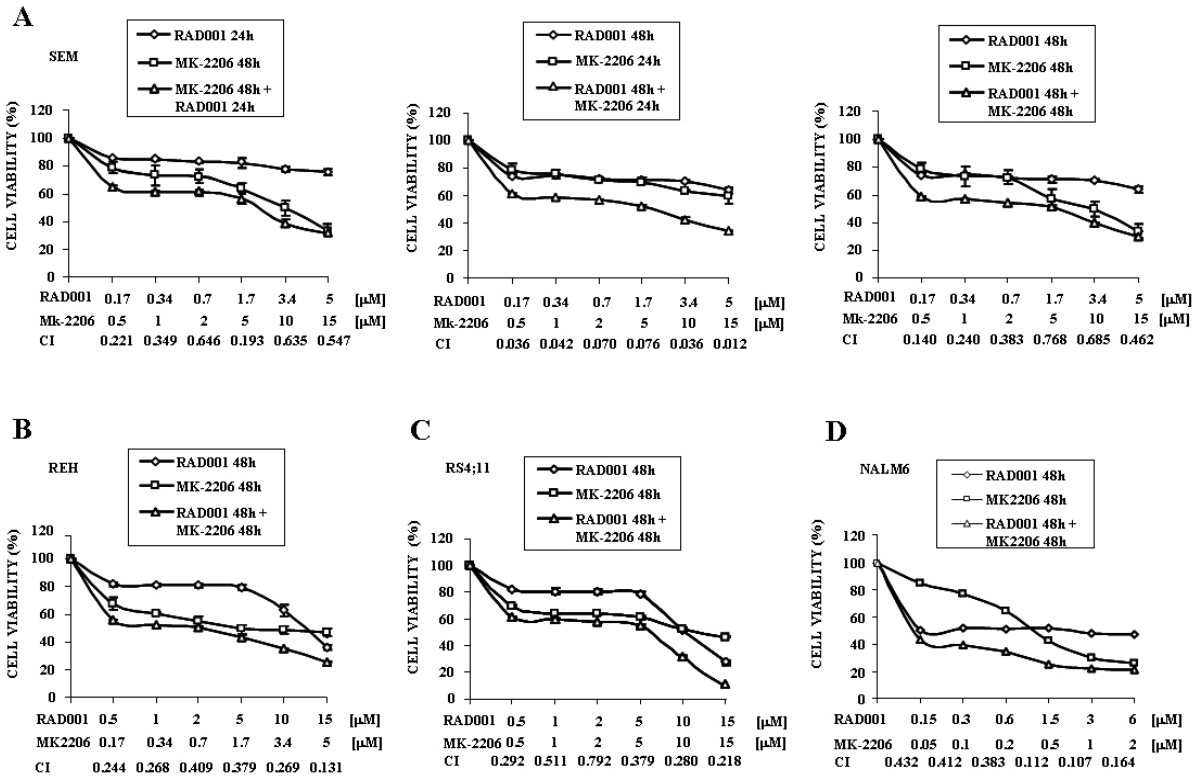


Figure 18. RAD001 and MK-2006 display a synergistic cytotoxic effect in B-pre ALL cell lines. **(a)** SEM cells were treated for 48h with either RAD001 or MK-2206 alone, or in combination or in sequential exposure. **(b)** REH, **(c)** RS4;11 and **(d)** NALM6 cells were treated with RAD001 and MK-2206 either alone or in combination for 48h. In **a–d**, one representative of three different experiments that yielded similar results is shown.

The effects of the RAD001/MK-2206 combination on the PI3K/Akt/mTOR pathway in SEM cells have been studied by western blotting analysis of the phosphorylation levels of 4EBP1 and GSK3-α/β. The cells were treated with 0.34 μM RAD001 for 48h and with 1 μM MK-2206 for the last 24h. The two proteins were much more efficiently dephosphorylated by the drug combination than that by either drug employed alone (Figure 19a).

Moreover, RAD001 and MK-2206, when administered together, increased apoptosis through extrinsic and intrinsic pathways, as documented by the increment of cleaved caspase-8, -9 and PARP (Figure 19b). Furthermore, increased levels of lipidated LC3A/B suggested that the drugs acted together for potentiating autophagy induction (Figure 19b). The effects of the RAD001 and MK-2206 combination were also analyzed in NALM6 cells. Also, in this cell line, GSK3-α/β and 4EBP1 were much more efficiently dephosphorylated by the drug combination than by either drug employed alone. Moreover,

up-regulated lipidation of LC3A/B indicated that the drugs synergized in potentiating autophagy induction (Figure 19c).

The increase in the percentage of G_0/G_1 phase cells and the concomitant decrease in both S and G_2/M phases were more evident when RAD001 and MK-2206 were used in combination in SEM cells (Figure 19d).

The tumor suppressor protein retinoblastoma (Rb) in the phosphorylated state (p-Rb) is active and exert its role by promoting cell cycle progression from G_1 to S phase [225]. Western blotting analysis documented that in SEM cells, dual treatment with RAD001 and MK-2206 maximally reduced the levels of phosphorylated Rb protein on Ser 807/811 and Ser 780 residues (Figure 19e). This effect was consistent with the arrest of B-pre ALL cells in G_0/G_1 phase of the cell cycle.

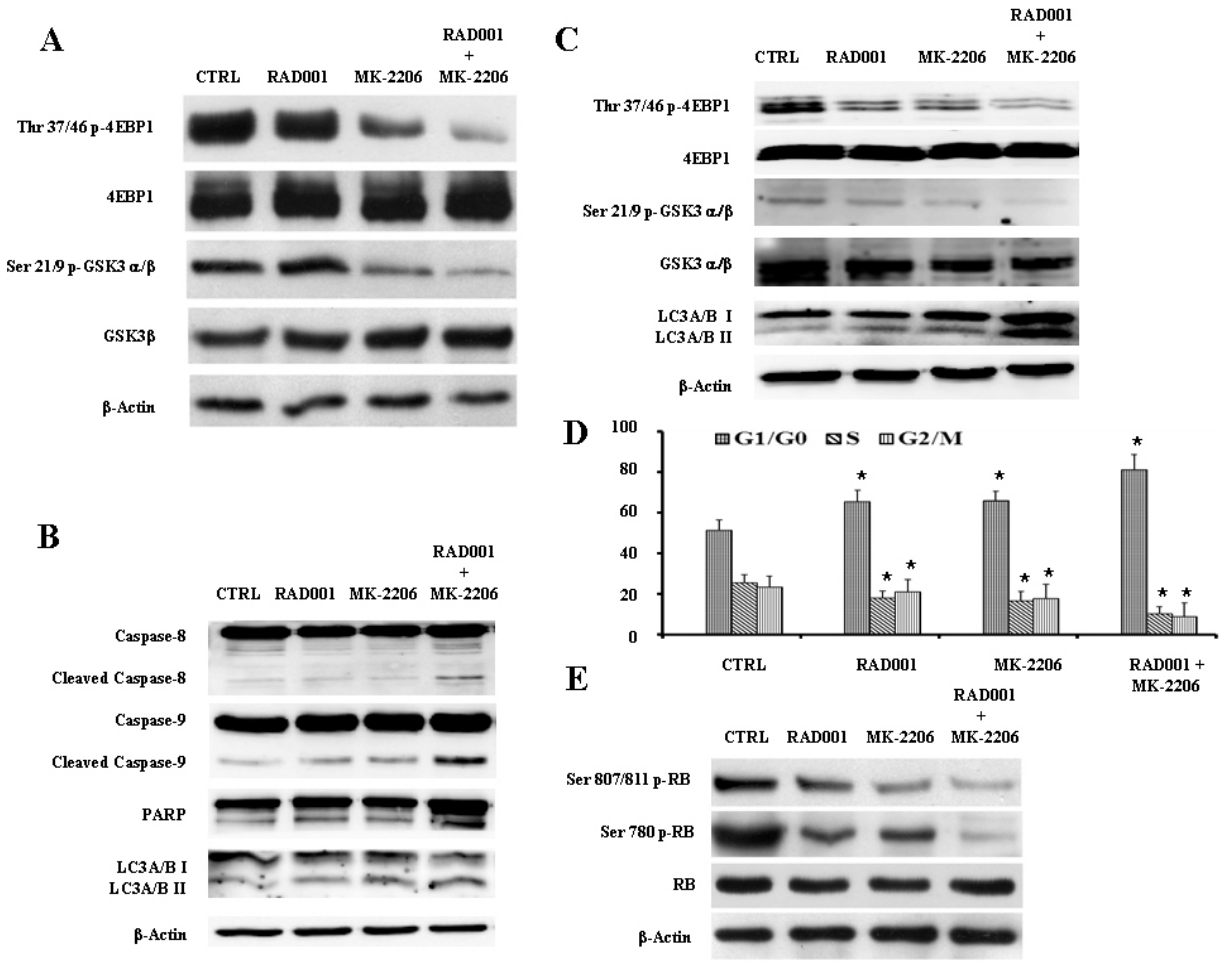


Figure 19. Effects of the RAD001/MK-2206 drug combination on phosphorylation of critical components of the PI3K/Akt/mTOR pathway, apoptosis, autophagy and cell cycle in SEM (**a, b** and **d, e**) and NALM6 (**c**) cells. (**a**) Western blot analysis on the phosphorylation status of 4EBP1 and GSK3-α/β. (**b**) Western blot analysis for caspase-8, -9 and PARP cleavage, and LC3A/B expression. (**c**) Western blot analysis on the phosphorylation status of 4EBP1 and GSK3-

α/β, and LC3A/B expression. (**d**) Flow cytometric analysis of the cell cycle performed using PI-stained samples. The results are the mean ± s.d. of three different experiments. Asterisks (*) indicate significant differences (P<0.05) in comparison with control. (**e**) Western blot analysis for the levels of total Rb and Ser 807/811 or Ser 780 p-Rb. In **a, b** and **d, e**), 0.34 μM RAD001 for 48h and MK-2206 at 1 μM for the last 24h were used. In panel **c**, 0.6 μM RAD001 and MK-2206 at 0.2 μM for 48h were used. CTRL, untreated cells.

To confirm these findings, we employed a different mTORC1/Akt inhibitor combination, consisting of CCI-779 and GSK690693. SEM and NALM6 cells were treated with the two drugs simultaneously for 48h. Then, MTT assays were performed. As shown in Figures 20a and b, the two drugs synergized to inhibit cell viability. The effects of the drug combination have been studied by western blotting analysis of the phosphorylation levels of 4EBP1 and GSK3-α/β. The two proteins were much more efficiently dephosphorylated by the drug combination than that by either drug employed alone (Figures 20c and d). As different inhibitors yielded similar results, it was concluded that there were no off-target drug effects implicated in the described findings.

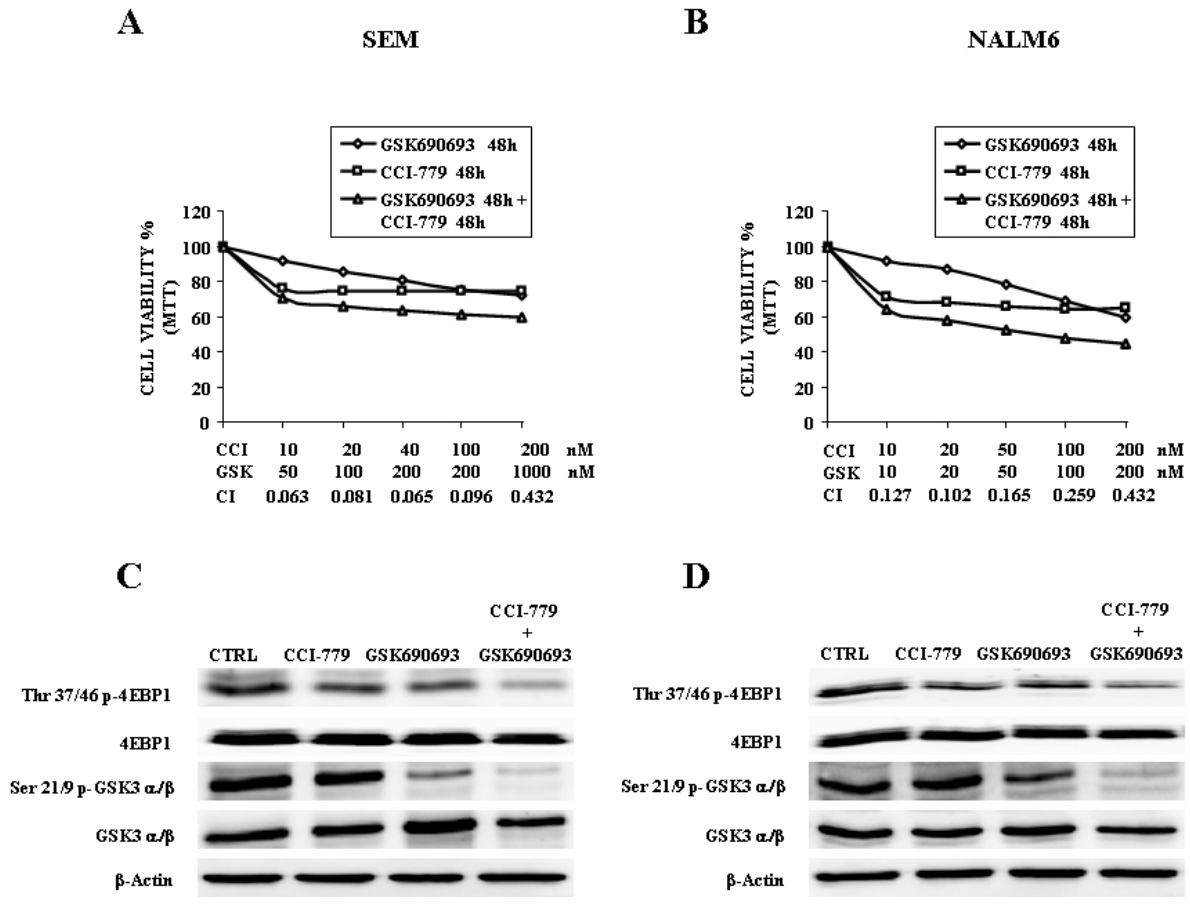


Figure 20. CCI-779 and GSK690693 display a synergistic cytotoxic effect in B-pre ALL cell lines and affect the phosphorylation status of critical components of the PI3K/Akt/mTOR pathway. (**a**) SEM and (**b**) NALM6 cells were treated for 48h with either CCI-779 or GSK690693 alone or in combination. In **a** and **b**, one representative of three different MTT experiments that yielded similar results is shown. (**c, d**) Western blot analysis on the phosphorylation status of 4EBP1 and GSK3-α/β in SEM and NALM6 cell lines, respectively.

4.3.b B-pre ALL lymphoblasts are sensitive to combined mTOR/Akt inhibition

To establish the efficacy of the inhibitors as potential therapeutic agents for B-pre ALL, we analyzed four B-pre ALL adult patient samples isolated from peripheral blood/bone marrow. Available patient characteristics are presented in Table 1.

Patient	Age (years)	Sex	WBC (x 10^9/L)	Ph+	Cytogenetics
No.1	35	M	14.4	-	Normal
No.2	25	M	12.8	-	Normal
No.3	27	F	19.6	-	11q23
No.4	31	F	22.7	-	Normal

Table 1: B-pre ALL adult patient characteristics.

Samples were treated with either RAD001 or MK-2206 alone or with the combination of 0.34 μM RAD001 and 1 μM MK-2206 for 72h. In the two patients shown in Figure 21a, RAD001 induced a down-regulation Ser 473 p-Akt, Ser 2448 p-mTOR and Thr 389 p-p70S6K levels. In the same two patients, the synergistic effects of RAD001 and MK-2206 on 4EBP1 and GSK3-α/β have been tested. The decrease in the phosphorylation levels of the two proteins was much higher when the two drugs were used in combination than as single agents. Autophagic induction in the primary samples was also more effective when the two drugs were combined together as indicated by the levels of lapidated LC3A/B (Figure 21b).

Dual targeting of mTOR and Akt also increased apoptosis in patient primary cells. Cells were stained by PI/Annexin V-FITC and analyzed by flow cytometry. After 48h of

treatment, when the two drugs were administered together, the apoptotic cells raised to 50%, whereas RAD001 and MK-2206 alone induced apoptosis in a percentage of 14% or 25%, respectively (Figure 21c).

In Figure 21d are shown the results of MTT assays of four representative patients analyzed for cell viability after in vitro treatment with either drugs used as single agents or combined together. In all the patients studied, the drug combination induced a stronger decrease in cell viability. Overall, these findings demonstrated that the combination consisting of RAD001 and MK-2206 reduced the growth of B-pre ALL primary cells.

