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Therapeutic Potential of Targeting Enzymatic Activity of PI3K/AKT/mTOR Signaling Pathway For the Treatment of Acute Lymphoblastic Leukemia

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Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignant hematological disorder arising in the thymus from T-cell progenitors. T-ALL mainly affects children and young adults, and remains fatal in 20% of adolescents and 25% of adults, despite progress in polychemotherapy protocols. Therefore, innovative-targeted therapies are needed for patients with poor prognosis. Aberrant activation of PI3K/Akt/mTOR signaling pathway is a common event in T-ALL patients and portends a poor prognosis. Recent findings have highlighted that constitutively active phosphatidylinositol 3-kinase PI3K/Akt/mammalian target of rapamycin (mTOR) signaling pathway upregulates cell proliferation, survival, and drug resistance. These observations lend compelling weight to the application of PI3K/Akt/mTOR inhibitors in the therapy of T-ALL. Preclinical studies have highlighted that modulators of PI3K/Akt/mTOR signaling could have a therapeutic relevance in T-ALL. However, the best strategy for inhibiting this highly complex signal transduction pathway, is still unclear, as the pharmaceutical companies have disclosed an impressive array of small molecules targeting this signaling network at different levels. In this study we have analyzed the therapeutic potential of the novel dual PI3K/mTOR inhibitor NVP-BGT226, an orally bioavailable imidazoquinoline derivative, which has entered clinical trials for solid tumors and an ATP-competitive mTORC1/mTORC2 inhibitor Torin-2, on both T-ALL cell lines and normal T-lymphocytes samples. We found that NVP-BGT226 and Torin-2 displayed the most powerful cytotoxic effects against T-ALL cell lines and primary mitogenically stimulated T-lymphocytes while quiescent cells were not affected. NVP-BGT226 and Torin-2 treatment also resulted in cell cycle arrest, apoptosis, and autophagy. Western blots showed a dose- and time-dependent dephosphorylation of Akt and mTORC1 downstream targets in response to both drugs in T-ALL cell lines. Nevertheless, the effect of both drugs also was documented in mitogenic stimulated primary lymphocytes, while no effects there detectable in quiescent cells. We also documented that dual targeting of this pathway was significantly cytotoxic in T-ALL cells. This effect was absent in quiescent T-lymphocyte but present in mitogenically activated T-lymphocytes, at variance requiring a higher concentrations of drugs. This observation indicates that vertical inhibition at different levels of the PI3K/Akt/mTOR network could be considered as a future innovative strategy for treating T-ALL patients.

Riassunto

La leucemia acuta linfoblastica a cellule T (LAL-T) è una malattia ematologica maligna aggressiva derivante nel timo da progenitori delle cellule T. La LAL-T colpisce soprattutto bambini e giovani adulti, e rimane fatale nel 20% degli adolescenti e nel 25% degli adulti, nonostante i progressi nei protocolli di polichemioterapia. Pertanto, terapie innovative mirate sono necessarie per i pazienti con prognosi infausta. L'iper attivazione della via di trasduzione segnale PI3K / Akt / mTOR è un evento comune in molti pazienti e fa presagire una prognosi infausta. Recenti scoperte hanno messo in evidenza che la via di trasduzione del segnale PI3K / Akt / mTOR costitutivamente attiva leucemie acute linfoblastiche LAL-T, fa aumentare la proliferazione cellulare, la sopravvivenza, e la resistenza ai farmaci. Queste osservazioni indirizzano verso l'uso di inibitori PI3K / Akt / mTOR nella terapia di LAL-T. Studi preclinici hanno evidenziato che modulatori di PI3K / Akt / mTOR potrebbero avere un valore terapeutico in LAL-T. Tuttavia, la migliore strategia per inibire questa via di trasduzione del segnale estremamente complessa non è ancora chiara. Le aziende farmaceutiche hanno sviluppato una serie impressionante di piccole molecole farmacologiche che hanno come bersaglio questa via di trasduzione del segnale a diversi livelli. In questo studio abbiamo analizzato il potenziale terapeutico del nuovo doppio inibitore di PI3K e di mTOR, NVP-BGT226, un'imidazoquinoline derivato biodisponibile per via orale, che è entrato in studi clinici per i tumori solidi, ed il potenziale terapeutico di un nuovo inibitore mTORC1 / mTORC2, ATP-competitivo, Torin-2, sia su linee cellulari leucemiche T sia linfociti T normali. Abbiamo osservato che NVP-BGT226 e Torin-2 hanno mostrato i più potenti effetti citotossici contro tutte le linee cellulari di LAL-T e contro i linfociti T stimolati con stimolo mitogenico, mentre i linfociti T quiescenti non sono stati affetti dal farmaco. NVP-BGT226 e il trattamento con Torin-2 hanno portato anche ad un arresto del ciclo cellulare, all' apoptosi e all' autofagia.

Le analisi in Western blot hanno mostrato una defosforilazione dose e tempo-dipendente dei bersagli a valle di Akt e mTORC1 in risposta a entrambi i farmaci in tutte le linee cellulari di LAL-T. Inoltre l'effetto di entrambi i farmaci è stata osservato anche in linfociti primari stimolati dalla proliferazione, mentre nessuna defosforilazione è stata rivelata in cellule quiescenti. Abbiamo anche osservato che i farmaci diretti contro 2 bersagli della via di trasduzione del segnale PI3K / Akt / mTOR hanno un'importante efficacia citotossica, rilevata anche in cellule stimolate alla proliferazione, ma non in

linfociti T quiescenti (non stimolati). Questi risultati indicano che l'inibizione a diversi livelli della via di trasduzione del segnale PI3K/Akt/mTOR potrebbe essere considerata come una futura strategia innovativa per il trattamento dei pazienti affetti da LAL-T.

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Dedication

To my parents.

The reason of what I become today.

Thanks for your great support and continuous care.

To my wife and lovely Basma.

I am grateful to all of you; you have been my inspiration, and my soul mates.

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1. Introduction

1.1 The PI3K/Akt/mTOR signal transduction pathway

1.1.1 The PI3K family

The pathways regulated following activation of the phosphoinositide 3-kinase (PI3K) family of enzymes have been intensively studied in recent years (Fig. 1). In the 1980s, investigators observed phosphoinositide kinase enzymatic activity that modified a specific site on phosphatidylinositides (PtdIns), showing that it was associated with tyrosine-phosphorylated proteins. Much of this work was initiated in the laboratories of Tom Roberts and Lewis Cantley [1-3]. In 1988, Whitman et al. provided biochemical proof that the kinase activity was able to specifically phosphorylate the 3 position of the inositol ring, thereby initiating the age of PI3K investigations. Subsequent characterization and molecular cloning revealed that a p110 catalytic subunit was bound to a regulatory subunit containing Src homology 2 (SH2) domains, p85, which was responsible for the association with tyrosine-phosphorylated proteins initiating PI3K activation. The p85 subunit, which was later shown to be one of the several forms of a regulatory subunit, associates with a p110 subunit responsible for the catalytic activity. Studies over several years revealed that the PI3K family consists of at least nine genes in mammalian systems, corresponding to various isoforms that are categorized into class I, II and III (Fig. 2). The class I subgroup is the most relevant when discussing growth-factor- or cytokine-activated PI3K.