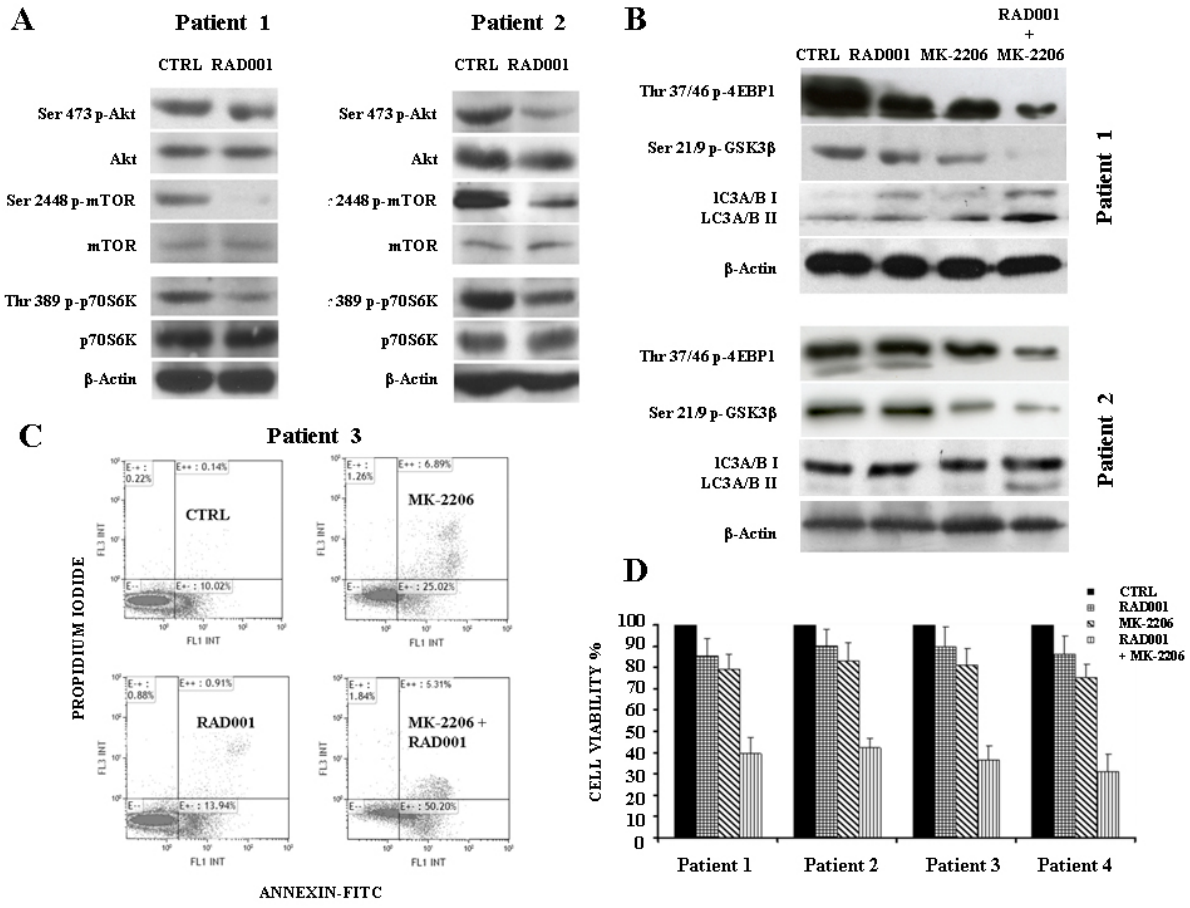


Figure 21. The RAD001/MK-2206 combination is cytotoxic to B-pre ALL lymphoblasts. (**a**) Effects of RAD001 alone on the phosphorylation status of Akt, mTOR and p70S6K in two representative patient samples. (**b**) Western blot analysis for 4EBP1, GSK3-α/β and LC3A/B in two patient samples after dual targeting with RAD001 and MK-2206. (**c**) Flow cytometric analysis of Annexin V-FITC/PI-stained patient lymphoblasts. One representative of three different experiments that yielded similar results is shown. (**d**) Cell viability analysis, as documented by MTT assays, performed on four patient samples. The results are the mean ± s.d. of three different experiments. CTRL, untreated cells. Asterisks (*) indicate significant differences ($P<0.05$) in comparison with control. In **a–d**, samples were treated with 0.34 μM RAD001 and 1 μM MK-2206 for 72h.

Moreover, also the combination of CCI-779 and GSK690693 caused a stronger decrease in cell viability than that by either drug employed alone (Figure 22).

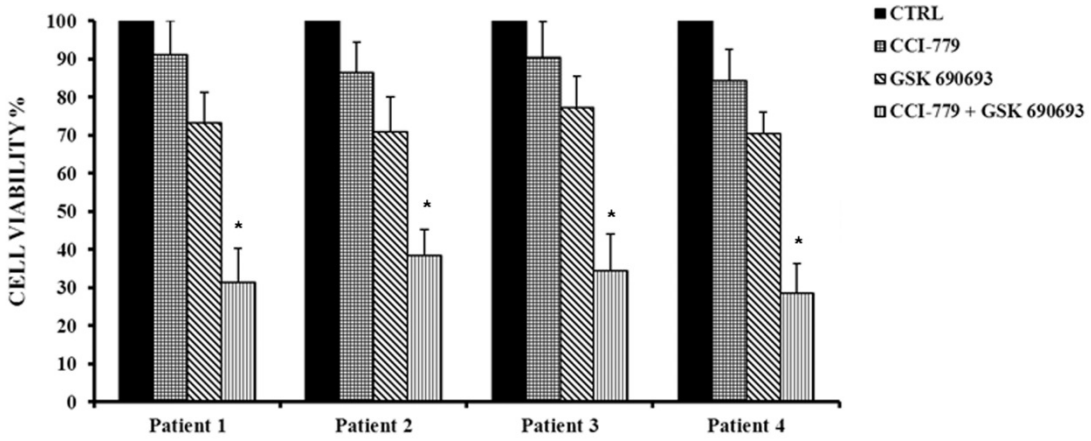


Figure 22: Cell viability analysis, as documented by MTT assays, performed on four patient samples treated with CCI-779 and GSK690693 alone and in combination for 72h. The results are the mean ± s.d. of three different experiments. CTRL, untreated cells. Asterisks (*) indicate significant differences ($P<0.05$) in comparison with control.

4.4 Treatment with Torin-2 affects B-pre ALL cells

4.4.a PI3K/Akt/mTOR pathway activation status in B-pre ALL cell lines

We first analyzed by Western blot the baseline expression of key components of the PI3K/Akt/mTOR pathway and their phosphorylation status in a panel of human B-pre ALL cell lines (NALM-6, SEM, REH, RS4;11, BV-173, SUP-B15, TOM-1). Three of these cell lines, BV-173, SUP-B15 and TOM-1 are Ph+, since they harbour the Bcr-Abl fusion protein. Despite some heterogeneity, all the B-pre ALL cell lines displayed phosphorylation at the Ser 2448 and Ser 2481 (readout for mTORC1 and mTORC2, respectively) residues of mTOR and at the Ser 473 residue of Akt, which are indicative of the constitutive activation of this signaling pathway (Fig. 23).

We further explored the basal condition of downstream targets of both kinases. In agreement with the hyperactivated status of mTORC1, the ribosomal protein S6 and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) were phosphorylated

at Ser 235/236 and Thr 37/46 respectively (Fig. 23). A readout for mTORC2 activity is represented by the phosphorylation at the Ser 473 site of Akt. Hyperactivation of Akt resulted in the phosphorylation at the Thr 32 site of Forkhead box O3A (FoxO3A) (Fig. 23).

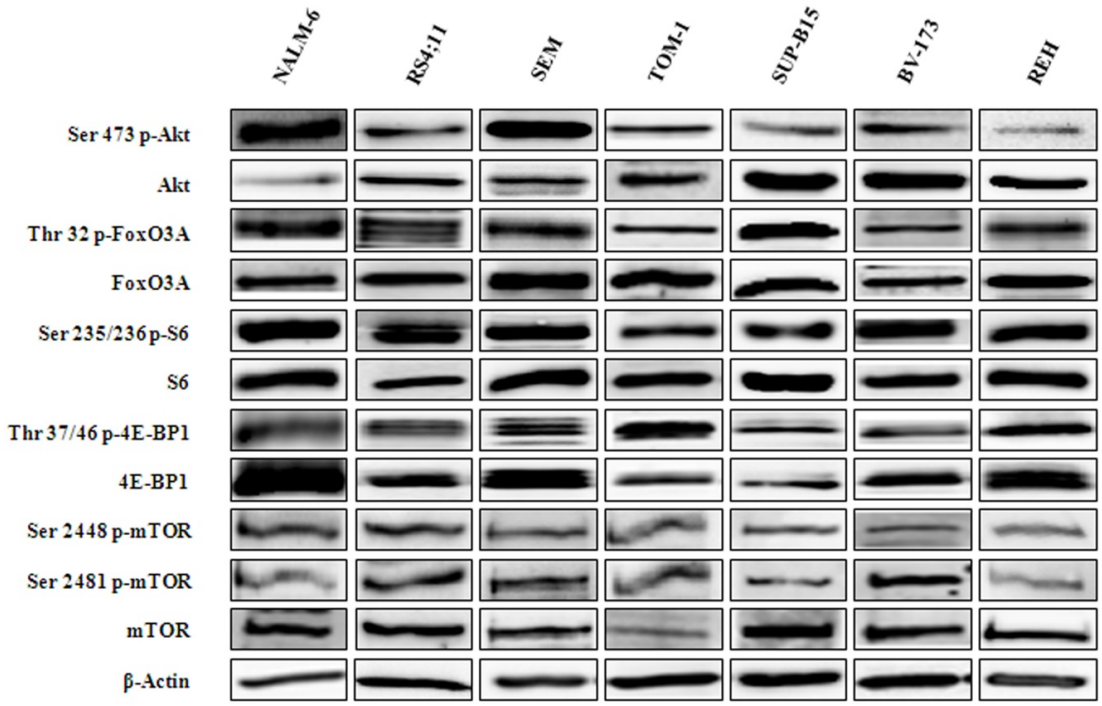


Figure 23: Expression and phosphorylation status of mTOR and Akt and their downstream targets in B-pre ALL cell lines. Western blot analysis of B-pre ALL cell lines to detect the expression and phosphorylation levels of Akt, mTOR and its downstream substrates. Twenty-five μg of protein were blotted to each lane. Antibody to β-actin served as a loading control.

4.4.b Torin-2 induces cytotoxicity, blocks cell cycle progression at the G_0/G_1 phase and induces autophagy

To determine whether Torin-2 could affect viability of B-pre ALL cell lines, cells were incubated in the presence of increasing concentrations of Torin-2 for 48h and then analyzed by MTT assays. All cell lines resulted sensitive to the drug, in a concentration-dependent fashion, with the IC_{50} value that ranged between 0.07 μM and 0.19 μM (Fig. 24a). Given the importance of the PI3K/Akt/mTOR signaling pathway in the regulation of cell proliferation, the effects of Torin-2 on cell cycle progression were also investigated. SEM and BV-173 cell lines were treated with increasing concentrations of Torin-2 for 24h,

then cells were harvested, fixed and stained with Propidium Iodide (PI) and analyzed with the Muse^{TM} Cell Analyzer. The assay showed in both cell lines a concentration-dependent increase of G_0/G_1 phase of cell cycle and a simultaneous decrease in the S phase (Fig. 24b). Autophagy can be a form of programmed cell death, but is also involved in protective mechanisms against apoptosis [226, 227]. To evaluate whether the treatment with Torin-2 could lead to autophagy, we detected the expression of LC3A/B I (non lipidated) and LC3A/B II (lipidated) by Western blot in BV-173, SEM and NALM-6 cells treated with increasing concentrations of Torin-2. The expression levels of LC3A/B II gradually increased in the three cell lines in a dose-dependent manner (Fig. 24c).

To verify whether autophagy was either a cell survival or a cell death mechanism, we used the autophagy inhibitor 3-MA (3-Methyladenine), which blocks an early stage of autophagy by inhibiting the class III phosphoinositide 3-kinase (PI3K) [228]. 3-MA alone did not affect cell growth, even at the concentration of 10 μM (cell viability was comparable to untreated cells), but cells treated with 3-MA become significantly more resistant to Torin-2 cytotoxic effect (Fig. 24d, upper panel). We also employed Bafilomycin A1, another well established autophagy inhibitor that inhibits vacuolar ATPase (V-ATPase) and promotes the accumulation of autophagic vacuoles [229]. As shown in the lower panel of Fig. 24d, the treatment with 4 μM Bafilomycin A1 confirmed that inhibition of autophagy significantly reduced the cytotoxic effect of Torin-2. These results indicated that autophagy is a critical determinant of the cytotoxic effects induced in B-pre ALL cells by Torin-2.

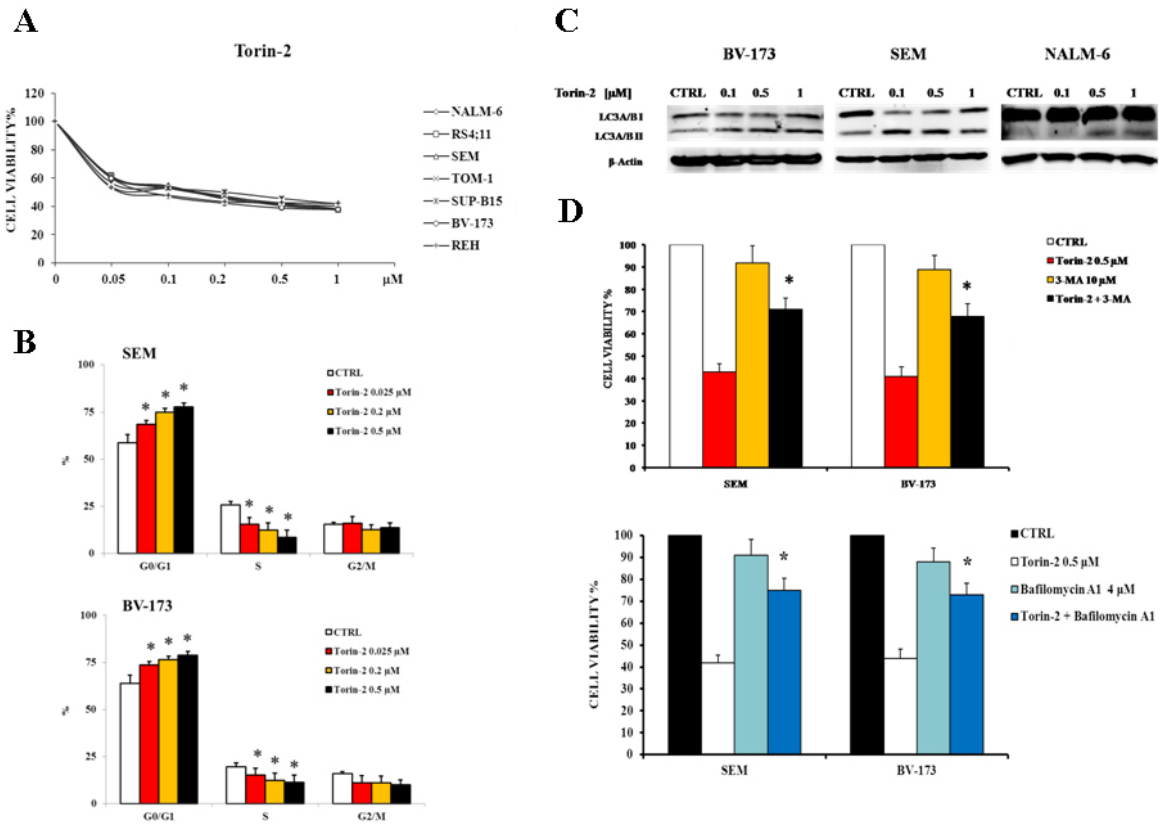


Figure 24. Torin-2 is cytotoxic, cytostatic and induces autophagy in B-pre ALL cell lines. (**a**) MTT assay of B-pre ALL cell lines treated with increasing concentrations of Torin-2 for 48h. One representative experiment is shown. (**b**) Flow cytometric analysis for SEM and BV-173 cells treated with increasing concentrations of Torin-2 for 24h. Asterisks indicate statistically significant differences with respect to untreated cells ($P<0.05$). Cell lines displayed are representative of the cell panel used in this study. (**c**) Effect of Torin-2 on autophagy in BV-173, SEM and NALM-6 cells, documented by the lipidation of the autophagy marker LC3A/B. Antibody to β-actin served as a loading control. (**d**) The activity of 3-MA in combination with Torin-2 is reported in the upper histogram, after SEM and BV-173 treatment for 24h. Below, the effect on cell viability of SEM and BV-173 cells after treatment with Torin-2 and the autophagy inhibitor Bafilomycin A1 is reported. Results are the mean of three different experiments ± SD. Asterisks indicate statistically significant differences with respect to untreated cells ($P<0.05$).

4.4.c Torin-2 causes pro-apoptotic effects on B-pre ALL cell lines

To investigate whether the decreased viability was related to apoptosis, cells were treated for 24h with increasing concentrations of Torin-2 and analyzed by both Western blot and DNA staining. Cleavage of poly(ADP-ribose)polymerase (PARP) in BV-173, SEM and NALM- 6 revealed the pro-apoptotic effect of Torin-2 (Fig. 25a). REH and SUP-B15 cells were treated with Torin-2 at 0.5 μM for 24h. DAPI staining revealed the morphological changes associated with apoptosis, such as chromatin condensation and nuclear

fragmentation (Fig. 25b). Apoptosis was further investigated by flow cytometric analysis of Annexin V-stained samples, that showed in NALM-6 and TOM-1 a significant and concentration-dependent increase of apoptotic cells (Fig. 25c, left panel). To determine whether activated caspases are involved in the apoptotic action of Torin-2, we examined the effects of the broad spectrum caspase inhibitor Z-VAD-fmk on cell apoptosis as determined by annexin-V FITC binding. Torin-2-mediated apoptosis was blocked markedly by 25 μM Z-VAD-fmk (Figure 25c, right panel). Therefore these results showed that Torin-2 induced caspase-dependent cell death.

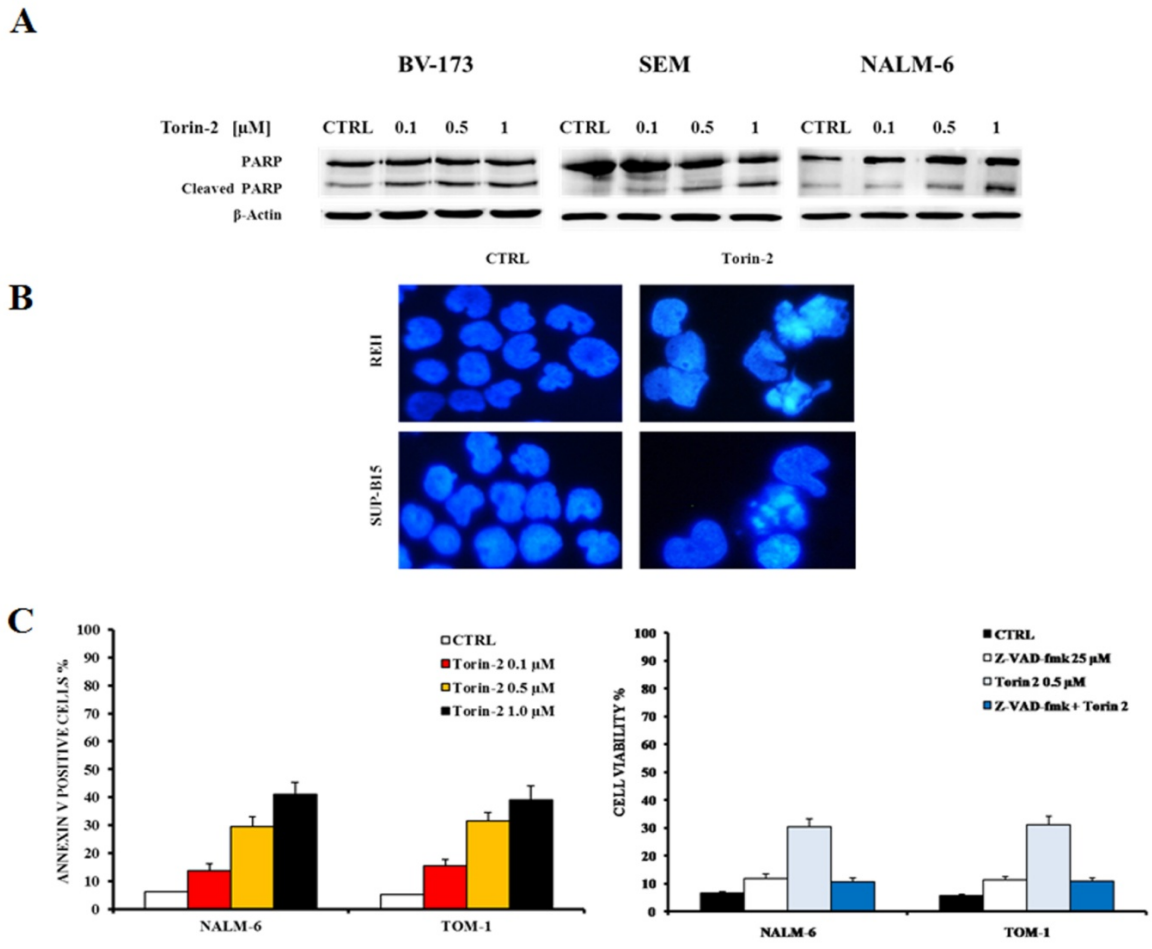


Figure 25. Torin-2 induces apoptosis. (**a**). Western blot analysis documenting a Torin-2 concentration-dependent PARP cleavage in BV-173, SEM and NALM-6 cells. Antibody to β-actin served as a loading control. (**b**) DNA staining of REH and SUP-B15 with the fluorescent dye DAPI is reported. In REH and SUP-B15 treated with 0.5 μM Torin-2 various aspects of nuclear shrinkage, fragmentation and chromatin margination, that are associated with the apoptotic mode of cell death, are observable. (**c**) On the left, flow cytometric analysis of NALM-6 and TOM-1 cell lines treated with increasing concentrations of Torin-2. Samples were incubated with Annexin V-fluorescein isothiocyanate. On the right, flow cytometric analysis of NALM-6 and TOM-1 cells treated with Torin-2 and Z-VAD-fmk, a pan caspase inhibitor, is showed. Results are the mean of three different experiments ± SD.

4.4.d Torin-2 affects the PI3K/Akt/mTOR pathway in B-pre ALL cells

To assess the effects of Torin-2 on the PI3K/Akt/mTOR signaling pathway, we studied the expression and activation status of critical components of the PI3K/Akt/ mTOR cascade. NALM-6, RS4;11, SEM, TOM-1 and BV- 173 cells were treated with increasing concentrations of Torin-2 for 2h and Western blot was then performed (Fig. 26).

Torin-2 decreased the phosphorylation levels of mTOR on both the Ser 2448 and Ser 2481 residues. It should be remembered that the phosphorylation of mTOR on Ser 2481 is a mTORC2-selective autophosphorylation site [230]. mTORC1 inhibition had functional effects on two well-known mTORC1 substrates, S6 and 4E-BP1. S6 was completely dephosphorylated on the Ser 235/236 residue already at 50 nM concentration of Torin-2 in all cell lines, whereas 4E-BP1 was fully dephosphorylated on the Thr 37/46 site starting from the 100 nM concentration. Total levels of all these proteins were instead unaffected by Torin-2.

mTORC2 inhibition had a readout in Ser 473 Akt dephosphorylation and it was observable in all the cell lines starting from Torin-2 concentration of 50 nM. Despite some differences, also the Akt downstream substrate FoxO3A was dephosphorylated in a dose dependent fashion on its Thr 32 residue in all cell lines (Fig. 26).

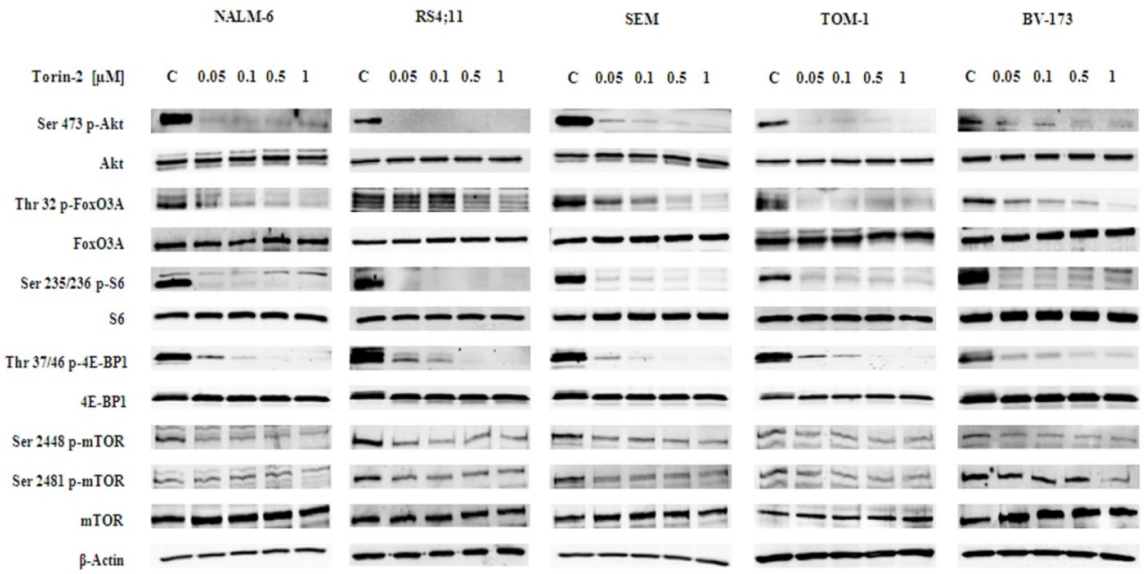


Figure 26: Torin-2 cytotoxicity is related to PI3K/Akt/mTOR signaling pathway inhibition. Western blot analysis for mTOR, Akt and their downstream targets S6, 4E-BP1 and FoxO3A in B-pre ALL cell lines. Twenty-five μg of protein

were blotted to each lane. In all samples 2h of Torin-2 treatment with increasing concentrations was performed. β-actin served as a loading control.

4.5 Comparison between RAD001 and Torin-2 in B-pre ALL cells

4.5.a Torin-2 prevents the reactivation of Akt upon mTOR inhibition in B-pre ALL cells

Since it has been previously described that in hematological malignancies [231, 232] and solid tumors [233, 234] with constitutive PI3K/Akt activation, the rapamycin derivative inhibitor everolimus (RAD001) increased Akt phosphorylation, we sought to explore if Torin-2 might prevent Akt re-activation after mTORC1 inhibition.

For this set of experiments we decided to use the concentration of 0.15 μM, that represents nearly the average IC_{50} of Torin-2 in the panel of cell lines employed. We prolonged the treatment with Torin-2 up to 48h and we compared it with the mTORC1 inhibitor, RAD001, employed at the concentration of 0.6 μM. This value has been chosen to mirror a concentration of a mTORC1 inhibitor (Temsirolimus) correspondent to the plasma concentration achievable in clinical trials [235]. As shown in Fig. 27a and b, in a range from 0.05 to 5 μM, RAD001 alone could not achieve the IC_{50}. Thus, RAD001 had a putative IC_{50} higher than 5 μM, whereas Torin-2 displayed an IC_{50} value < 0.2 μM in all cell lines. Interestingly, we found that either mTORC1 and mTORC2 substrates, including Akt, after 48h of Torin-2 treatment remained dephosphorylated (Fig. 27c). On the contrary, samples treated with RAD001 already after 24h showed a re-phosphorylation of Akt. Direct (FoxO3A) or indirect (S6) downstream targets of Akt displayed the same behaviour (Fig. 27c).

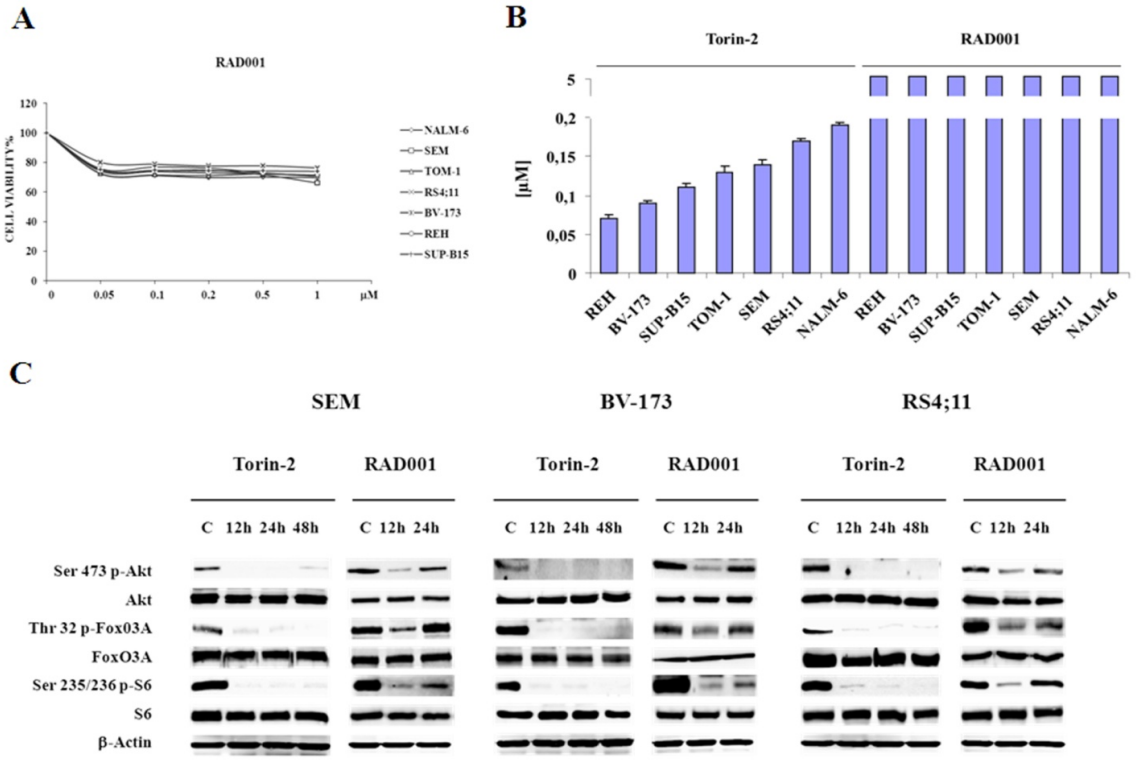


Figure 27. Torin-2 prevents Akt reactivation in B-pre ALL. (**a**) MTT assay of B-pre ALL cell lines treated with increasing concentrations of RAD001 for 48h. One representative experiment of three is shown. (**b**) IC_{50} values for Torin-2 and RAD001 on the viability of B-pre ALL cell lines after 48h. Results are the mean of three different experiments ± SD. (**c**) Western blot analysis for PI3K/Akt/mTOR signaling pathway in SEM, BV-173 and RS4;11 cells. Cells were treated with 0.15 μM Torin-2 and 0.6 μM RAD001 for different times of incubation. In RAD001 treated samples after 24h is evident the re-phosphorylation of Akt, FoxO3A and S6. β-actin served as a loading control.

4.6 Comparison between RAD001 and Torin-2 administered in B-pre ALL cells with MK-2206

4.6.a MK-2206 synergizes with RAD001 but not with Torin-2

For therapeutic targeting of the PI3K/Akt/mTOR pathway, the combined inhibition at different points of the cascade often leads to more effective results than the use of a drug that acts on a single or dual targets [236]. To better assess the potential therapeutic value of Torin-2 in B-pre ALL, we analyzed its synergistic potential with MK-2206, an orally active, allosteric Akt inhibitor, which is currently tested in phase II clinical trials. We also compared this drug combination with a second one consisting of the association of MK-

2206 and RAD001. The drugs were used at a fixed ratio (1:1 both for Torin-2/MK-2206 and RAD001/MK-2206).

After 48h of treatment, MTT assays were performed. The dual targeting of mTORC1/mTORC2 and Akt with Torin-2 and MK-2206 did not show a synergistic effect at any concentration, whereas the administration of RAD001 and MK-2206 together resulted in a relevant synergistic cytotoxic effect in RS4;11 and BV-173 cell lines (Fig. 28a). This phenomenon was more relevant in the range between 0.5 and 1 μM, as confirmed by the combination index (CI) values. Similar results were obtained with other B-pre ALL cell lines (Fig. 29).

It should be noted that a comparison between the IC_{50} values obtained from the two different treatment at 48h (Torin-2/MK-2206 and RAD001/MK-2206), showed that the IC_{50} values were very similar and comparable in each cell line (Fig. 28b).

We next studied the effects of the two drug combinations on the phosphorylation levels of Akt, FoxO3A and S6 protein. Torin-2 was used at 0.15 μM, RAD001 at 0.6 μM and MK-2206 at 0.5 μM for 30 minutes and then Western blot analysis was performed.

The concentration of MK-2206 was selected for either being in the range of maximal synergy and to be comparable to the plasma concentration that has been obtained in clinical trials in acute myelogenous leukemia [237]. Both SEM and SUP-B15 cell lines did not show a synergistic effect of Torin-2 and MK-2206 on the phosphorylation levels of Akt, FoxO3A and S6. Interestingly, there was an additional down-regulation of protein phosphorylation only when the dual treatment RAD001/MK-2206 was administered, thus confirming the synergistic effect in modulating the PI3K/Akt/mTOR pathway (Fig. 28c).