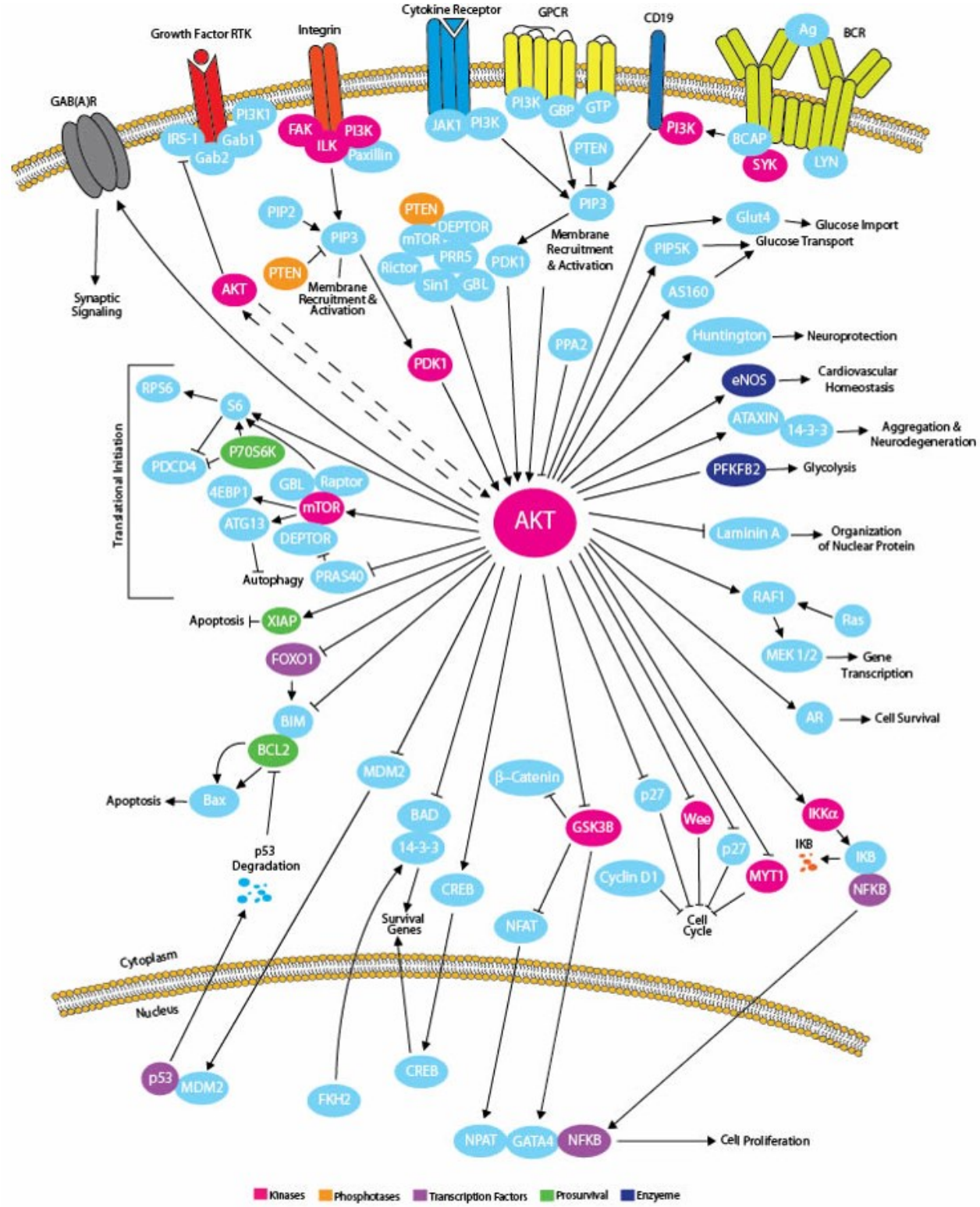


Figure 1. Schematic representation of the PI3K/Akt/mTOR pathway

Class, I, II and III PI3Ks: Unlike the other PI3K isoforms, class I PI3Ks are thought to exclusively phosphorylate PtdIns (4,5)P2 to generate PtdIns(3,4,5)P3 in vivo, even though they can utilize other lipids such as PtdIns and PtdIns4P when assayed in vitro. The class I enzymes consist of p110α, p110β, p110γ and p110δ catalytic subunits. The α, β, and δ subunits are referred to as class IA and associate with regulatory subunits of the p85 type, which contain SH2 domains. Following activation of tyrosine kinases, the SH2 domains of p85 mediate activation of PI3K by binding to a consensus YXXM motif, when the tyrosine residue is phosphorylated [4]. In an alternative pathway of class IA PI3K activation, activated p21-Ras has been shown to directly bind and activate the p110 catalytic subunits [5]. The potential importance of Ras-mediated regulation of PI3K was recently reinforced in Downward's laboratory using a murine model to show that Ras-driven tumorigenesis requires the Ras-binding site on p110α [6]. The less-well-characterized p110γ isoform of PI3K associated with a regulatory subunit known as p101, and is activated in response to G-protein-coupled receptor (GPCR) stimulation [7, 8]. To date, the exact molecular mechanism involved in regulation by the p101 subunit is not fully defined, but it has been shown to have sites of interaction with βγ subunits of the trimeric G-proteins[9]. On the other hand, earlier studies suggested that, in vitro, the βγ subunits may activate p110γ directly [10]. Knockout studies showed that the function of p110γ was most important for T-cells and neutrophils and was essential for the GPCR-mediated generation of PtdIns (3,4,5)P3 in these cells [11-13]. The p110α and p110β forms of PI3K are essential for normal mammalian development, as well as p85α [14]. Interestingly, loss of the p85β subunit has a milder phenotype, resulting in increased sensitivity to the insulin receptor. Therefore, although there are unique roles for the various isoforms of PI3K, it has also become clear that this family plays a very important role in normal mammalian development. Key functions for the various PI3K enzymes have been demonstrated in numerous model systems, utilizing cellular responses to different agonists. In fact, virtually every hormone and growth factor investigated has been shown to have effects on PI3K activity. There are two major lipid products of class I PI3Ks: PtdIns(3,4,5)P3 which results from phosphorylation of PtdIns(4,5)P2, and PtdIns(3,4)P2 that is a product of 5-phosphatases, such as SH2-containing inositol phosphatase-1 (SHIP-1) or SHIP-2, removing one phosphate residue from the 5 position on the inositol ring of PtdIns(3,4,5)P3[15]. The mechanism by which the lipids mediate their cellular effects was clarified by the identification of pleckstrin

homology (PH) domains in a variety of proteins as docking modules for these lipids[16]. Thus, the lipid products of PI3K can be considered as second messengers, representing the internal chemical signal that is generated in response to an external signal. These lipids transmit information thus orchestrating the recruitment of cytosolic proteins to the plasma membrane where they can dock to the poly phosphorylated lipids and are activated. 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 [17].

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 amino acid availability and the regulation of autophagy in response to cellular stress, indicating the importance of class III PI3K in controlling cell growth and survival [18]. The most extensively investigated PI3Ks are class I, especially class IA PI3Ks. Figure 2 reports the scheme of the PI3K classes and their modular structure.

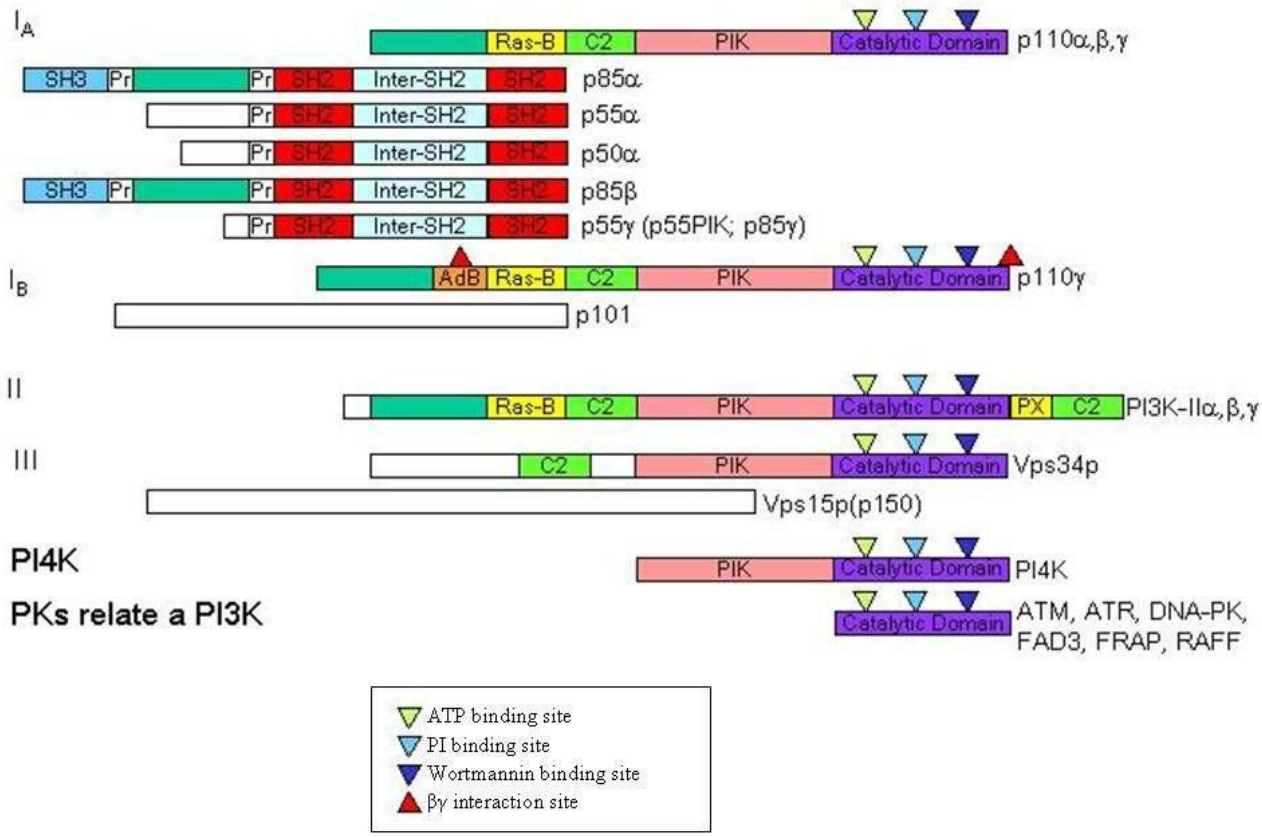


Figure 2. Scheme of the PI3K classes and modular structure.