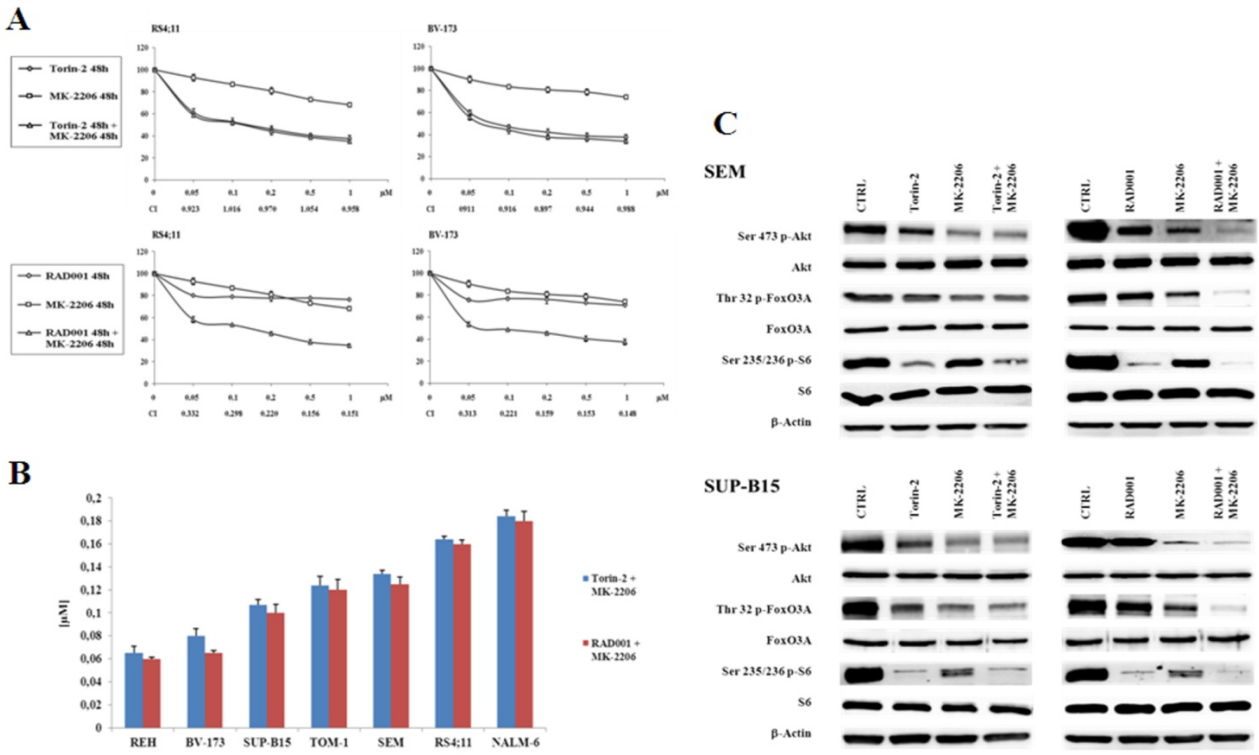


Figure 28. Dual administration of Torin-2 or RAD001 with MK-2206 in B-pre ALL cell lines. (**a**) B-pre ALL cell lines were treated for 48h with Torin-2 or RAD001, either alone or in combination with MK-2206. Results are the mean of three different experiments ± SD. Combination index (CI) value for each data point was calculated with the appropriate software for dose effect analysis (Calcusyn). (**b**) IC_{50} values for Torin-2 or RAD001 in combination with MK-2206 on the viability of B-pre ALL cell lines at 48h. Results are the mean of three different experiments ± SD. (**c**) Western blot analysis for PI3K/Akt/mTOR in SEM and SUP-B15 cells. Cells were treated for 30 minutes with 0.15 µM Torin-2 or 0.6 µM RAD001 in combination with 0.5 µM MK-2206. β-actin served as a loading control.

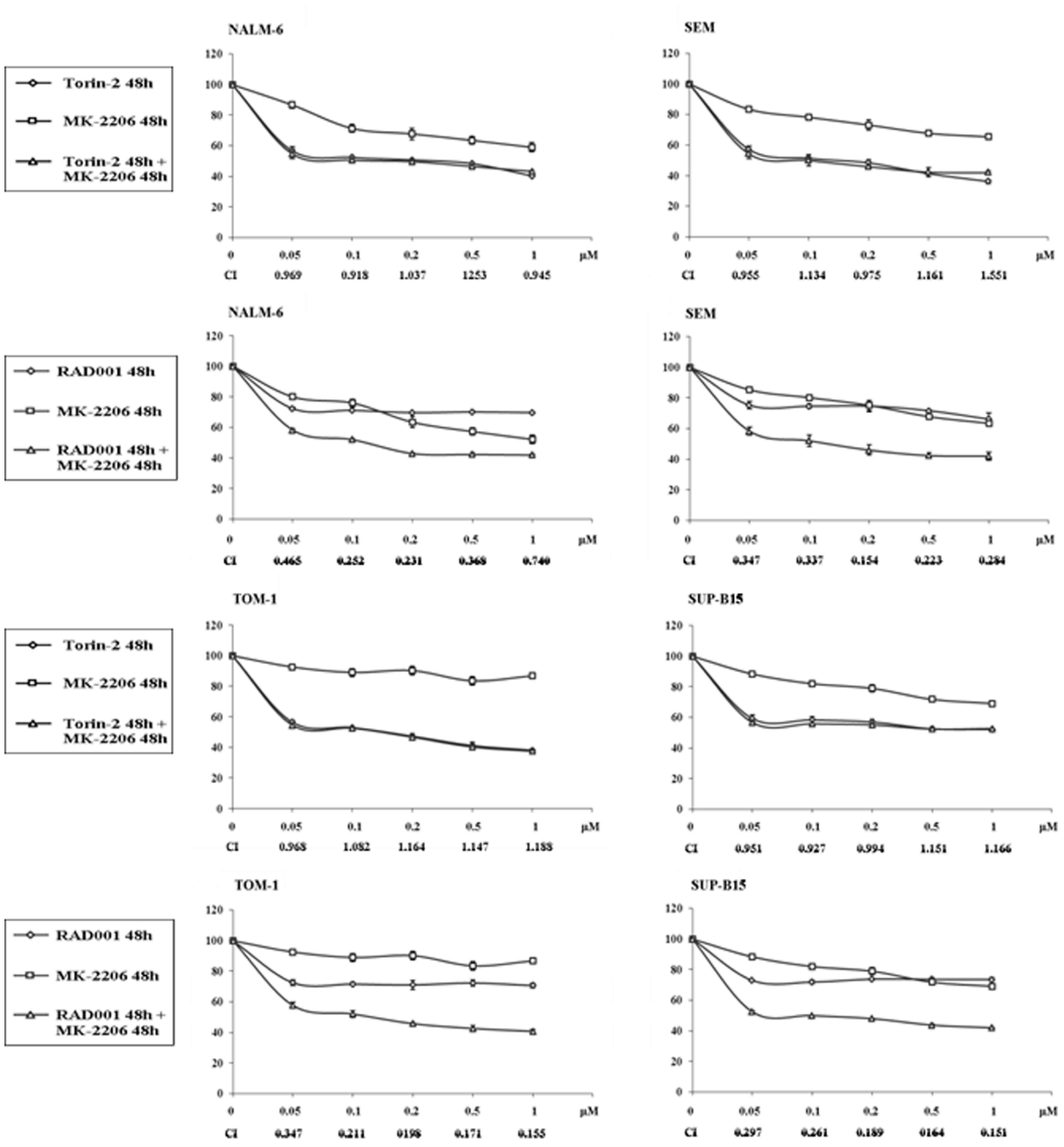


Figure 29: Dual administration of Torin-2 or RAD001 with MK-2206 in B-pre ALL cell lines. NALM-6, SEM, TOM-1 and SUP-B15 cells were treated for 48h with Torin-2 or RAD001, either alone or in combination with MK-2206. Results are the mean of three different experiments ± SD. Combination index (CI) value for each data point was calculated with the appropriate software for dose effect analysis (Calcusyn).

To further assess these findings, we explored the effects of dual treatments on cell cycle, by flow cytometric analysis of PI-stained samples in RS4;11 and BV-173 cells cultured for 24h. Both drug associations yielded comparable results. The treatment with Torin-2 and

MK-2206 was similar to the single administration of Torin-2 alone. At variance, the dual administration of RAD001 and MK-2206 increased the percentage of cells in the G_0/G_1 phase of the cell cycle in comparison with single drug administration (Fig. 30a).

We also examined the pro-apoptotic effect of the two drug association on NALM-6 and TOM-1 cells. Cells were treated with 0.15 μM Torin-2 or 0.6 μM RAD001 and 0.5 μM MK-2206 for 24h and then analyzed by MTT assays. Using these drug concentrations, nearly 20% of apoptotic cells were observed in either cell line with both drug combinations. However, apoptosis induced by the treatment with Torin-2 and MK-2206 was comparable with that observable with the administration of Torin-2 alone, whereas the apoptosis obtained with the dual administration of RAD001 and MK-2206 was the consequence of a synergistic effect (Fig. 30b).

Finally, the effect of Bafilomycin A1 on dual targeting the PI3K/Akt/mTOR pathway with a combination of Torin-2/MK-2206 or RAD001/MK- 2206 was analyzed. A consistent inhibition of autophagy increased the viability of B-pre ALL cells treated with Torin-2/MK-2206 (Fig. 30c, left panel) and to a lower extent with RAD001/MK-2206 (Fig. 30c, right panel). This difference may rely on the different mechanism through which autophagy is recruited by the drugs: those who act on mTOR, activated autophagy as a cell death mechanism [238], whereas the drug that inhibit Akt, induced autophagy as a cell protection mechanism [179].

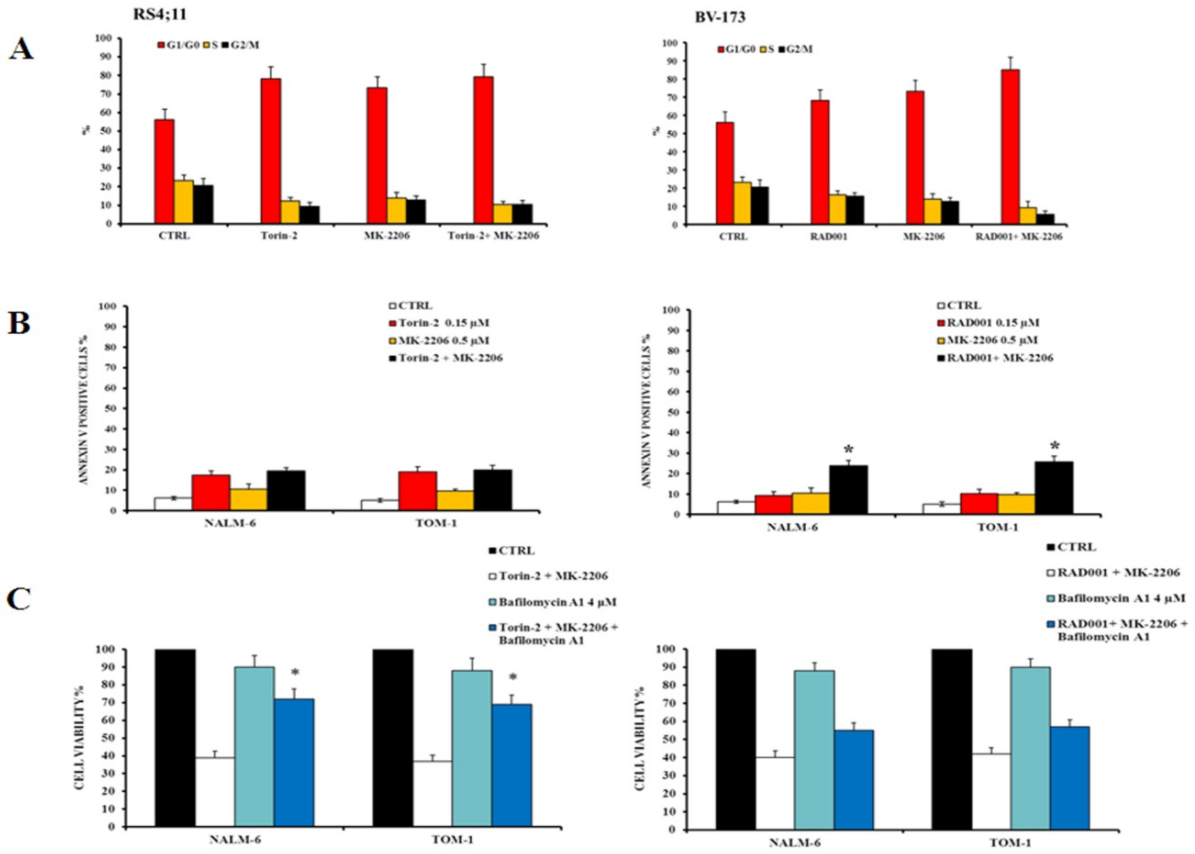


Figure 30. Effects of dual administration of Torin-2 or RAD001 with MK-2206 in B-pre ALL cell lines. (**a**) Flow cytometric representation of the effects of Torin-2/MK-2206 and RAD001/MK-2206 combinations on the cell cycle of RS4;11 and BV-173 cell lines after 24h of treatment. Results are the mean of three different experiments ± SD. (**b**) Flow cytometric analysis of NALM-6 and TOM-1 cell lines treated with increasing concentrations of Torin-2 or RAD001, alone and in combination with MK-2206, for 24h. Samples were incubated with Annexin V-fluorescein isothiocyanate and then analyzed for apoptosis. Results are the mean of three different experiments ± SD. Asterisks indicate statistically significant differences with respect to untreated cells ($P<0.05$). (**c**) MTT assay representation of the autophagy effects on NALM-6 and TOM-1 cells treated with Torin-2/MK-2206 or RAD001/MK-2206 combinations or plus the administration of the autophagy inhibitor Bafilomycin A1. Results are the mean of three different experiments ± SD. Asterisks indicate statistically significant differences with respect to untreated cells ($P<0.05$).

4.6.b Torin-2 suppresses Doxorubicin-induced cell cycle checkpoint activation

When DNA is damaged by DNA intercalating agents, such as Doxorubicin, double stranded breaks (DSBs) trigger recruitment of ATM and ATR to the damage site which in turn phosphorylates histone H2AX leading to foci formation [239].

Torin-2 also exhibited potent biochemical and cellular activity against PIKK family kinases including ATM, ATR, and DNA-PK, whose inhibition sensitized cells to

irradiation [199]. The ATR pathway is known to transmit DNA damage signals through the ATR-CHK1 kinase cascade and activation of cell cycle checkpoint regulators such as CHK1 and CHK2 has a critical role in promoting cell cycle arrest in response to cytotoxic agents, including doxorubicin [240].

We tested if the combination of Doxorubicin and Torin-2 may exert additional cytotoxic activity than the two drugs administered alone. In Figure 30a are shown the results of MTT assays of two representative cell lines (SEM and TOM-1) analyzed for cell viability after treatment with the drugs used either as single agents or combined together. In both cell lines, the drug combination induced a stronger decrease in cell viability (Fig. 31a). Doxorubicin alone induced phosphorylation of CHK1 (Ser 345, a marker for ATR activity) and CHK2 (Thr 68, a readout for ATM activity). In contrast, Torin-2 (0.25 μM) alone did not increase CHK1 and CHK2 phosphorylation, whereas it dramatically decreased the phosphorylation induced by Doxorubicin (Fig. 31b). One prominent chromatin modification in response to DNA damage is phosphorylation of histone H2AX on Ser 139, which is referred to as γ-H2AX [241].

Torin-2 inhibited the DNA damage response induced by Doxorubicin, as documented by the effects on the levels of phosphorylated γ-H2AX on the Ser 139 residue (Fig. 31b).