1.1.2 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 [19] plus a fourth isoform defined Akt-γ1, have been identified in humans (Fig. 3). Different genes with 80% sequence homology codify them. 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 phospholipids; the central kinase domain, which contains a 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 bind to PtdIns 3, 4,5P3 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 [20]. 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 the 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 the cytoplasm to the nucleus, where signaling events appear independent from those on the plasmatic membrane [21]. Akt phosphorylates a plethora of targets [22-24] on R-X-R-X-X-S/T consensus motifs [25]. 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 [26]. 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 [27].

Thus, the heterogeneity of proteins potentially phosphorylated by Akt supports the key role of this kinase in different fundamental cell processes.

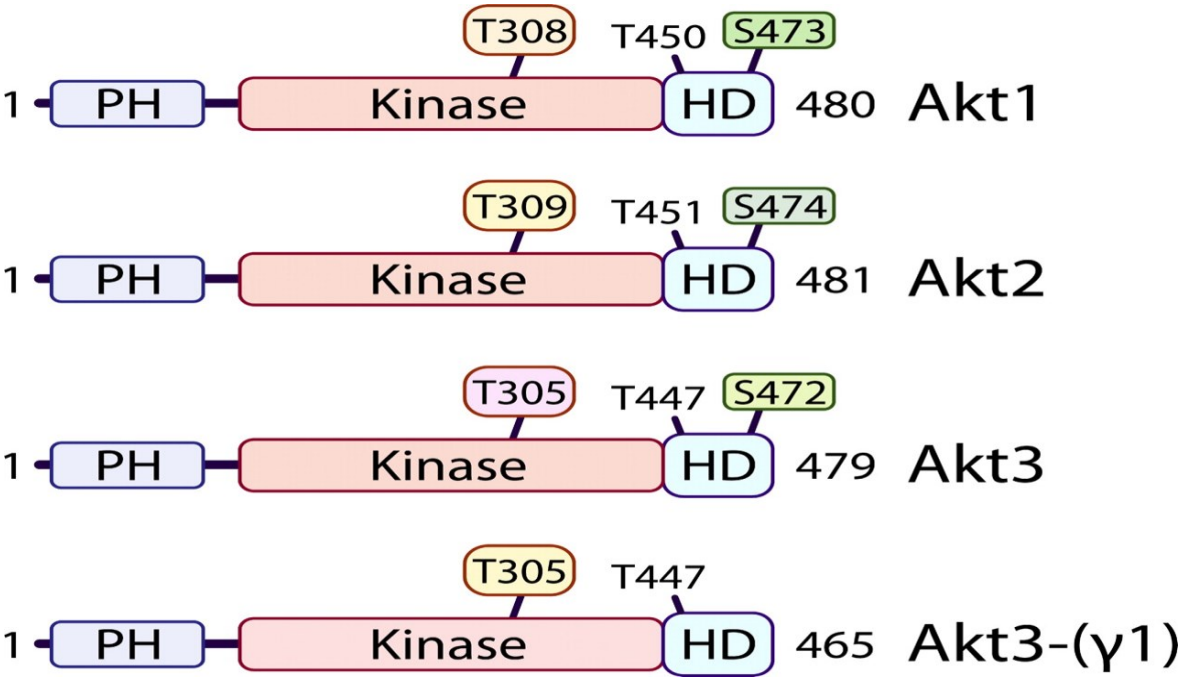


Figure 3. Scheme of the Akt isoforms structure.

1.1.3 mTOR

mTOR is a 289-kDa serine/threonine kinase which belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family[28]. 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 (MEK)/extracellular signal-regulated kinase (ERK) 1/2 signaling network, and the liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) cascade [29, 30].

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 [31, 32]. 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 [32]. 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 [33, 34].

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[35]. Furthermore, p70S6K phosphorylates the eukaryotic initiation factor 4B (eIF4B) which is critically involved in translation [36, 37]. However, eIF4B is a downstream target also of MEK/ERK signaling [38]. 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[39]. 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[33, 39, 40].

Moreover, mTORC1 represses autophagy, a lysosome-dependent degradation pathway that allows cells to recycle damaged or superfluous cytoplasmic content, such as proteins, lipids, and organelles [41]. Consequently, cells produce metabolic precursors for macromolecular biosynthesis or ATP generation. In cancer cells, autophagy fulfills 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 [42].

The mechanisms that control mTORC2 activity have only begun to be revealed [43], however, mTORC2 activation by growth factors requires PI3K, as pharmacological inhibition of PI3K decreased mTORC2 activity *in vitro* [44]. 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 [33]. 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 [45, 46]. 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* [47]. 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[48, 49]. 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 [50]. Protein phosphatase 2A (PP2A) downregulates Akt activity directly, by dephosphorylating it at Thr 308 and several lines of evidence indicate that PP2A is a tumor suppressor [51]. 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 [52, 53].

1.2 Downstream targets of PI3K/Akt/mTOR network

1.2.1 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 [54]. 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 [55].

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 [56-61]. GSK3 has been implicated to play roles in cancers, which are resistant to chemo-, radio-, and targeted therapy [62]. Targeting GSK3 may be a means to overcome the resistance of these cancers to certain chemotherapeutic drugs, radiation and small molecule inhibitors [63-65].

1.2.2 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 [66]. 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 tumor metastasis[67, 68]. 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 effector proteins [69, 70]. Akt that promotes their export from the nucleus abolishing FOXO-dependent gene transcription, thus ensuring that FOXO activity is suppressed [71], directly phosphorylates the FOXO transcription factors. 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 is considered to be among the most promising strategies in drug development for cancer therapy [72].

1.2.3 S6K

mTORC1 controls the hydrophobic motif of p70 ribosomal S6 kinase[31, 73]. 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 [74]. 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 [75].

p70S6K is probably one of the best characterized downstream effectors of mTORC1 [76]. 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 [77].

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[78]. The best-characterized sites are Thr 229 in the activation loop and Thr 389 in a conserved hydrophobic motif [77]. 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 [36, 79]. More recently, it has been found that Ser 371, which resides within a turn motif, is essential for Thr 389 phosphorylation and S6K1 activity [77, 79]. However, it remains unclear how S371 phosphorylation is regulated. One report suggested that this site is also regulated by mTOR [80], 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.

1.2.4 4EBP1

Protein synthesis is controlled primarily at the step of mRNA translation initiation [81]. 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 [82].

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 [36]. 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 [83]. 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 [36, 82, 84]. 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 [85].

1.2.5 The antagonist: PTEN-mediated inhibition of the pathway

The PI3K/Akt activity is negatively modulated by the Phosphatase and Tensin homolog detected on chromosome 10 (PTEN) and SH2 Inositol 5-Phosphatase (SHIP) inhibitors. PTEN is a 3'-phosphatase that 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 [18]. 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. 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 [86]. Similarly, inactivation of Akt by dominant-negative mutants inhibits the survival advantage provided by activated class I PI3K [87]. These and other results point out the essential role of PTEN in modulating and turning off the PI3K/Akt network [88-92].

1.3 PI3K/Akt/mTOR and cancer

The PIK3CA gene, encoding for the p110α catalytic subunit of PI3K, is amplified in different tumors [93, 94]. PI3Kα mutations are found in more than 30% of solid tumors including colorectal, gastric, pancreatic, esophageal, breast, certain brain tumors, ovarian, cervix, thyroid, lung, and hepatocellular carcinomas[68, 89, 95-104].

The gain of function of PI3K mutations has been frequently observed in many human cancers, in particular in ovarian, breast, gastric, and hepatocellular carcinoma [105]. An increased copy number of PIK3CA was observed in approximately 40% of ovarian cancer occurrences [93]. PIK3CA is also found to be amplified and over-expressed in several other types of cancers including cervical, gastric, ovarian, and breast cancers through a large-scale mutational analysis [106-110]. In addition, somatic mutations of PIK3CA were found in 25% to 32% of colorectal cancers, 27% of glioblastomas, 25% of gastric cancers, 3% to 8% of breast, and 4% of lung cancer [111]. The PIK3CA mutations were also found in ovarian, hepatocellular, thyroid, endometrial cancers, and acute leukemia, as well as in malignancies of the central nervous system [97, 111-114]. These mutations of PIK3CA constitutively increased PI3K activities in the cells. PI3K regulatory subunit p85 dimerizes with catalytic subunit p110 and inhibits PI3K activity in normal cells. The deletion and somatic mutations of the p85α regulatory subunit (PIK3R1) were found in primary human glioblastoma, colon, and ovarian cancers. The deletion of p85 protein that lacks the inhibitory domain, and loss of the autophosphorylation site at the p85 inhibitory domain, commonly increase PI3K activity [115, 116]. The mutations of PI3KCA in human cancers are summarized in Table 1.

PI3Kα mutations are mainly localized in two hot-spots: the helical and the catalytic domain[111]. In the helical domain, residues Glu542 and Glu545 are often mutated to lysine, whereas His1047 residue localized in the kinase domain is changed to arginine. A rationale for the molecular mechanism on how the above mutations activate PI3Kα has been provided on the basis of the structural resolution of a co-crystallized p85 inter-SH2 domain interacting with the N-terminus of the PI3Kα catalytic subunit[117]. The three PI3Kα mutant proteins described above

(Glu542Lys-PI3K, Glu545Lys-PI3K and His1047Arg-PI3K) display elevated lipid kinase activity as compared to wild-type PI3Kα and have the potential to transform chicken embryo fibroblasts and NIH3T3 cells. Moreover, PIK3CA mutant cells have an increased migratory and invasive capacity in vitro and in vivo. Altogether, these data suggest that expression of a constitutively active form of PI3Kα allows cells to survive and even migrate in suboptimal environmental conditions and that PI3Kα contributes to tumor formation and metastasis. Thus, the importance of PI3K in cell transformation and cancer occurrence is now well established [118-120] and is supported by the key role of PTEN, the PI3K antagonist, that acts as tumor-suppressor gene [121, 122].

PI3K can be activated by receptor protein tyrosine kinases (RTKs) in response to growth factors. RTKs include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), insulin-like growth factor 1 receptor (IGF-1R), interleukin receptors, vascular endothelial growth factor receptor (VEGFR), interferon receptors, and integrin receptors. RTKs interact with the p85 regulatory subunit of PI3K while Ras protein directly interacts with the p110 catalytic subunit of PI3K in a GTP-dependent manner. Activated receptors interact with p85 Src homology 2 (SH2) domains, and localize PI3K to the plasma membrane. In addition to RTKs, intracellular proteins such as protein kinase C (PKC), SHP1, Rac, Rho, and Src can also activate PI3K in the cells [72]. Upon activation, PI3K phosphorylates the D3 hydroxyl of PI(4,5)P2 to produce PI(3,4,5)P3 as a second messenger which activates these downstream targets with lipid-binding domains at the membrane [18, 72, 123, 124]. In addition, the mutations of PI3K upstream elements such as the epidermal growth factor (EGFR), ErbB2, and IGF-1R, as shown in Table 1, can also increase PI3K activity in various cancer cell types [125-129].

Table 1. Alterations of the PI3K/Akt network in cancer.

GENE ALTERATION	CANCER
PIK3CA amplification	Ovarian, uterine cervical, breast, gastric, thyroid, lung cancers, oral, head and neck squamous cell carcinoma.
PIK3CA mutations	Colorectal, gastric, breast, lung, ovarian, hepatocellular, thyroid, endometrial cancers, glioblastomas, nasopharyngeal carcinoma, acute leukemia and other nervous system malignancies.
Akt1 amplification	Glioblastoma, gliosarcoma, gastric cancer.
Akt2 amplification	Head and neck squamous cell carcinoma, pancreatic, ovarian, breast cancers.
Increased Akt3 mRNA	Breast, prostate cancers.
PTEN mutation and loss	Head and neck squamous cell carcinoma, glioblastoma, gastric, breast, prostate, ovarian, lung, kidney cancers, uterine endometrioid, cervical carcinomas, astrocytoma, melanoma.
EGFR, ErbB2, IGF-1R, Ras	Glioblastoma, gliosarcoma, lung, ovarian, prostate, cervical, endometrial, liver, breast cancers, esophageal and Barrett's adenocarcinomas.

1.4 The acute lymphoblastic leukemia

1.4.1 Clinical Presentation

Normal T-cell development is a strictly regulated, multistep process in which hematopoietic progenitor cells differentiate into functionally diverse T-lymphocyte subsets after their migration into the thymus microenvironment. Diverse transcriptional regulatory networks and transitions between epigenetic states in response to cytokine receptor activation orchestrate the different checkpoints covering thymic colonization lineage commitment and definitive differentiation. During this fine-tuned developmental process, inappropriate activation of T-cell acute lymphoblastic leukemia (T-ALL) oncogenes and loss of tumor suppressor gene activity will coordinately push thymic precursors into uncontrolled clonal expansion and cause T-ALL[130-132].

T-cell acute lymphoblastic leukemia (T-ALL) is one of the aggressive hematologic malignant disorders of lymphoid progenitor (Fig.4), which is characterized by the accumulation of immature undifferentiated thymocytes that acquired multiple genetic aberrations and overproduction of immune white blood cells [133]. 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. The symptoms of ALL are indicative of a reduced production of functional blood cells because leukemia wastes the resources of the bone marrow that are normally used to produce new functioning blood cells. These symptoms can include fever, increased risk of infection (especially bacterial infections like pneumonia, due to neutropenia; symptoms of such an infection include shortness of breath, chest pain, cough, vomiting, changes in bowel or bladder habits), increased tendency to bleed (due to thrombocytopenia), and signs indicative of anemia, including pallor, tachycardia (high heart rate), fatigue, and headache [134, 135].

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 [136, 137]. Both oncogenes and tumor suppressor

genes, cooperate to alter the normal signaling pathways that regulate proliferation, differentiation, and survival of developing T-cells, this process known as T-cell neoplastic transformation[138-141].