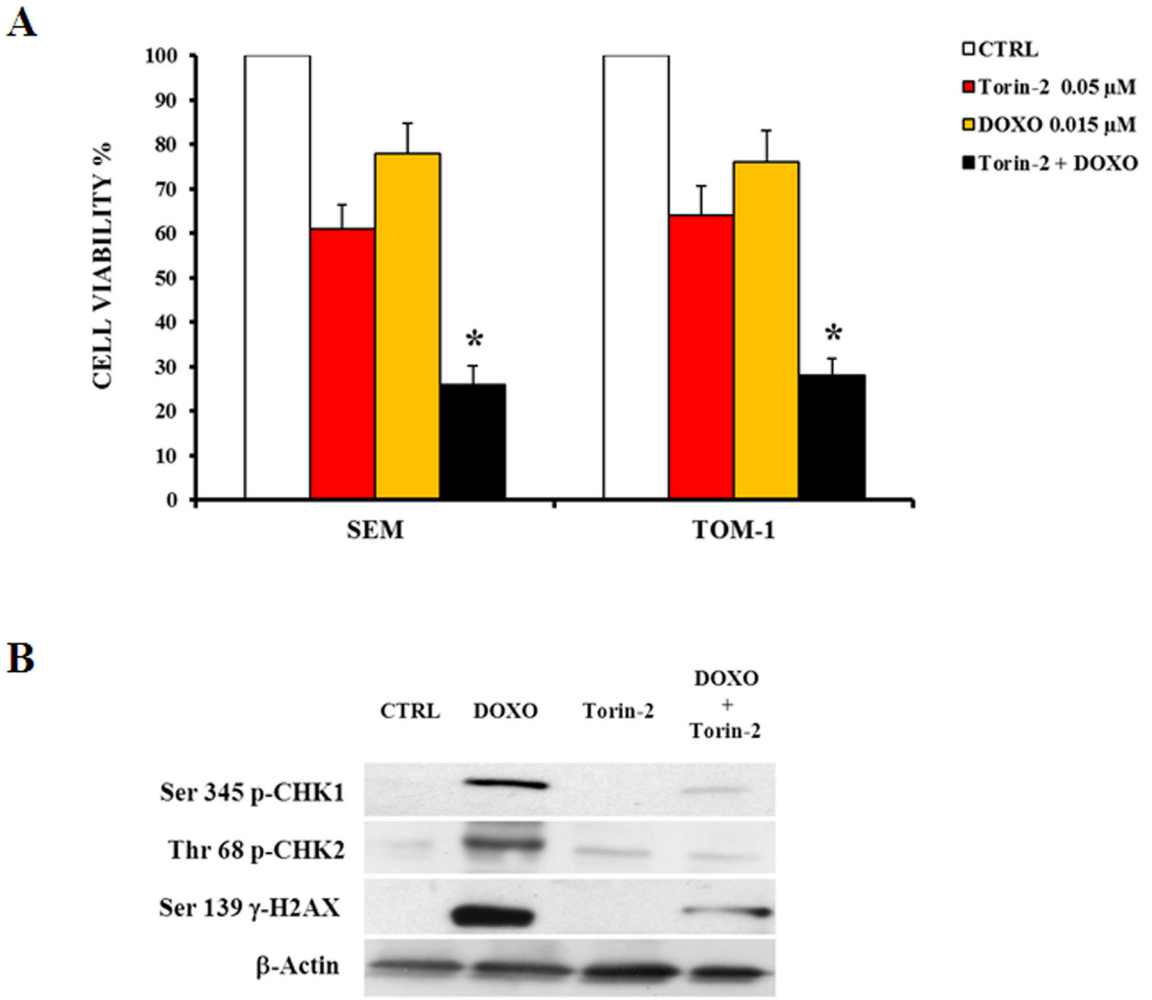


Figure 31: Torin2 suppresses Doxorubicin-induced cell cycle checkpoint activation. (**a**) MTT assay showing the cytotoxic effect of Torin-2 and Doxorubicin (0.015 µM) alone or in combination in SEM and TOM-1 cells. DOXO, Doxorubicin-treated cells. Results are the mean of three different experiments ± SD. Asterisks indicate statistically significant differences with respect to untreated cells (*$p<0.05$). (**b**): Western blot analysis of SEM cells showing the activation by Doxorubicin treatment of cell cycle checkpoint regulators CHK1 and CHK2, markers for ATR or ATM activity, respectively. Torin-2 alone did not modify CHK1 and CHK2 phosphorylation, whereas it dramatically decreased after Doxorubicin administration. Torin-2 also influenced the levels of phosphorylated γ-H2AX, inhibiting its phosphorylation after Doxorubicin treatment. β-actin served as a loading control.

4.7 Triple hit Akt affects T-ALL cells

4.7.a PI3K/Akt/mTOR pathway activation status in T-ALL cell lines

By western blotting analysis, we first evaluated the phosphorylation status of key proteins of the PI3K/Akt/mTOR pathway in a panel of T-ALL cell lines (JURKAT, MOLT-4, CEM-S, CEM-R, PEER and BE-13). PEER and BE-13 cells are characterized by the NUP214-ABL1 fusion gene mutation, that is the most frequent and highly specific ABL1 fusion protein for T-lineage acute lymphoblastic leukemia, that transforms T-cells and is a constitutively active tyrosine kinase with oncogenic potential [242].

Whereas PEER and BE-13 cells did not displayed phosphorylated Akt, the other four cell lines displayed an hyperphosphorylated form, which is indicative of constitutive activation of PI3K signaling pathway (Fig. 32).

Moreover, we confirmed as previously reported [120, 243] the absence of PTEN protein expression in most T-ALL cell lines analyzed, except for PEER and BE-13.

Accordingly to the above observations, Ser 2448 and Ser 2481 p-mTOR (readout of mTORC1 and mTORC2 activity) were hyperphosphorylated in NUP214-ABL1 negative cells.

In PEER and BE-13cell lines, PTEN was phosphorylated at Ser 380, a marker of PTEN post-translational inactivation and consequent PI3K pathway activation [134]. However, despite this observation neither Akt nor mTOR appeared hyperphosphorylated.

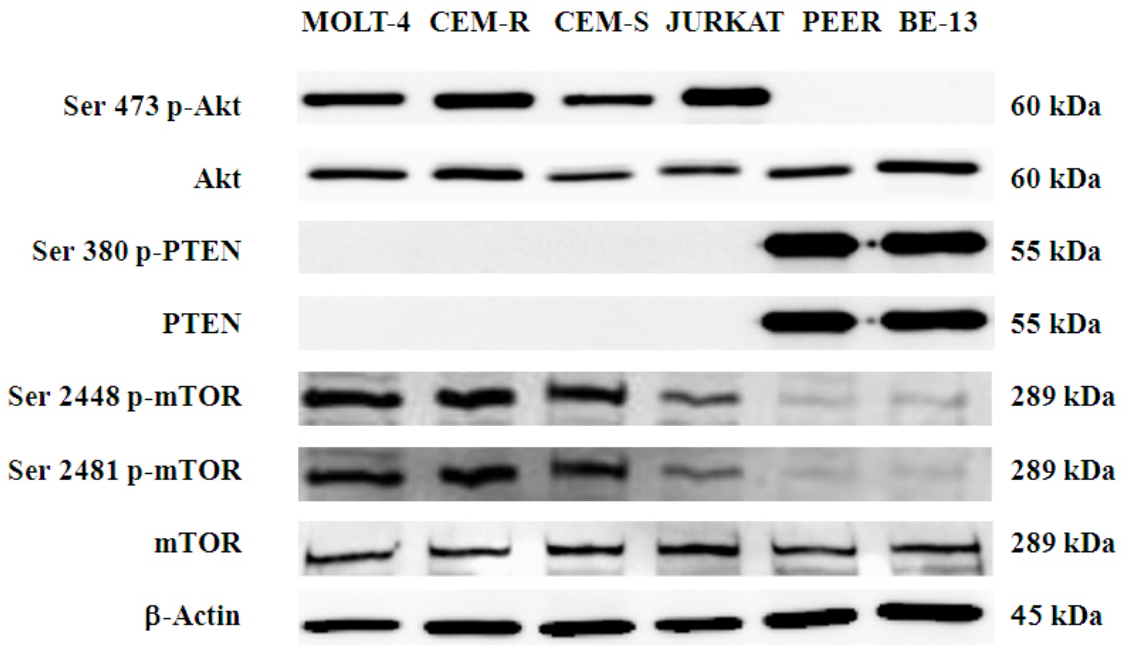


Figure 32. Expression and phosphorylation status of Akt, PTEN and mTOR in T- ALL cell lines. Western blot analysis of T-ALL cell lines to detect the expression and phosphorylation levels of Akt, PTEN and mTOR. Twenty-five μg of protein was blotted to each lane. Twenty-five μg of protein was blotted to each lane. Antibody to β-actin served as a loading control.

4.7.b Multiple Akt targeting had higher cytotoxic effect and synergized only in Ser 473 p-Akt expressing cells

We examined by MTT assay the IC_{50} values of each drug on the six T-ALL cell lines. After 24h of treatment, cell lines displayed different sensitivity to the single drugs. GSK690693 ranged from 0.31 or 0.21 μM, in MOLT-4 and JURKAT, to 7 or 5 μM in CEM-R and -S, respectively. MK-2206 IC_{50} ranged from 1.7 to 6.9 μM. Perifosine required higher concentration to obtain IC_{50} and ranged between 9.35 and 14.65 μM (Fig. 33) and this phenomenon is well known and has already been described [182, 244]. In both PEER and BE-13 the IC_{50} values of all the drugs was higher than 15 μM (Fig. 33).

[µM] Cell lines	GSK690693	MK-2206	Perifosine
MOLT-4	0.31	1.7	14.65
JURKAT	0.21	4.5	9.35
CEM-R	7	4.1	10
CEM-S	5	6.9	10
PEER	>15	>15	>15
BE-13	>15	>15	>15

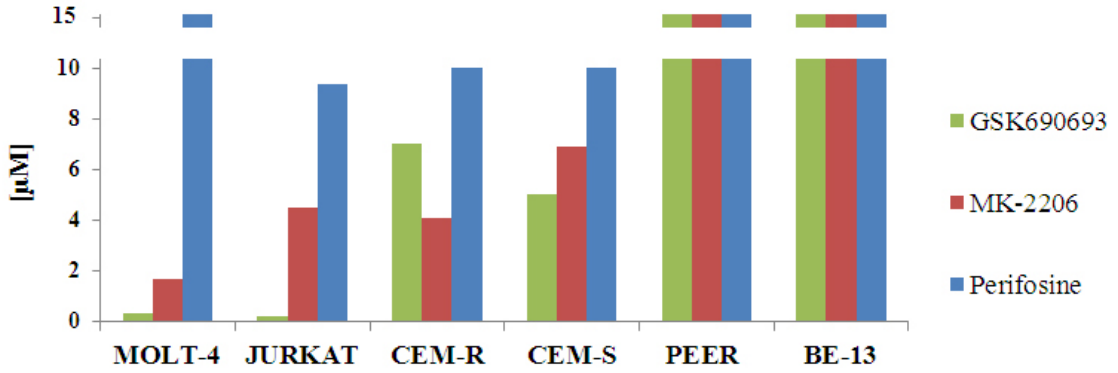


Figure 33. Cytotoxicity of Perifosine, MK-2206 and GSK690693 in T-ALL cell lines. MOLT-4, JURKAT, CEM-R, CEM-S, PEER and BE-13 cell lines were treated for 24h with increasing concentrations of the drugs, ranging from 0.1 to 20 µM, to determine the IC_{50} value. Results are the mean of three different experiments. SD was less than 10%.

We next studied if the simultaneous administration of GSK690693, MK-2206 and Perifosine could lead to a similar cytotoxic effect on the T-ALL cell lines with a significant decrease of the concentration of every single drug due to the synergy of the three compound combination.

Therefore we treated cells with drugs administered together for 24h, using the IC_{50} value as the highest one and decreasing progressively up to 1/20 of the IC_{50} value. MTT assays were then performed.

As shown in Figure 34, all the four more responsive cell lines showed the synergistic cytotoxicity of the triple drug combination, very significant in MOLT-4 and JURKAT cells. We calculated the cell index value (CI) with Calcusyn Software, to quantify the combined effects of the drugs, such as synergism or interference. We did not maintain a constant ratio since it was necessary to fine tune each drug concentration to better understand the synergistic or interfering effect and to avoid a too high cytotoxicity

depending on a single drug administered at a fixed constant ratio. The data analysis (not shown) indicated strong synergisms in all cell lines, even more evident in MOLT-4 cells, with the best CI value of 0.101 corresponding to the combination of 0.05 μM GSK690693, 0.2 μM MK-2206 and 2 μM Perifosine.

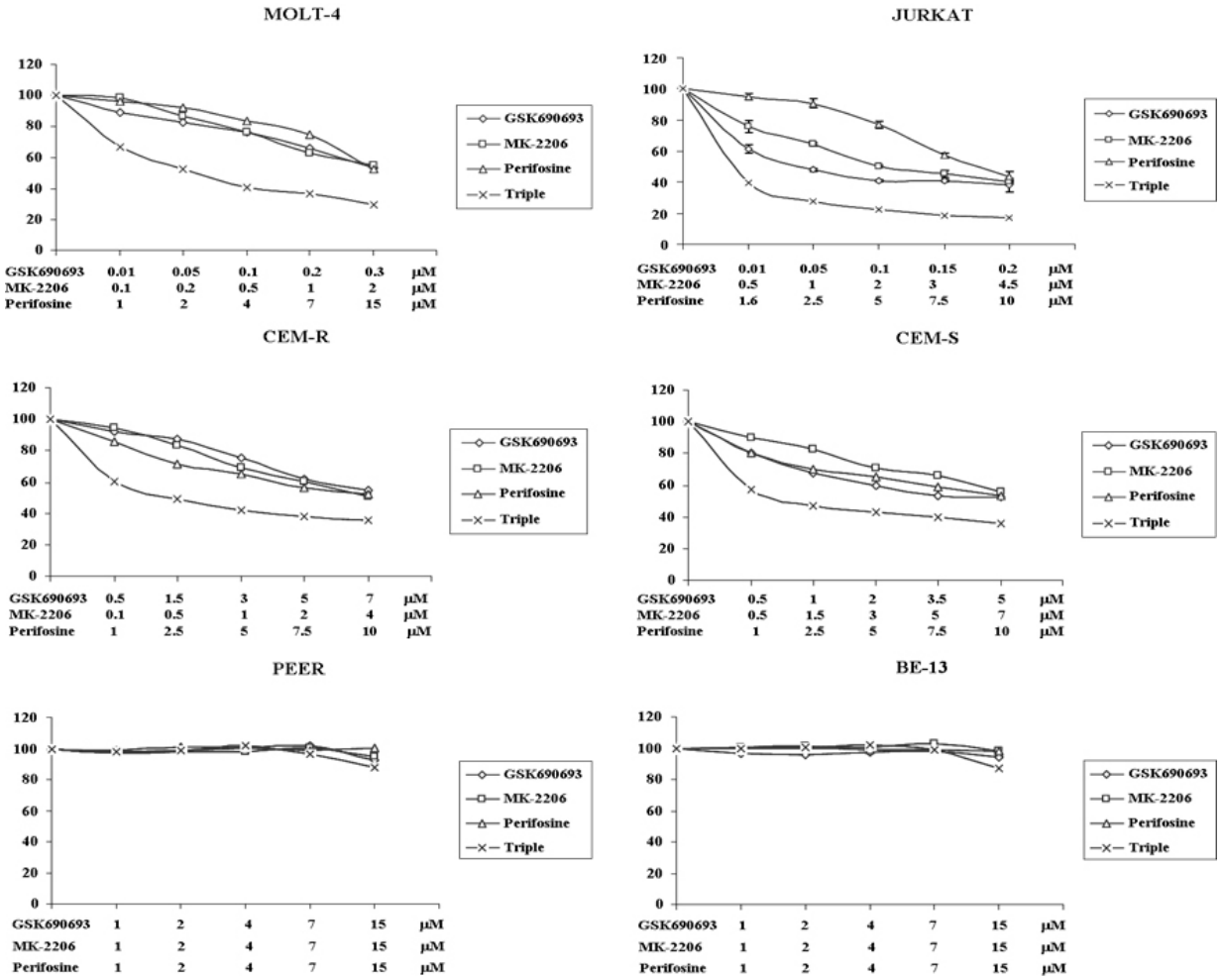


Figure 34. Cytotoxicity of Perifosine, MK-2206 and GSK690693 in T-ALL cell lines. MTT assay of T-ALL cell lines treated with Perifosine, MK-2206 and GSK690693, either alone and in triple combination for 24h. Concentration of each drug is reported under the graph. One representative experiment of three is shown.

4.7.c Triple Akt hit increases the inhibition of the PI3K/Akt/mTOR signaling pathway

To verify if the multiple and simultaneous in vitro treatment with MK-2206, GSK690693 and Perifosine could lead to a modulation of PI3K/Akt/mTOR pathway, we checked the phosphorylation status of key components of this signaling cascade in our panel of more responsive cell lines. In particular we analyzed p-Akt, its downstream target, GSK3-α/β, and the ribosomal protein S6 kinase, readout of mTORC1 activity, after 30 min of drug exposure.

GSK690693 and Perifosine were used at 1/2 of the IC_{50} concentration, whereas MK-2206 was used at 1/5 of IC_{50}, since half of MK-2206 IC_{50} concentration was enough to completely abolish the Ser 473 Akt phosphorylation already at 30 minutes.