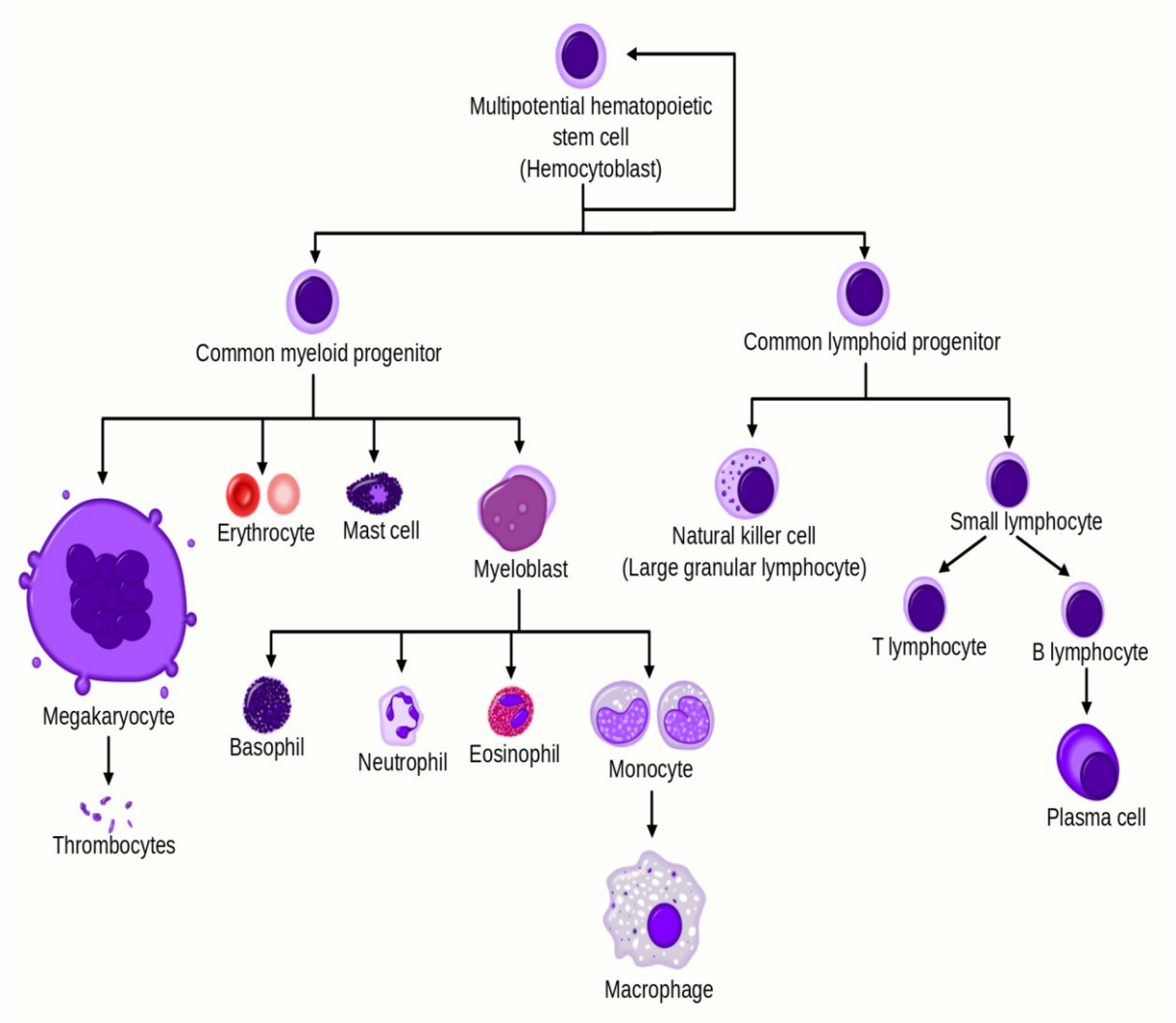


Figure 4. Scheme of myeloid and lymphoid cells maturation

1.4.2 Etiology

Some possible risk factors are genetic syndromes (Down, Noonan, trisomy 9), high birth weight (> 3.5 kg), previous abortion, maternal behavior (use of antihistamine, metronidazole, dipyrone, estrogen, alcohol consumption, use of marijuana and hallucinogenic drugs, radiation, and exposure to insecticides and pesticides). ALL, like cancer in general, is likely to arise from interactions between exogenous or endogenous exposures, genetic (inherited) susceptibility, and chance. These factors account for the approximately 1 in 2000 risk of childhood (0–15 years) ALL. The challenge is to identify the relevant exposures and inherited genetic variants and to decipher how and when they contribute to the multi-step natural history of ALL from its initiation (usually in utero) through its largely covert evolution to overt disease [142]. Exogenous and endogenous factors like (infection, inflammation and oxidative stress exposures respectively), normal allelic variation in inherited genes and chance all play roles in the covert natural history of childhood ALL leading ultimately to overt disease and clinical diagnosis. Cancer causation is riddled with a chance, for example, incidental “external” exposure, incidental damage to a relevant oncogene in a relevant cell (stem or progenitor cell) and chance events at conception involving parental gene shuffling and recombination (Figure 5). [142-144]

1.4.3 Diagnosis

Morphological identification of lymphoblasts by microscopy and immunophenotypic determination of lineage commitment and developmental stage by flow cytometry are essential for correct diagnosis of ALL. Chromosomal analysis still plays an important role in the initial cytogenetic work-up. RT-PCR, FISH/multiplex ligation-dependent probe amplification, and flow cytometry are used to identify leukaemia-specific translocations, submicroscopic chromosomal abnormalities, and cellular DNA content, respectively. After genome-wide analysis becomes time- and cost-effective, it may replace many current diagnostic techniques [145].

1.4.4 Treatment

Treatment of ALL typically spans 2–2.5 years, comprising 3 phases: remission induction, intensification (or consolidation) and continuation (or maintenance). Most of the drugs used were developed before 1970. However, their dosage and schedule of administration in combination chemotherapy have been optimized, and based on leukemic-cell biological features, response to therapy (MRD), patient pharmacodynamic and pharmacogenomics findings which are resulting in the current high survival rate. Central nervous system (CNS)-directed therapy is administered to prevent relapse caused by leukemia cells sequestered in this sanctuary site. Allogeneic hematopoietic stem-cell transplantation is considered for patients at very high risk [145].

Approximately 10% of all cancers affecting children under 15 years correspond to those diagnosed in the first year of life. Leukemia is the second most common cancer in children under 1 year of age, and acute lymphoblastic leukemia (ALL) the most frequently observed type [146]. Moreover, 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 the survival rates at 5 years for children is 76-86% whereas the rate for adults below 60 years old is 35-40%, and 10% above this age[145, 147, 148]. However, these therapies are with a high efficiency and toxicity and patients who get relapse will be susceptible to the development of extreme resistance to chemotherapy and gain a poor prognosis[149].

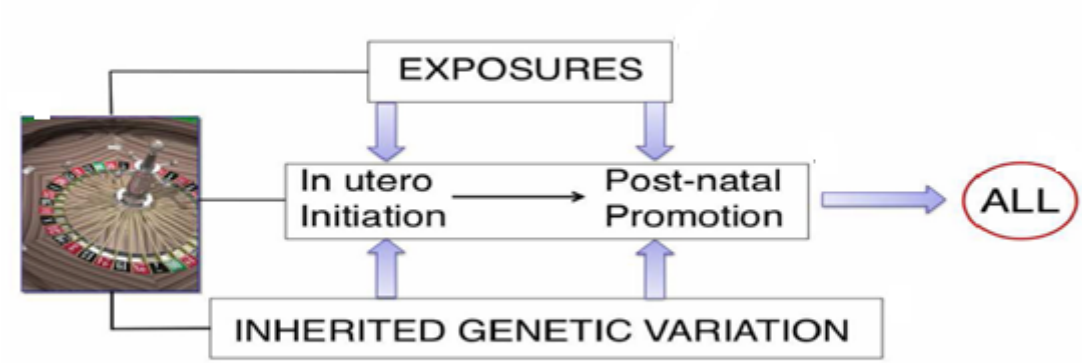


Figure 5. Composite causality of childhood ALL

1.4.5 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 [150], 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 documented that PTEN gene expression was inactivated in T-ALL cell lines and patients displayed Notch-1 activating mutations, through a repressive mechanism mediated by Hairy and Enhancer of Split homolog-1 (HES-1) [151-153]. In T-ALL cell lines, PTEN loss correlated with resistance to Notch inhibitors, raising concerns that patients with the PTEN-negative disease could not respond to Notch inhibitor therapy [152]. However, it has been subsequently demonstrated that PTEN loss did not relieve primary T-ALL cells of their “addiction” to Notch-1 signaling [154]. 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 [155]. Furthermore, in a zebrafish model of T-ALL, c-Myc, which is typically overexpressed downstream of activated Notch-1 in T-ALL [156], caused PTEN mRNA down-regulation [157].

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 [150].

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 [158, 159].

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 [160]. 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 [160]. 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 [161], and IL-7 [162, 163]. 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 [164]. A source for IL-7 could be represented also by thymic epithelial cells [165]. 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 [166].

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) [167]. CXCL12 is produced by bone marrow stromal cells in T-ALL patients [168] and has been recently demonstrated to be involved in PI3K/Akt activation and drug-resistance in T-ALL cells [169].

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 [162]. 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 [170].

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

1.5.1 Advances in targeting the PI3K/Akt/mTOR pathway

The PI3K/Akt/mTOR pathway is also involved in drug resistance, sensitivity to therapy and metastasis [29, 171-179]. PIK3CA mutations may act as driver mutations in certain cancers responsible for metastasis [180]. Novel PI3K-alpha inhibitors have been isolated and they inhibit metastasis [181]. Most PI3K inhibitors are cytostatic rather than cytotoxic and it has been questioned whether treatment with a single PI3K inhibitor will be effective [182].

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 [183].

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 [184]. 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.

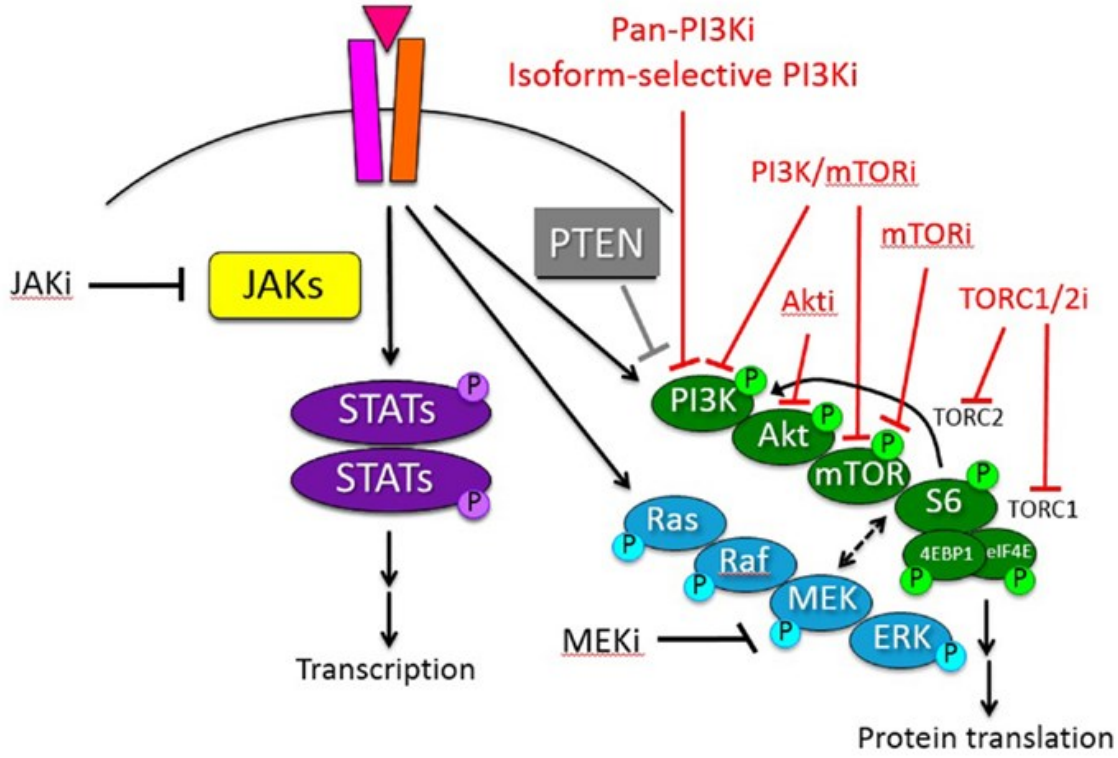


Figure 6. Schema of PI3K/Akt/mTOR signaling pathway inhibitors (i) in hematologic malignancies.

1.5.2 PI3k Inhibitors

PI3K inhibition may represent a potential therapeutic strategy for cancer treatment. Recent study shows that inhibition of PI3K suppresses angiogenesis and tumor growth [185, 186]. Several therapeutic strategies targeting PI3K pathway are now in development. In a number of pre-clinical studies, the use of specific PI3K pharmacologic inhibitors such as LY294002 and wortmannin, and natural compounds with PI3K inhibitory capacities such as resveratrol, increased apoptosis and arrested the cell cycle in T-ALL cells [187, 188]. PI3K inhibition is also important to abolish chemo-resistance to drugs used in current therapeutic regimens or that are being tested in clinical trials [152]. The PI3K inhibitors wortmannin and LY294002 are commonly used to inhibit cancer cell proliferation and tumor growth, and sensitize tumor cells to the treatment of chemotherapeutic

drugs and radiation. Unlucky, the poor solubility and high toxicity of these inhibitors limit the clinical application.

NVP-BGT226 8-(6-methoxypyridin-3-yl)-3-methyl-1-(4-(3-(trifluoromethyl) piperazin-1-yl) phenyl)-1H-imidazol [4,5-c]quinolin-2(3H)-one) (Fig.7), one of an imidazoquinoline derivative. An ATP-competitive dual PI3K/mTORC1/C2 inhibitor it is a potent pan-class I PI3K inhibitor (p110a, ß, & d with a preference for the a-isoform -wild type and mutated-) and is an mTORC1/2 catalytic inhibitor [189, 190].In cellular assays, it could produce nearly complete inhibition of PI3K signaling at low nanomolar concentrations. Flow cytometric analysis revealed an accumulation of cells in the G0–G1 phase with a concomitant loss in the S-phase. TUNEL assay and the analysis of Caspase 3/7 and PARP indicated that BGT226 induced cancer cell death through an apoptosis-independent pathway. BGT226 induced autophagy as indicated by the aggregation and upregulation of the microtubule-associated protein light chain 3B-II, and p62 degradation. It is in phase I/II clinical trials for the treatment of advanced solid tumors [191-194].

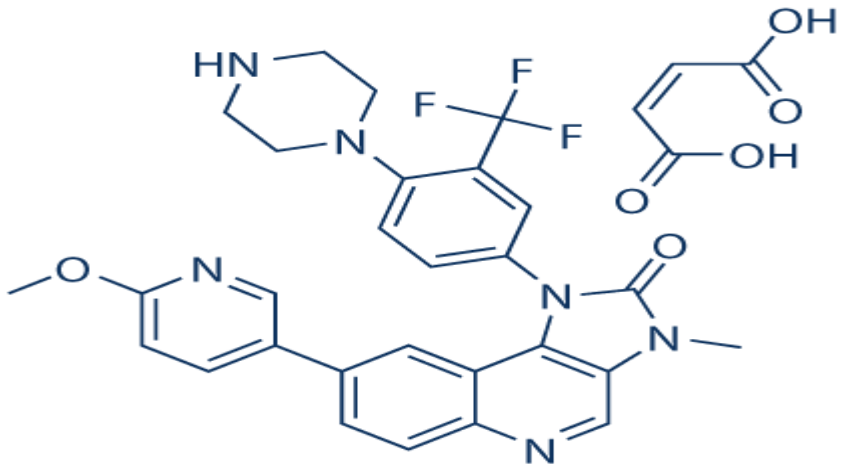


Figure 7. Chemical structure of NVP-BGT226.

A novel s-triazine derivative, ZSTK474 [2-(2-difluoromethylbenzimidazol-1-yl)-4,6-dimorpholino-1,3,5-triazine], strongly inhibited the growth of tumor cells (Fig.8). A molecular target for ZSTK474 is PI3K. ZSTK474 directly inhibits PI3K activity more efficiently than the PI3K inhibitor LY294002. At concentrations of 1μM, ZSTK474 and LY2194002 reduced PI3K activity to 4.7% and 44.6% respectively, of the

untreated control level. Molecular modeling of the PI3K-ZSTK474 complex indicated that ZSTK474 could bind to the ATP-binding pocket of PI3K. ZSTK474 inhibited phosphorylation of signaling components downstream from PI3K, such as Akt and glycogen synthase kinase 3β, and mediated a decrease in cyclin D1 levels. ZSTK474 administered orally to mice had strong antitumor activity against human cancer xenografts without toxic effects in critical organs. Akt phosphorylation was reduced in xenograft tumors after oral administration of ZSTK474. ZSTK474 is a new PI3K inhibitor with strong antitumor activity against human cancer xenografts, without toxic effects in critical organs [195-197].

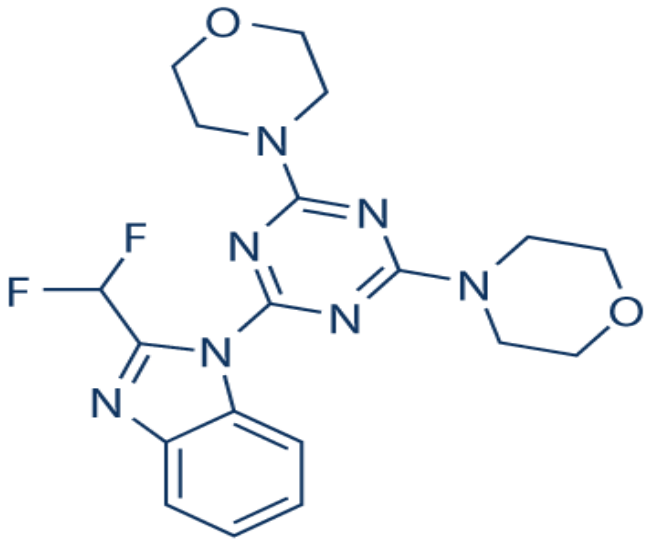


Figure 8. Chemical structure of ZSTK-474

1.5.3. Akt Inhibitors

Akt inhibition may represent a potential therapeutic strategy in acute lymphoblastic leukemia. Many attempts to develop Akt inhibitors 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. 9). 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 G0/G1 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 [198]. 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.

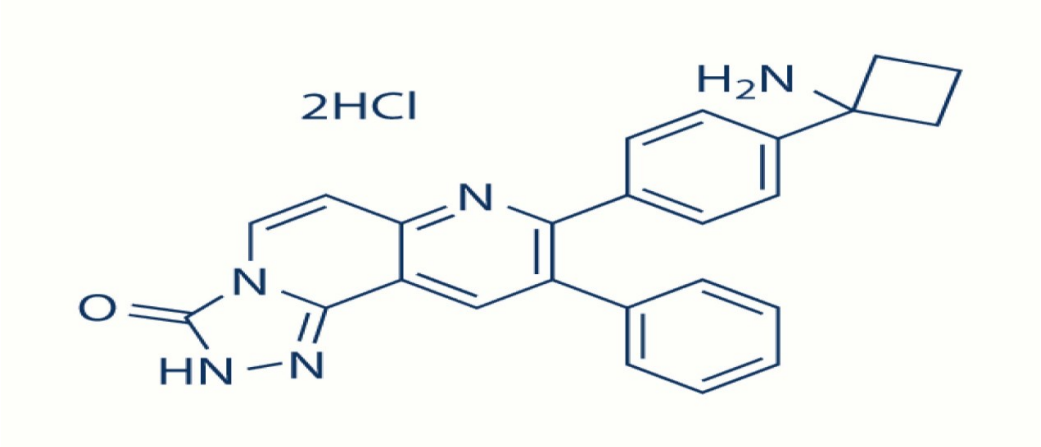


Figure 9. Chemical structure of MK-2206

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. 10). 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 [199]. 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.