Akt phosphorylation was affected in different ways by single drug administration: in all cell lines MK-2206 very significantly reduced p-Akt, Perifosine only slightly reduced it and GSK690693 on the contrary increased the protein phosphorylation. The latter one is an already described phenomenon [245]. This increase of Akt phosphorylation diminished the observable effect of double or triple compound combination, since p-Akt was not significantly reduced, unless when using MK-2206 in double exposure (Fig. 35a).

On the contrary, even after such a short time of treatment, in all of the four cell lines it was very evident the efficacy of the multiple hit on Akt. The triple administration of the drugs completely abolished the phosphorylation on the downstream targets, Ser 21/9 p-GSK3-α/β and Ser 235/236 p-S6, with a much superior efficacy of the triple exposure when compared with the single or with the different double combinations (Fig. 35a). The total amount of the proteins was unchanged in all the treatments (Fig. 35a).

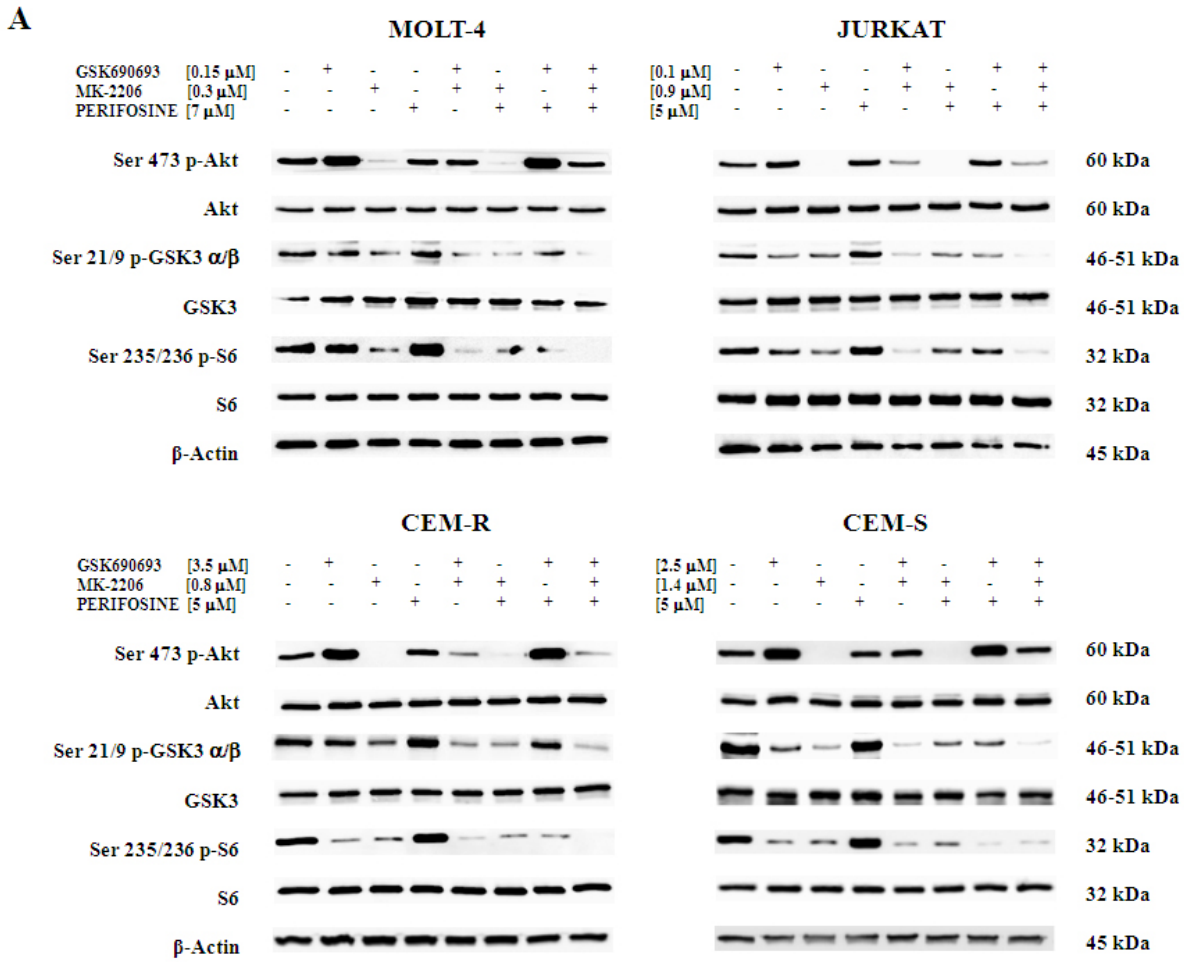


Figure 35: (a) Multiple Akt inhibition affects PI3K/Akt/mTOR pathway and the Akt inhibition is time-dependent. Western blot analysis of Akt drug sensitive T-ALL cell lines for total and phosphorylated form of Akt and of its downstream substrate GSK3-α/β and of mTOR downstream target S6. Samples were treated for 30 minutes with GSK690693, MK-2206 and Perifosine, alone or in double or triple combinations.

4.7.d Pre-treatment with Perifosine enhances synergistic effect

Given that the GSK690693 drug alone led to Ser 473 p-Akt increase, whereas MK-2206 alone almost turn off the signal, we sought to explore if we can find a compound combination capable of synergistically dephosphorylate Akt.

We first tested if there is any concentration capable to modulate Akt phosphorylation in JURKAT and MOLT-4 cells. Therefore GSK690693 was administered at 1/2 of the IC_{50} value (0.15 µM for MOLT-4 cells and 0.1 µM for JURKAT) and MK-2206 was contemporary given at increasing concentrations (0.3 – 0.5 – 1 µM). After 30 minutes of

exposure, western blot was performed. The best drug combination to observe p-Akt modulation resulted to be 0.5 μM for MOLT-4 and 1 μM MK-2206 for JURKAT cells (Fig. 35b).

We then analyzed by western blot the phosphorylation levels of Akt after treatment with 7 μM Perifosine at different time points. In both cell lines the drug affected in a time-dependent manner the Ser 473 Akt phosphorylation (Fig. 35c).

Finally, we merged the two previous assays pre-treating cells for 6h with Perifosine before a 30 min administration of GSK690693 and MK-2206.

As shown in Fig. 35d, in 6h Perifosine pre-treated cells, the administration of GSK690693 reduced Ser 473 p-Akt hyperphosphorylation. The combination of all three drugs allowed to obtain a full Akt dephosphorylation in both MOLT-4 and JURKAT cells, thus showing that full Akt inhibition with low drug doses is not only concentration but also time and drug sequence dependent.

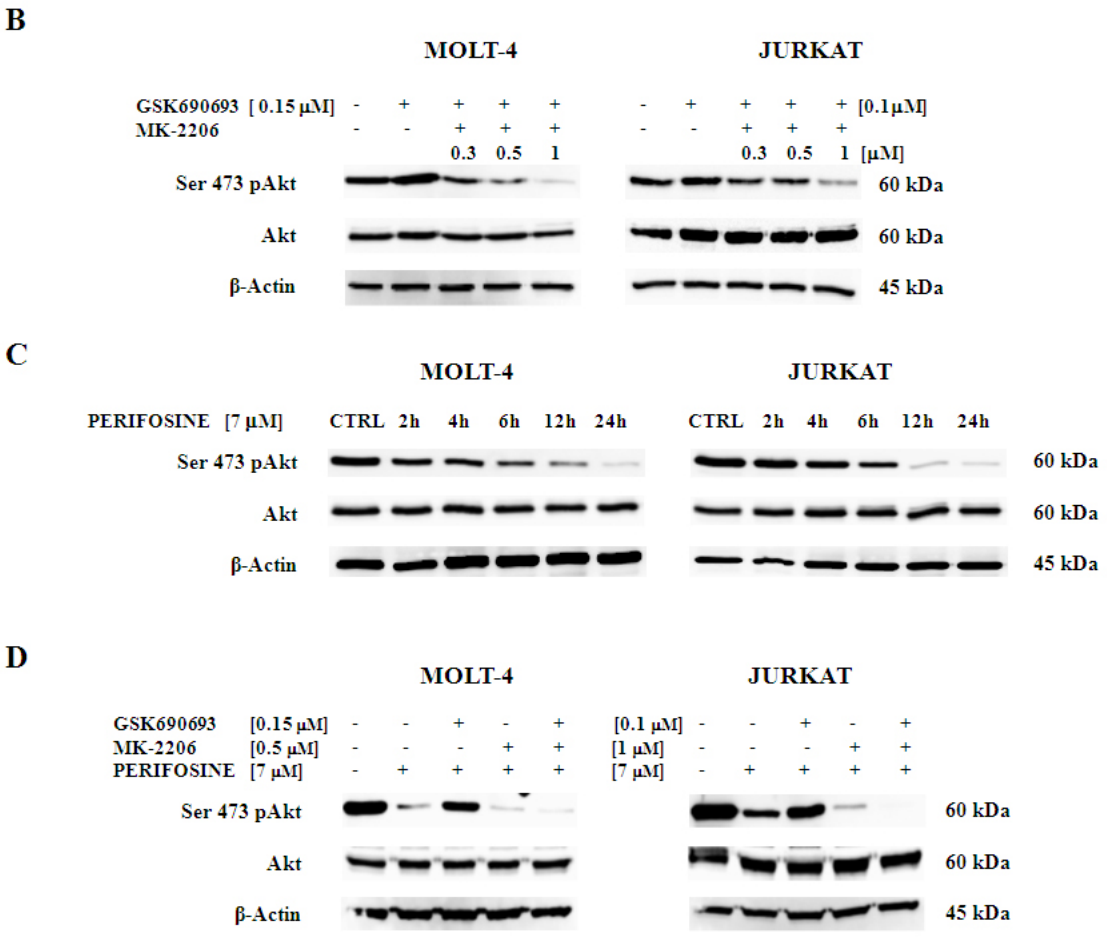
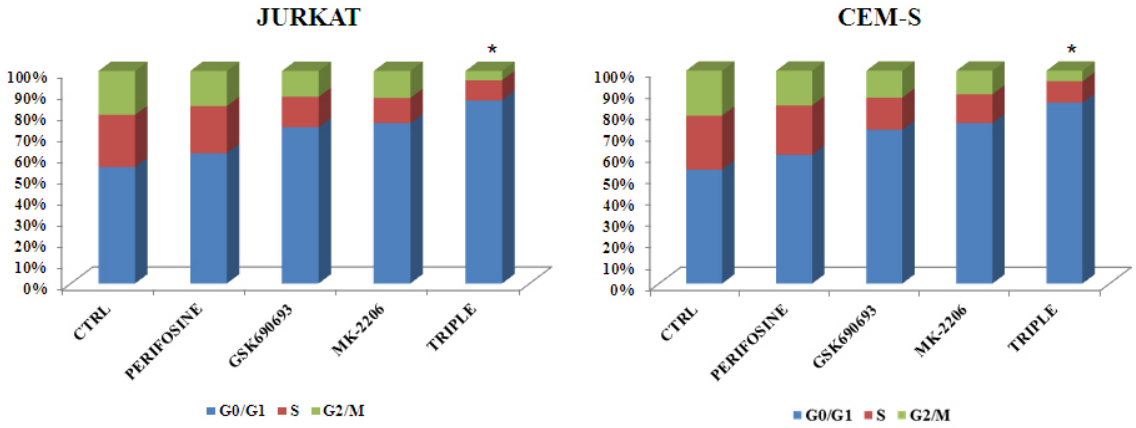


Figure 35: Multiple Akt inhibition affects PI3K/Akt/mTOR pathway and the Akt inhibition is time-dependent. **(b)** Akt

protein inhibition as detected by its phosphorylation status. MOLT-4 and JURKAT cells were treated for 30 minutes with a combination of a fixed concentration of GSK690693 and three different concentrations of MK-2206. (**c**) Akt phosphorylation levels in cells treated with 7 μM Perifosine at different time points. (**d**) p-Akt status in MOLT-4 and JURKAT cells pre-treated for 6h with 7 μM Perifosine followed by GSK690693 and MK-2206 administered for 30 minutes. Twenty-five μg of protein was blotted to each lane. β-actin served as a loading control.
For all panel one representative experiment of three is shown as well as cell lines are representative also of the others if not shown.

4.7.e The triple Akt inhibition induces cell cycle arrest and causes autophagy and pro-apoptotic effects in T-ALL cells

The significant in vitro antitumor activity of the triple anti Akt smi drug combination on T-ALL cells led us to investigate the mechanisms of its antileukemic efficacy. To assess the effects of the combined treatment on the PI3K pathway, we analyzed the effect of triple treatment on cell cycle progression, given the importance of the PI3K/Akt/mTOR signaling pathway in the regulation of cell proliferation [246].
Flow cytometric analysis of PI-stained samples in JURKAT, CEM-S and MOLT-4 cells was performed. Cells were treated with single and triple administration of drugs for 24h. The multiple anti Akt treatment increased the percentage of cells in G_0/G_1 phase of cell cycle, with a parallel decrease of both S and G_2/M phases (Fig. 36).



	JURKAT			CEM-S			MOLT-4		
	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
CTRL	54.8	24.6	20.6	53.5	25.4	21.1	52.3	26.9	20.8
Perifosine	61.3	22.2	16.5	60.4	23.3	16.3	61.7	22.3	16
GSK690693	73.5	14.3	12.2	72.2	15.1	12.7	73.6	14.4	12
MK-2206	75.4	11.9	12.7	75.3	13.6	11.1	76.1	12.4	11.5
Triple	86.1	9.5	4.3	84.9	10.1	5	87.3	8.6	4.1

Figure 36. Multiple Akt inhibition induces cell cycle arrest. Flow cytometric analysis of PI-stained samples was performed on JURKAT, and CEM-S cells after 24h of treatment with Perifosine, GSK690693 and MK-2206 administered both alone or in triple combination, always used at the IC_{50} value. In the table are reported the values of every cell cycle phase obtained in all treatments, with the addition of MOLT-4 cells. Asterisks indicate statistically significant differences with respect to untreated cells (*$p<0.05$). One representative experiment of three is shown as well as cell lines are representative also of the others not shown.

It has been reported that Akt targeted drugs induce autophagy in human glioma and T-ALL cells [179, 213]. Therefore, we investigated whether the triple treatment enhanced autophagy in our T-ALL cell panel. The exposure of cells to 24h of the combined treatment increased the amount of lipidated (14-kDa form) LC3A/B isoform, a well-established autophagy marker, detected by western blot in MOLT-4, JURKAT and CEM-S cells (Fig. 37a). Interestingly it has been previously reported that inhibiting autophagy with Chloroquine sensitises non small cell lung cancer cells (NSCLC) to combined treatment with Akt inhibitors [247]. We then inhibited autophagy using Bafilomycin A1 or Chloroquine and measured cell viability after 24h of treatment by MTT assay. Bafilomycin A1 or Chloroquine, when used alone, did not significantly affect cell viability. However when each drug was combined with triple Akt inhibition, it was possible to detect a

significantly increased cytotoxicity in CEM-S and MOLT-4 cells (Fig. 37b). These findings suggest that autophagy could protect T-ALL cells by the cytotoxic effects of the Akt inhibitors.

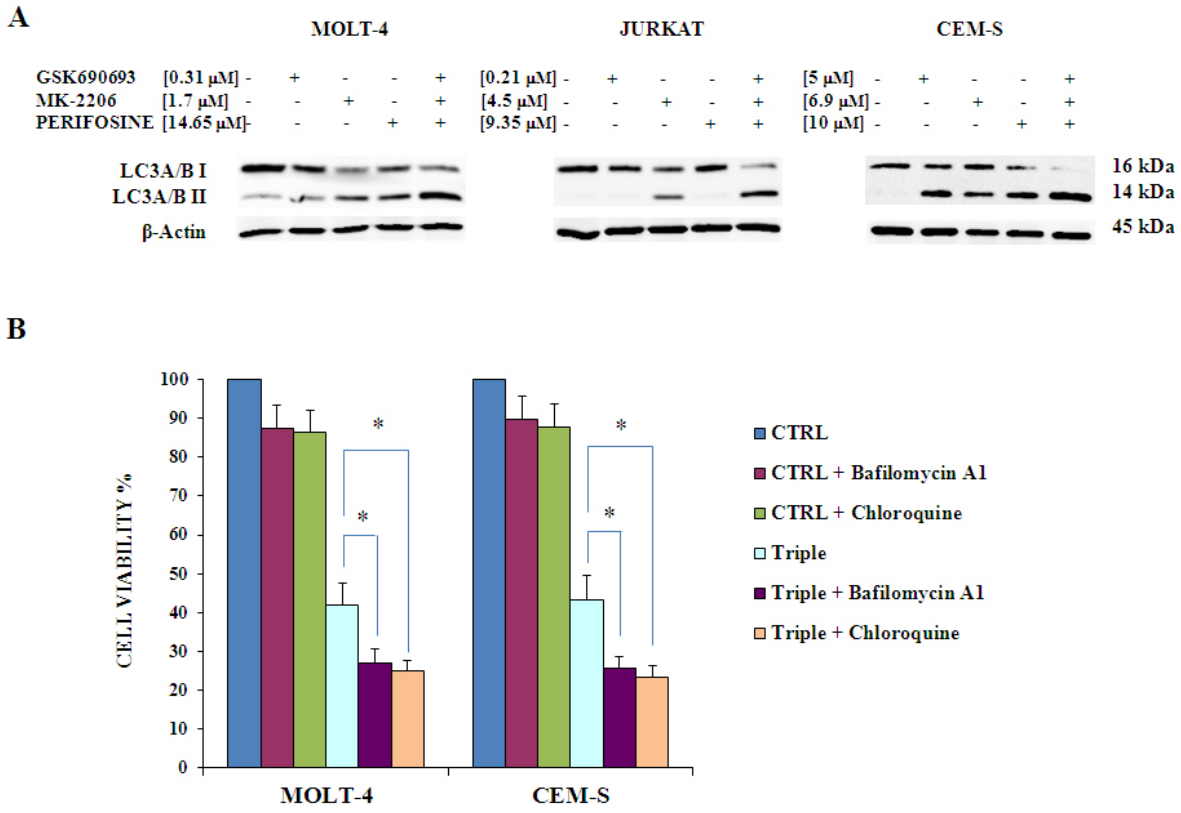
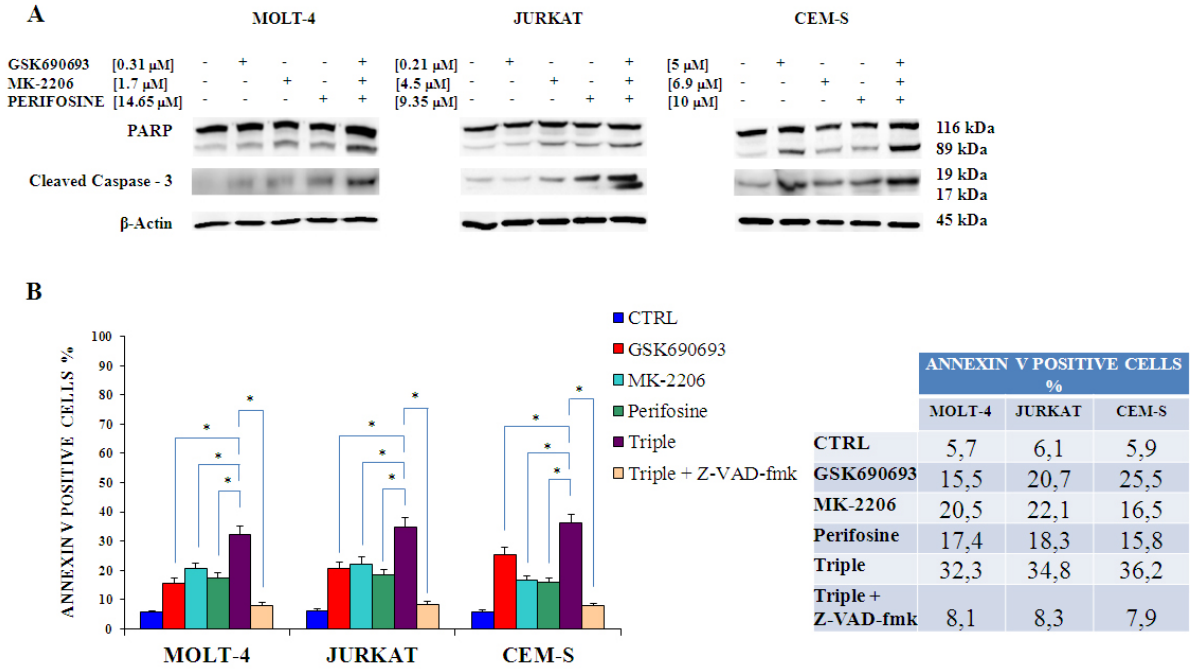


Figure 37: Triple Akt inhibition induces enhanced autophagy. **(a)** The effect of the three drugs administered alone or in combination on autophagy, after 24h of treatment, in MOLT-4, JURKAT and CEM-S cell lines, documented by the lipidation of the autophagy marker LC3A/B. The increase in the triple treatment is well evident. Antibody to β-actin served as a loading control. **(b)** MTT assays documenting the effects of Bafilomycin A1 and Chloroquine on viability of MOLT-4 and CEM-S cells treated for 24h with Perifosine, GSK690693 and MK-2206 administered in combination. Results are mean of three different experiments ± s.d. Asterisks indicate significant differences (*$p<0.05$). One representative experiment of three is shown as well as cell lines are representative also of the others not shown.

We next verified the induction of cleaved PARP, a well established apoptotic marker [248], in JURKAT, MOLT-4 and CEM-S cell lines by Western blotting after 24h of treatment. The apoptotic effect of the single drug administration was visible in each cell lines compared to the control, but was even more evident in MOLT-4 treated with MK-2206 and CEM-S treated with GSK690693. Interestingly, the triple administration of the drugs displayed a higher cleaved PARP in all the three cell lines (Fig. 38a). To further

strengthen this observation we also analyzed caspase-3, which plays an essential role during apoptotic cell death [249]: also with this protein the triple exposure to drugs induced an increased caspase-3 cleavage (Fig. 38a). The percentages of apoptotic cells was examined by flow cytometry after staining with annexin-V-FITC and PI. Flow cytometric analysis confirmed that multiple treatment induced a greater, statistically significant, increase in apoptosis compared to single drugs (Fig. 38b). In addition we studied if Z-VAD-fmk, a pan caspase inhibitor would affect the apoptotic process, as detected by annexin V staining: cell death was sensitive to the administration of 50 μM Z-VAD-fmk, that inhibited apoptosis in triple drug exposed cells.



	ANNEXIN V POSITIVE CELLS %		
	MOLT-4	JURKAT	CEM-S
CTRL	5,7	6,1	5,9
GSK690693	15,5	20,7	25,5
MK-2206	20,5	22,1	16,5
Perifosine	17,4	18,3	15,8
Triple	32,3	34,8	36,2
Triple + Z-VAD-fmk	8,1	8,3	7,9

Figure 38: Combined Akt inhibition increases apoptosis**. (a)** Western blot analysis documenting the increase of PARP or of caspase-3 cleavage in MOLT-4, JURKAT and CEM-S cell lines, after 24h of multiple drugs administration when compared with single exposure. Antibody to β-actin served as a loading control. **(b)** Flow cytometric analysis of MOLT-4, JURKAT and CEM-S cell lines treated with GSK690693, MK-2206 and Perifosine administered alone and in combination for 24h is reported. Triple drug exposure was also performed in the presence of 50 μM Z-VAD-fmk, a pan-caspase inhibitor. In the table are reported the percentages of annexin V positive cells after each treatment. Samples were incubated with Annexin V-fluorescein isothiocyanate. Results are the mean of three different experiments ± SD. Asterisks indicate statistically significant differences (*$p<0.05$). One representative experiment of three is shown as well as cell lines are representative also of the others not shown.

4.7.f The multiple treatment has the capability to inhibit ERK pathway

We finally then focused our attention on the MEK/ERK signaling pathway, which plays an important role in cell proliferation and growth [250] and potentially mediates resistance to drug-induced growth inhibition [251].

For this reason, we examined the p-ERK 1/2 phosphorylation status in JURKAT, MOLT-4 and CEM-S cells, after 24h of treatment with the three drugs.

Interestingly, the multiple treatment was capable to downregulate the phosphorylation state of Tyr 202/204 p-ERK 1/2 with a superior efficacy than single drugs, whereas the amount of total protein remains unchanged (Fig. 39).

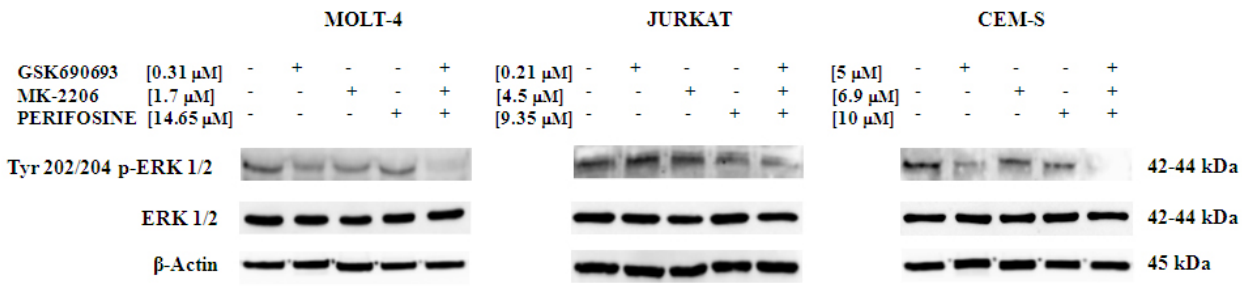


Figure 39: Multiple anti Akt drug treatment affects also MEK/ERK pathway. Western blot analysis for Tyr 202/204 p-ERK 1/2 phosphorylation status in MOLT-4, JURKAT and CEM-S cells, after 24h of treatment with the three drugs alone and in combination. The triple treatment almost abolish p-ERK staining. Twenty-five μg of protein was blotted to each lane. β-actin served as a loading control. One representative experiment of three is shown as well as cell lines are representative also of the others not shown.

5. Discussion

The PI3K/Akt/mTOR signaling cascade is a pivotal pathway that is deregulated in a wide variety of human cancers and strongly contributes to cancer cell survival, promotes chemotherapy resistance through disruption of apoptosis and initiates cap-dependent translation of mRNAs essential for cell cycle progression, differentiation and growth [252]. Activation of this pathway is a common feature of a wide range of human cancers [253], including hematological malignancies, and is an indicator of poor prognosis [254-258]. Although the survival of younger patients with acute leukemia has improved in the early 21st century, mutations event can occur at any stage of the disease and negatively influences the response to therapeutic treatments because lead to resistance to therapy. A major challenge remains the lifelong morbidity suffered by patients treated with current chemotherapy regimens. For long-term survivors, acute and lasting toxicities remain important issues underlining the critical need of more effective and selective personalized and targeted therapies, and treatment strategies.

Therefore, owing to the fundamental role of PI3K/Akt/mTOR pathway in tumor development and progression, there is a significant interest in developing inhibitors against components of this pathway, up to having now many compounds currently under evaluation in clinical trials.

A large variety of inhibitors have been widely used both in vitro and in vivo in preclinical settings of acute leukemias, where they blocked cell proliferation and induced, sometimes, apoptosis and/or autophagy [158, 259-261]. Several studies have highlighted that both PI3K and mTOR modulators could synergize with a wide range of drugs that are currently in use for treating acute leukemias, including chemotherapeutic drugs [262, 263]. Drugs dual targeting PI3K/Akt/mTOR pathway at various points of the signaling are under evaluation in preclinical models and clinical trials, but the observation that not even the combined inhibition of Akt and mTOR is enough to completely turn off the pathway during chronic treatment is very intriguing [264].

5.1 Treatment with MK-2206 affects T-ALL cells

Considering the crucial role had by aberrantly activated Akt in the pathogenesis of T-ALL [265, 266], we studied the efficacy of MK-2206, a novel allosteric Akt inhibitor [267], as a

potential therapeutic agent. MK-2206 decreased the viability of T-ALL cell lines, in a concentration-dependent manner. All these cell lines are phosphatase and tensin homologue deleted on chromosome 10 negative and display a nonfunctional p53 pathway [268]. The efficacy of MK-2206 in decreasing the viability of T-ALL cell lines was due to both cell cycle arrest and caspase-dependent apoptosis.

MK-2206 dephosphorylated Akt on both Thr 308 and Ser 473, its downstream targets, GSK3-α/β and FoxO3A and two mTORC1 downstream targets, that is, p70S6K and 4EB-P1. Down-regulation of Ser 2448 p-mTOR levels were indicative of an inhibition of mTORC1 (data not shown), whereas decreased phosphorylation of Ser 473 p-Akt indicated an indirect targeting of mTORC2 by MK-2206.

The mechanisms that control mTORC2 activity have only begun to be revealed. mTORC2 activation requires PI3K and the TSC1/TSC2 complex [269]. As Akt is upstream of TSC1/TSC2, MK-2206, by inhibiting Akt, could also down-regulate mTORC2 activity.

In addition to apoptosis, MK-2206 caused autophagy in T-ALL cell lines. Autophagy induction by MK-2206 could be related to mTORC1 inhibition, as mTORC1 inhibits autophagy through phosphorylation of two autophagy-promoting factors, unc-51-like kinase 1 and autophagy-related gene 13 [270]. However, the induction of autophagy, as indicated by cleavage of LC3A/B, was much stronger in MOLT-4 and CEM-S cells than in drug-resistant CEM-R cells. As down-regulation of Bcl-XL by siRNA increased LC3A/B cleavage in CEM-R cells, we could infer that the differential induction of autophagy in CEM-R vs CEM-S cells was related to the fact that CEM-R cells express Bcl-XL to a much higher extent than CEM-S cells. This observation is consistent with findings obtained in other cell types [271].

Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation development and homeostasis. Tumor cells can exploit autophagy as a survival mechanism to tolerate metabolic stress and to contrast apoptosis [272].

Interestingly, both Bafilomycin A1 and Chloroquine, two inhibitors of autophagy, sensitized CEM-S cells to MK-2206, by increasing apoptosis. This finding demonstrated that autophagy is a pro-survival mechanism that protected CEM-S cells from MK-2006-induced apoptosis. Therefore, the use of autophagy inhibitors in combination with MK-2006 should be considered in future clinical trials.

We have also documented that MK-2206 synergized with Doxorubicin. Analysis of the results demonstrated that in MOLT-4 the synergism of MK-2206 with Doxorubicin was

relevant at low concentrations (10–30 nM of doxorubicin) at 48h of treatment. The sequential exposure to MK-2206 after Doxorubicin treatment for a total of 48h resulted in relevant synergism at concentrations ranging from 10 to 100 nM Doxorubicin, with an even stronger synergism using the reverse sequence. In CEM-R cell line, if the two drugs were administered together for 48h, the synergism was evident at intermediate concentrations. The sequential exposure to MK-2206 after doxorubicin treatment for a total of 48h resulted in relevant synergism. Nevertheless, it is remarkable that in MOLT-4 cells a synergism was detected at concentrations that were well below the MK-2206 IC_{50} for this cells line. This finding could have a clinical relevance for T-ALL patients, as a combination of MK-2206 and Doxorubicin increased the cytotoxic activity of MK-2206 and allowed for the use of a much lower concentration of the inhibitor. This could considerably attenuate the possible MK-2206 toxic side effects. However, MK-2206 is generally well tolerated at doses that result in plasma concentrations portending activity in preclinical models [273].

In conclusion, our preclinical findings strongly suggest that MK-2206 could be a valuable compound for treating those T-ALL patients displaying activation of PI3K/Akt signaling and who are still facing a poor prognosis.

5.2 Treatment with RAD001 affects B-pre ALL cells

Targeting mTOR using the orally active rapamycin derivative RAD001 can effectively contribute to treat tumors with hyperactivation of PI3K/Akt/mTOR signaling. Here, we demonstrated the efficacy of RAD001 as a potential therapeutic inhibitor of the PI3K/Akt/mTOR pathway in B-pre ALL cells.

RAD001 was cytotoxic to B-pre ALL cell lines in a concentration-dependent manner, which was demonstrated by both MTT assays and flow cytometric analysis of Annexin V-stained samples. Cytotoxicity was independent from the t4;11 translocation, which occurs in SEM and RS4;11 cell lines.

In B-pre ALL cell lines, RAD001 not only down-regulated mTORC1 activity (as documented by dephosphorylation of Ser 2448 p-mTOR, Thr 389 p-p70S6K and Thr 37/46 p-4EBP1) but also decreased mTORC2 activity, as demonstrated by decreased phosphorylation of Ser 473 p-Akt and Ser 2481 p-mTOR.

The cytotoxic effects of RAD001 on B-pre ALL cell lines could be related to cell cycle arrest in G_0/G_1, and induction of either apoptosis or autophagy (or both). Remarkably, both apoptosis and autophagy were more evident in SEM cells, consistently with the highest sensitivity of this cell line to RAD001.