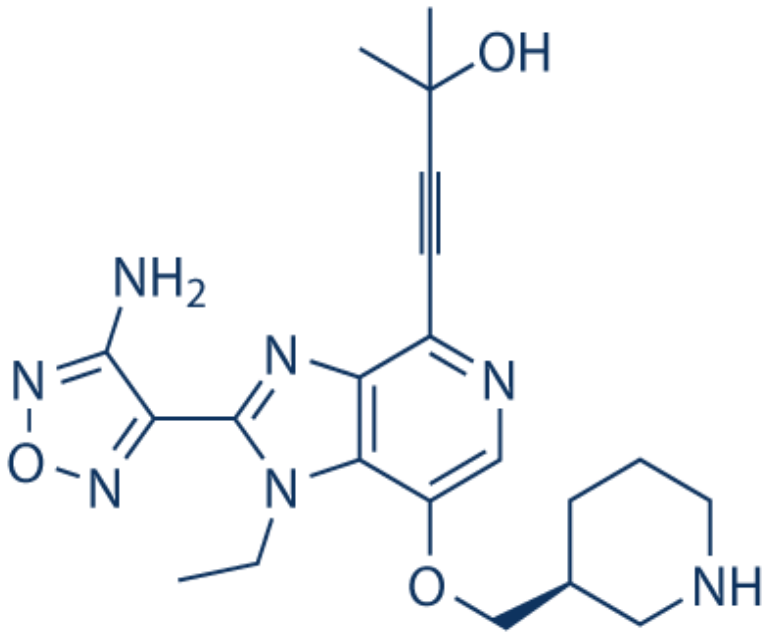


Figure 10. Chemical structure of GSK690693.

Alkylphospholipids and alkyl phosphocholines (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 alkyl phospholipid (Fig. 11) which inhibits the translocation of Akt to

the cell membrane, blocking the growth of several different human cancers [200]. 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 for many different tumor types. Perifosine induces caspase-dependent apoptosis and downregulates P-glycoprotein expression in multidrug resistant T-ALL cells by a JNK-dependent mechanism [201]. 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.

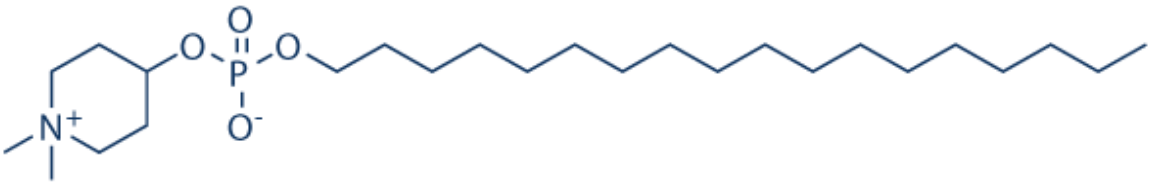


Figure 11. Chemical structure of Perifosine.

1.5.4 mTOR Inhibitors

The mTORC1/mTORC2 dual inhibitors (Fig. 12) are the second generation of mTOR inhibitors designed to compete with ATP for 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 [202, 203]. 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 [204]. 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 in human cancer. In fact, they have been proven to be more potent than rapalogs [203].

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. 12). It potently targets 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 [205].

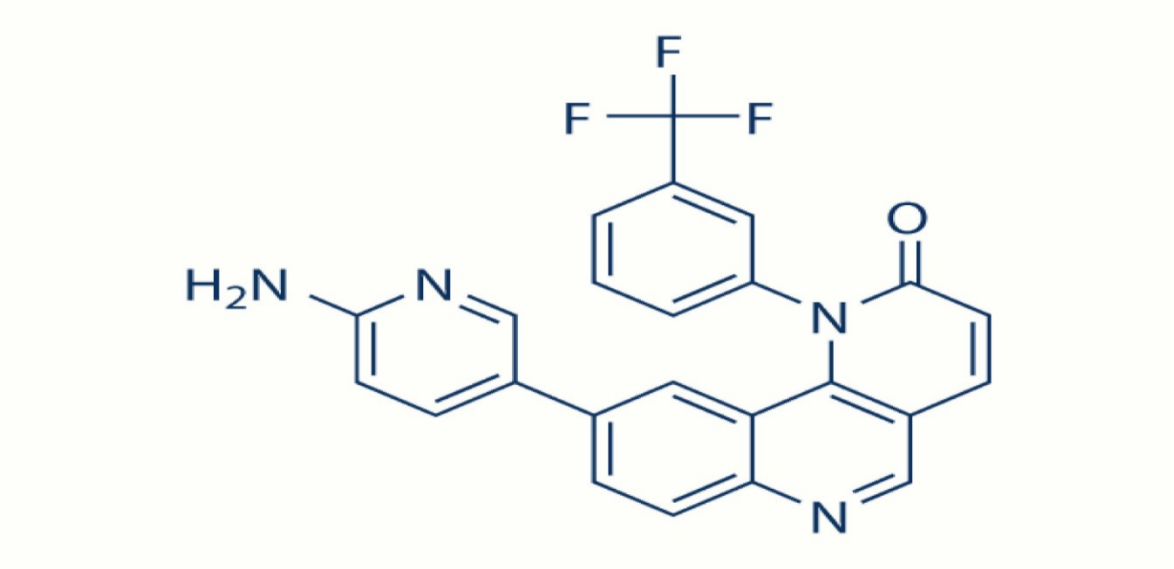


Figure 12. Chemical structure of Torin-2.

2. Aim of the Study

Since, 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, investigation of small molecule inhibitors of this complex signaling network is an active area of oncology drug development. It is very plausible that the oncogenic signature of some acute leukemia cases embraces 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. Hence, this research aimed to investigate the effect of PI3K/Akt/mTOR signaling pathway inhibitors in acute lymphoblastic leukemia cells and primary T-lymphocytes in both status (quiescent and mitogenically activated). In particular, we wanted to verify the use of specific inhibitory compounds directed against key proteins of this pathway at different points. These pharmacological strategies allow inhibition of the PI3K/Akt/mTOR pathway and could represent a new promising and innovative therapeutic treatments in acute lymphoblastic leukemia.

3. Materials and Methods

3.1 Materials

RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Lonza Milano SRL (Milan, Italy). Torin-2, NVP-BGT226, MK2206, and ZSTK474 were obtained from Selleck Chemicals (Houston, TX, USA). For cell viability determination, Cell Proliferation Kit I (MTT) was purchased from Roche Applied Science (Basel, Switzerland). Annexin V/7-ADD detection kit and cell cycle kits were from Merck-Millipore (Darmstadt, Germany). For western blot antibodies for total Akt-1, Ser473 p-Akt-1 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, Z-VAD-FMK, 3-Methyladenine (3- MA), Ficoll-Paque Plus and phytohemagglutinin were purchased from Sigma-Aldrich (Milan, Italy). Dyna beads T-cell separation kit was from Invitrogen life Technologies (Monza MB, Italy). Signals were detected using ECL Plus reagent from Perkin Elmer (Boston, MA, USA).

3.2 Cell lines

T-acute lymphoblastic leukemia cell lines obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). JURKAT and MOLT-4 were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin at a density of 0.5 to 2 x 10^6 cells/ml and incubated at 37ºC with 5% CO_2.

3.3 Primary samples

Peripheral Blood CD4+ T lymphocytes from healthy donors were obtained with informed consent according to institutional guidelines, isolated with Ficoll-Paque and magnetic beads labeling protocols (Dynabeads, Monza MB, Italy). Whole blood or buffy coat were diluted with PBS containing 0.1% BSA and 0.6% Sodium

citrate or 2 mM EDTA (without Ca2+ and Mg2+) in ratio 1:1, up to 35 ml of the diluted sample was layered over 15 ml of Ficoll-Paque solution and centrifuged at 600 g for 30-40 minutes at 18-20 ºC. The polymorph nuclear cell (PMNC) layer was transferred to clean centrifuge tube containing, at least, three volumes of balanced salt solution, centrifuged at 60-100 g for 10 minutes at 18-20 ºC, this process repeated two times. The supernatant was discarded and the cells were suspended in the complete RPMI-1640 medium. 500 µl PMNC were transferred into a test tube, at a density of 5 x 10^7cells/ml supplemented with100 µl of heat inactivated FBS and antibody mix and incubated at 2-8 ºC for 20 minutes. Followed by addition of 4 ml isolation buffer and centrifuged at 350 g at 2-8 ºC for 8 minutes. The supernatant discarded, and the pelleted cells were suspended in 500 µl of isolation buffer, added with 500 µl of pre-warmed Dyna beads and incubated for 15 min at 18-25°C, with gentle tilting and rotation. The cells bound to beads were resuspended using 4 ml isolation buffer by thoroughly pipetting (>10 times) and avoid foaming. The human CD4+ T-lymphocytes fished out from the supernatant by placing the resuspended cells in the magnet for 2 minutes and transferred to new tube. The untouched human CD4+ T-lymphocytes were grown in complete RPMI-1640 medium with/without 10µg/ml phytohemagglutinin at a density of 0.5 to 2 x 10^6 cells/ml, incubated in a CO_2 incubator at 37ºC for 24 h[206].