Cancer cells can gain benefit of autophagy as a survival mechanism, which gives them a selective advantage to tolerate metabolic stress and to contrast apoptosis [272]. We found that silencing Beclin-1, a protein essential for the assembly of the autophagy initiation complex that leads to autolysosomes formation [220], resulted in a lower sensitivity of both SEM and REH cells to the cytotoxic effects of RAD001. Therefore, RAD001-induced autophagy is very important for the reduction of cell viability. Although the induction of autophagy by RAD001 in B-pre ALL cells has been previously reported [261], this is the first time that the functional significance of autophagy was explored.

5.3 Two hit pathway with RAD001 and MK-2206 leads to synergistic effects

We aimed in investigating the effect of combined Akt and mTOR inhibition on B-pre ALL cells using the novel allosteric, clinically available pan-Akt inhibitor MK-2206 [179]. This combination has been demonstrated to be effective in hepatocellular carcinoma cell lines [274], cholangiocarcinoma cell lines [224] and in an in vivo model of castration-resistant prostate cancer [275]. Moreover, we used a second combination consisting of CCI-779 and GSK690693.

There is a strong rationale for targeting Akt in B-pre ALL cells, as a recent work has documented that Akt activation is associated in this leukemia subset with poor prognosis and resistance to a set of chemotherapeutic drugs, including prednisolone, dexamethasone, vincristine and Adriamycin [258].

We observed a synergistic cytotoxic effect of the RAD001/MK-2206 drug combination. The combination caused a dose-dependent decrease of cell viability, and this was visible even at low concentrations in all cell lines, but especially in SEM cells. After testing different strategies for combining the two drugs together, we observed that the highest synergistic effects were detectable in SEM cells when RAD001 was administered for 48h and MK-2206 was added after 24h.

The combination consisting of RAD001 and MK-2206 was more effective than either treatment alone in inducing cell cycle arrest in G_0/G_1, and this correlated with a more effective dephoshorylation of Rb on both Ser 807/811 and Ser 780. These two residues are key determinants of Rb activity. Indeed, it has been documented that a Rb mutant with alanine substitutions at Ser 807/811 had enhanced growth-suppressing activity [276]. Moreover, phosphorylation of Ser 807/811 led to an inactivation of Rb tumor suppressor activity in uveal melanoma [277]. The Ser 807/811 and Ser 780 residues are targets of cyclin E/CDK2 and cyclin D/CDK4, respectively [278]. It should be reminded here that mTORC1 controls the translation of several proteins that are of critical importance for cell cycle progression, including CDK2 [279]. It is important to emphasize that Rb gene activation is an unfavorable prognostic predictor in initial and relapsed childhood ALL [280]. Therefore, drugs that affect the Rb pathway activity may be important for development of individual-targeted therapies.

The dual mTOR/Akt inhibition was more effective than single drugs in lowering cell viability and affecting phosphorylation of critical components of the PI3K/Akt/mTOR pathway, also if we employed a different drug combination consisting of CCI-779 and GSK690693.

These findings may have a clinical relevance for B-pre ALL patients, because the synergistic combination of the drugs led to an increase of the cytotoxic activity at lower concentrations of the inhibitors. If this would be translated into the clinic, an attenuation of toxic side effects could be expected.

RAD001 alone displayed anti-leukemic effects also against B-pre ALL patient samples, acting on Ser 473 p-Akt, Ser 2448 p-mTOR and Thr 389 p-p70S6K.

Consistently, also in B-pre ALL lymphoblasts, we observed a striking synergistic effect of the combination of RAD001/MK-2206 that resulted in increased dephosphorylation of key components of the PI3K/Akt/mTOR pathway, as well as in enhanced apoptosis and decreased cell viability. Also the CCI-779 and GSK690693 combination affected B-pre ALL lymphoblasts viability more effectively than either drug alone.

In conclusion, we have documented that RAD001 is cytotoxic to B-pre ALL cells, and that autophagy has an important role in decreasing cell viability. Moreover, our findings suggest that rapamycin derivatives can be effectively used in combination with other inhibitors targeting key nodes of the PI3K/Akt/mTOR pathway, in order to reduce drug concentration and hence systemic side effects. Therefore, dual targeting of

PI3K/Akt/mTOR signaling pathway might represent a new promising therapeutic strategy for treatment of adult B-pre ALL patients.

5.4 Treatment with Torin-2 affects B-pre ALL cells

Second generation ATP-competitive mTOR kinase inhibitors represent a promising new approach to target the PI3K/Akt/mTOR pathway with potentially greater tolerability than dual PI3K/mTOR inhibitors [198, 281, 282]. It has been previously reported that first generation mTOR kinase inhibitors had improved efficacy compared to rapamycin in models of Ph+ B-ALL [283].

Here, we evaluated for the first time the efficacy of the novel mTORC1/mTORC2 second generation, ATP-competitive inhibitor, Torin-2 [284] in pre-clinical settings of Ph+ and Ph- B-pre ALL.

Torin-2 was both cytotoxic and cytostatic in a nanomolar range to B-pre ALL cell lines in a concentration-dependent mechanism, as demonstrated by MTT assays, flow cytometric analysis of Annexin V-stained samples and of PI-stained samples. Apoptosis resulted to play a determinant role in the killing mechanism, since the treatment with a pan caspase inhibitor protected the cells from Torin-2 cytotoxic effect. These results are in agreement with those observed with other drugs in acute myeloid leukemia [285, 286]. Torin-2 also induced autophagy, as documented by increased expression of the lipidated form of LC3. In order to demonstrate whether autophagy was either a survival or a death mechanism, B-pre ALL were co-treated with the autophagic sequestration inhibitors, 3-MA and Bafilomycin A1. We found that either treatment with 3-MA and Bafilomycin A1, resulted in a lower sensitivity of both SEM and BV-173 cells to the cytotoxic effects of Torin-2 and indicated that Torin-2-induced autophagy was very important for the cytotoxic effects of the drug. The phosphorylation status of the key elements of the PI3K/Akt/mTOR pathway, assessed by Western blot, was equally sensitive to Torin-2 inhibition in either cells harboring or not the Bcr-Abl fusion protein.

5.5 Comparison between RAD001 and Torin-2 in B-pre ALL cells

RAD001, when used in the nanomolar range against this panel of cell lines, was much less cytotoxic than Torin-2, as it displayed $IC_{50s} > 5$ μM. This superior efficacy of the second generation inhibitors, has been also reported for MLN0128, another mTORC1/mTORC2 inhibitor, which displayed an improved anti-leukemic activity when compared to first generation inhibitors, in Ph- B-ALL derived from both adult and pediatric subjects [230]. It has been reported that in solid tumor models, activation of mTORC1 drives p70S6K-mediated degradation of the IR/IGF-1R adaptor protein IRS-1, and is therefore a negative regulator of PI3K [287]. Accordingly, drugs targeting mTORC1 block this feedback and trigger reactivation of the pathway and re-phosphorylation of Akt on Ser 473 residue in acute myelogenous leukemia cells [231, 288]. The issue of Akt reactivation in response to mTORC1 inhibition, has not been throughly investigated in B-pre ALL. It is worth highlighting that Torin-2 maintained a prolonged suppression of mTORC1/mTORC2 with a sustained anti-proliferative effect, overcoming the limitations of rapalogs such as RAD001, as demonstrated by our findings, which resulted in re/ hyper-phosphorylation of Akt and could hamper their anti-tumor action and enhance resistance to antineoplastic therapy, thus resulting in a poor outcome [289].

Akt activity is directly down-regulated on Thr 308 by the protein phosphatase PP2A [56], which is also critically involved in regulation of cell cycle progression [290] and DNA damage response [291]. Accumulating evidence indicates that PP2A acts as a tumor suppressor and impairment of PP2A activity may result in loss of this function [292]. The capacity of Torin-2, but not of RAD001, to inhibit both mTORC1 and 2 may represent a possible mechanism for why Akt is not re-phosphorylated with this drug on the Ser 473 residue and this phosphorylation may be independent form PP2A activity.

The Torin-2 prolonged suppression of Akt phosphorylation may overcome the occurrence of PP2A oncosuppressor altered function and may render Akt inhibition independent from the activity of PP2A, thus proposing this therapeutic options as a potential tool that could act functionally on either impaired Akt and PP2A functions.

5.6 Comparison between RAD001 and Torin-2 administered in B-pre ALL cells with MK-2206

Overall, Torin-2 alone was as potent as a combination consisting of RAD001 and MK-2206, in terms of reduction of cell viability, apoptosis induction, and cell cycle block. In contrast, when MK-2206 was combined with Torin-2, it did not display any synergistic effect. Interestingly, the presence of synergism or its lack was evident also when the phosphorylation status of key elements of the pathway was analyzed by Western blot.

Both ATM and ATR play a central role in coordinating the DNA damage response, including cell cycle checkpoint control and apoptosis [293]. Since the results by Liu et al. [199], using purified enzymes, suggested that Torin-2 was also an inhibitor of several PIKK family members including ATR, ATM, and DNA-PK, we investigated if this was also true in B-pre ALL cell lines. Western blot analysis documented that indeed Torin-2 inhibited ATM and ATR in intact cells and, by inhibiting DNA repair, potentiated the cytotoxic effect of Doxorubicin. Torin-2 displayed an obvious advantage over RAD001, which also induced a similar phenomenon, as Torin-2 could be used in the nanomolar range, whereas RAD001 required a concentration as high as 16 μM [294], which could not be attained *in vivo*. In addition, very recently it has been reported that Akt inhibition with CCT12893 increased the phosphorylation of CHK1/CHK2 and γ-H2AX [295].

These findings open a new very interesting field to be further explored in the future regarding the therapeutic effect of PI3K/Akt/mTOR inhibitors involving DNA damage sensors and cell cycle checkpoints such as CHK1 and CHK2.

In conclusion, our data indicate that the novel mTORC1/mTORC2 kinase inhibitor Torin-2 can suppress the growth of both Ph+ and Ph- B-pre ALL cells and extend the finding that the antiproliferative and pro-apoptotic effects of PI3K/Akt/mTOR pathway inhibitors are independent from ABL-translocation, as reported in long-term cultures of Ph+ and Ph- B-precursor ALL cells from patients [289]. Remarkably, Torin-2, even after a 48h incubation, blocked the reactivation of Akt, thus confirming the new therapeutic hopes that this second generation of inhibitors is developing. In addition, Torin-2 could be also effective in combination with chemotherapeutic DNA-damaging agents, in light of its capacity of blocking DNA repair.

Interestingly, with the aim of improving B-pre ALL treatment, we also came to the conclusion that low concentrations of RAD001 and MK-2206 (which can be attained *in*

vivo) may achieve a therapeutic efficacy comparable to an mTORC1/mTORC2 inhibitor. These therapeutic strategies could be particularly effective when combined with targeted next generation sequencing of tumor samples, since genomic alterations can be detected and help to identify refractory patients with aberrations putatively activating the PI3K/Akt/mTOR pathway [296, 297]. These pharmacological options targeting PI3K/Akt/mTOR at different points of the signaling pathway cascade or in combination with conventional chemotherapy might represent a new therapeutic potential for treatment of B-pre ALL patients.

5.7 Triple hit Akt

A large variety of inhibitors have been widely used both in vitro and in vivo in preclinical settings of acute leukemias, where they blocked cell proliferation and induced, sometimes, apoptosis and/or autophagy [158, 259-261]. Several studies have highlighted that both PI3K and mTOR modulators could synergize with a wide range of drugs that are currently in use for treating acute leukemias, including chemotherapeutic drugs [262, 263]. Drugs dual targeting PI3K/Akt/mTOR pathway at various points of the signaling are under evaluation in preclinical models and clinical trials, but the observation that not even the combined inhibition of Akt and mTOR is enough to completely turn off the pathway during chronic treatment is very intriguing [264].

Despite several chemotherapy combinations were tested in vitro and are in clinical trials for the treatment of acute lymphoblastic leukemia, a new and promising innovative idea, for the individualization of the therapies, could be represented by hitting the same target with multiple specific drugs with different mechanisms of action.

Here, we demonstrated the efficacy of multi-inhibition of the same target, i.e. the Akt protein as a pivotal molecule of the PI3K/Akt/mTOR signaling pathway with three drugs with a totally different mechanism of action.

The triple administration of GSK690693, MK-2206 and Perifosine in cells with Ser 473 p-Akt hyperphosphorylation was cytotoxic and synergic at lower doses when compared with the IC_{50} values. We showed the relevance of a fine tuning of the single drug concentration to obtain the best synergistic effect. Single inhibition of Akt was lower when compared with every dual inhibition, which in turn was lower than the triple one.

We also demonstrated the importance of two issues: the compound concentration and the timing of drugs administration. About the first one, two of the drugs, MK-2206 and GSK690693 were very efficient acting synergistically even at low doses, whereas Perifosine administered together did not add its efficacy in the 10-20 μM range. The second issue disclosed that MK-2206 and GSK690693 may act very rapidly (minutes), but Perifosine requires a longer period of time (hours), to enter in action and to really synergize with MK-2206 and GSK690693.

These findings indicate that Akt inhibition mechanism may be compatible with a fine tuning of the concentrations of the single drugs, that may help with low doses to reduce the side effects of the therapy, and with crucial time points of administration of the drugs, that otherwise may improve the efficacy of the therapy. These observations are present in several reports [237, 298-302]. Further studies can sort out this issue.

We also documented the increasing of cleaved PARP and caspase-3, well known markers of apoptosis, thus showing this as a mechanism for the cytotoxicity of anti Akt drugs.

Both autophagy and apoptosis are well-controlled biological processes for programmed cell death, that play essential roles in development, tissue homeostasis and disease, with interactions among components of the two pathways [303]. Autophagy is a cellular catabolic degradation process that results in the autophagosomic-lysosomal degradation of cytosolic proteins and other cellular components [304].

Interestingly, here we demonstrated the increment of autophagy in multiple anti Akt drugs treated samples. Moreover, either the Bafilomycin A1 or the Chloroquine autophagy inhibitors, sensitized cells to treatment. These findings demonstrated that autophagy is a pro-survival mechanism that protected cells from Akt inhibitors induced cytotoxicity and the use of autophagy inhibitors may be considered in future drug combinations, also in clinical trials.

Compensatory up-regulation of parallel signaling through the MEK/ERK1/2 pathway in response to PI3K/Akt inhibition, is an emerging theme in cancer cell signal transduction, because it potentially mediates resistance to drug-induced growth inhibition [251]. Indeed, several recent reports have highlighted the importance of functional crosstalks between the MEK/ERK1/2 and PI3K/Akt signaling networks, in response to individual pathway inhibitors [171, 204, 251, 292, 305, 306].

The MEK/ERK1/2 pathway often reflects the rebound of Akt inhibition, developing or amplifying hyperactivation. Unexpectedly, our results displayed an inhibition of ERK activation in correspondence of multiple drugs administration. It is tempting to speculate

that the multi-Akt inhibition could represent an effective treatment to block crosstalk between PI3K/Akt/mTOR and Raf/MEK/ERK reducing tumor growth and cells proliferation.

Genomic DNA mutational analysis of Akt1 exons and adjacent splice sites evidenced a somatic point mutation in the Pleckstrin Homology domain (PH) of Akt1 gene: a lysine substitution for glutamic acid at the amino acid 17 (E17K). This genetic alteration was originally identified in solid tumors [307, 308]. Cystallography studies demonstrated that E17K mutation modified Akt1-PH conformation and disrupted ionic interaction with Lys14 in the lipid-binding pocket of Akt1 [309]. This mutation have effects on sensitivity to targeted pathway inhibitors, since it has been recently reported that the Akt1- E17K mutant cells maintained higher levels of Akt1 Ser 473 phosphorylation even with increasing concentrations of MK-2206 [310].

Very recently a total of five Akt1 variants such as E17K, E17S, E319G, L357P, and P388T were found to exert deleterious effects on the protein structure and function. Furthermore, the molecular docking study indicated the lesser binding affinity of inhibitor with the mutant structure than the native type. Moreover, the findings strongly indicated that screening for Akt1, E17K, E17S, E319G, L357P, and P388T variants may be useful for disease molecular diagnosis and also to design the potential Akt inhibitors [311].

Therefore it appears of great importance to hit Akt as a pivotal molecule in cancer development and progression with drugs acting with multiple mode of action, to overcome the potential resistance due to mutations in the site of action of the single drug.

There is growing clinical interest in determining whether mutations in the PI3K signaling pathway can serve as biomarkers to predict sensitivity to drugs targeting the pathway. Thus, the presence of Akt mutations or of PI3K/Akt/mTOR hyperactivation should be taken in account when establishing a therapeutic regimen for the patient. This is an important issue toward the personalized medicine, since it is useless to activate a treatment if the correspondent pivotal cytotoxic target is absent [312].

In conclusion, our preclinical finding strongly demonstrated that a multiple inhibition of Akt could represent a new promising therapeutic strategy to overcome relapse or resistance in the treatment of T- ALL patients with hyperactivation of PI3K/Akt/mTOR signaling pathway.

6. References

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