3.4 MTT

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

MTT (3-(4,5-dimethylthythiazol-2-yl)-2,5-diphenyltetrazolium bromide) is cleaved to formazan by enzymes of the endoplasmic reticulum. This intracellular bioreduction in viable cells is linked to NADPH 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. The cells cultured in triplicate at a density of 5 x 10^4 cells/ml in flat-bottomed 96-well plates were treated with different drugs at scalar concentrations and untreated control. The cultures were incubated for 24 and/or 48h at 37°C with 5% CO_2, 10 µl of MTT solution added and incubated with for approximately 4 hours. After incubation, a water-insoluble formazan dye produced and solubilized in another step by addition of 100-µl solubilization buffer followed by an overnight incubation at 37°C. After solubilization, the formazan dye quantified using a scanning multi-well spectrophotometer (ELISA reader) at the wavelength of 550-600 nm. The measured absorbance directly correlates to cells number [207].

3.5 Cell cycle analysis

The cell cycle analysis was performed using the Muse™ Cell Analyzer (Merck Millipore, Milan, Italy) and/or propidium iodide (PI)/RNaseA staining by flow cytometry according to the manufacturer protocol. The cells were harvested after 24h of treatment with the requested drugs, centrifuged at 300 g for 5 minutes and washed with 1X PBS. Then, the cells were fixed with 70% cold ethanol for nearly 3h at -20°C, centrifuged at 300 g for 5 min and washed with 1X PBS. 200 µl of Muse™ Cell Cycle reagent was added to each sample, incubated at room temperature in a dark place for 30 min. Finally analyzed according to the standard protocol. At least 15000 events/sample were acquired

3.6 Western blot

For protein extraction 4 × 10^6 cells were washed twice in PBS and lysed with RIPA buffer (50 mM Hepes pH 7.5, 5 mM EDTA pH 8.0, 10 mM MgCl2 , 150 mM NaCl, 50 mM NaF, 20 mM β-glicerophosphate, 0.5% NP40, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail, Roche Applied Science, (Mannheim, Germany). Samples were incubated for 30 min in ice. Cell extracts placed in a sonicator for 10 minutes, 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 polyacrylamide gel for electrophoresis separation (8% or 15%) and transferred to a nitrocellulose transfer membrane. Membranes were blocked with TBS containing 5% fat-free dry milk and 0.1% Tween-20 for 1h at RT followed by 3 washes using TBS 0.1% Tween-20, incubated at 4°C overnight with the primary polyclonal antibodies (1:1000 dilutions). After three washes with TBS 0.1% Tween-20, samples were incubated for 60 min at RT with secondary antibody after that washed as previously described. Specific horseradish peroxidase- conjugated secondary antibodies (anti-mouse or anti-rabbit) were used. Blots were incubated with mouse anti-ß-actin antibody (Sigma-Aldrich, St Louis, MO, USA) as a loading control. Signals were detected with ECL Plus reagent (Amersham Biosciences; Buckinghamshire, UK) and an Image Quant LAS4000 detection system (GE Healthcare Europe GmbH, Freiburg, Germany) [208].

3.7 PI/Annexin V assay

Analysis of apoptosis or cell death was performed by staining with Annexin V/7-ADD, using Muse™ Cell Analyzer. Cells were harvested after treatment with increasing concentrations of BGT226 or TORIN-2 for 24 h. The cell suspension was labeled in dark for 20 min with an equal volume (100µl) of the Muse™ Annexin-V Dead cell reagent (Merck Millipore). Subsequently, quantitative detection of Annexin-V/7-AAD positive cells was performed using the Muse™ Cell Analyzer. Unstained and single stained controls were included in each experiment.

3.8 Statistical evaluation

The data are presented as mean values from three separate experiments ± SD. Data were statistically analyzed by a Dunnet test after one-way analysis of variance (ANOVA) at a level of significance of $P<0.05$ vs control samples [209].

4. RESULTS

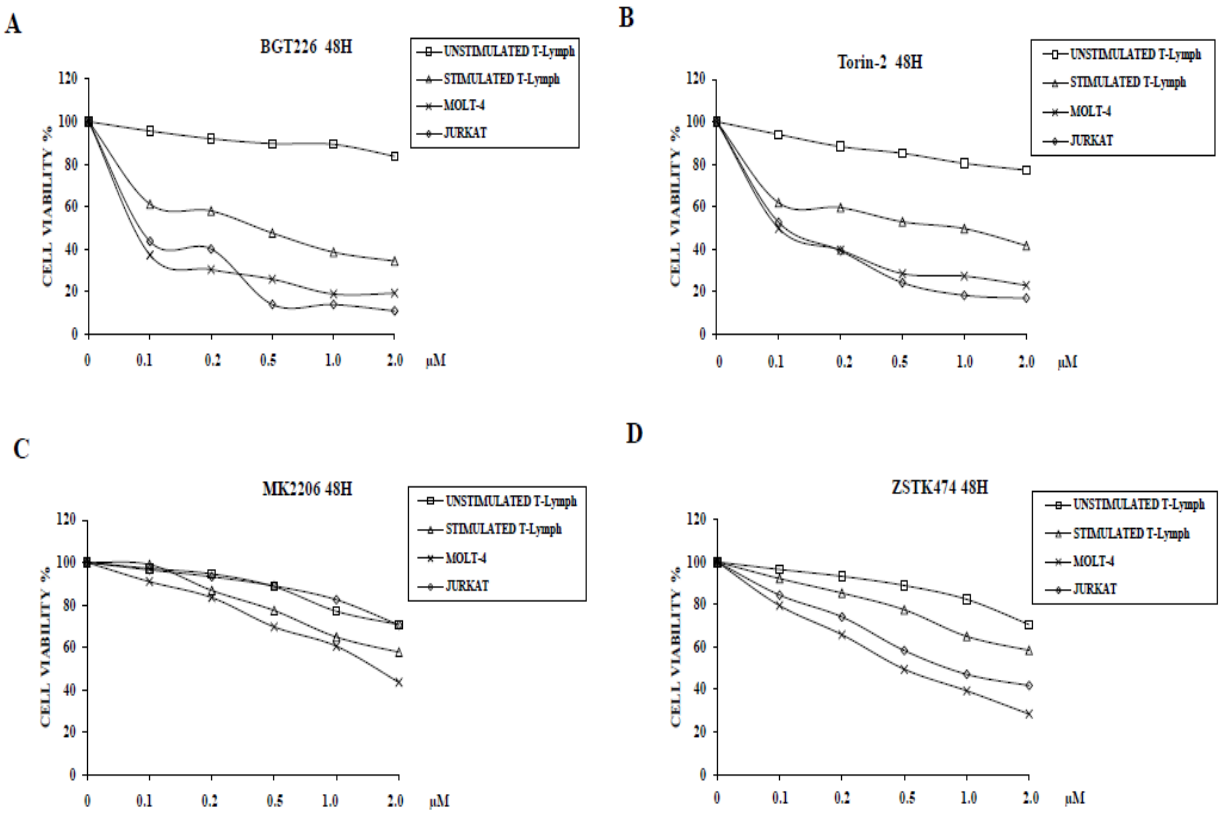
4.1 BGT226, Torin-2, MK-2206 and ZSTK474 display cytotoxic effects on mitogenic stimulated T-lymphocytes and T-ALL cell lines

BGT226 is an ATP-competitive dual PI3K/mTORC1/2 inhibitor used for the treatment of advanced solid tumors [191, 192]. Our group recently published a work reporting an excellent activity of this inhibitor on a panel of hepatocarcinoma cells, in normoxic and hypoxic conditions [210]. Torin-2 potently targets mTORC1/2 and is a potent inhibitor of ATR, ATM and DNA-PK [205].

The efficacy of MK-2206 has been tested in various preclinical models of human cancer, including leukemias [211-213], as well as the pan-PI3K inhibitor ZSTK474 [195].

To determine how these drugs could affect the viability of the primary T-Lymphocytes (Unstimulated & mitogenic stimulated) and T-ALL cell lines (MOLT-4 & JURKAT), MTT assays were performed. Cells were incubated for 48h with increasing concentrations of all the drugs and cell survival was analyzed (Fig. 13). BGT226 and Torin-2 turned out to be the most sensitive drugs in these cell lines, except for the unstimulated T-Lymphocytes that resulted unaffected. For BGT226, cell viability impairment was more evident in T-ALL cell lines, with IC_{50} values of 0.06 μM for MOLT-4, 0.08 μM for JURKAT and 1.61 μM for mitogenic stimulated T-lymphocytes. The same results were obtained after 48h of treatment with Torin-2, with IC_{50} values of 0.1 μM for both JURKAT and MOLT-4 and 1.8 μM for mitogenic stimulated T-cells. For Unstimulated T-Lymphocytes, there was no relevant inhibition with all drugs (IC_{50} > 2 μM).

As it concerns MK-2206, the cells displayed higher values of IC_{50} (> 2 μM) except for MOLT-4 with an IC_{50} of 1.58 μM. For the pan PI3K inhibitor ZSTK474 the same response for the primary T-lymphocytes is reported, while in MOLT-4 and JURKAT the sensitivity of the drug is evident, with IC_{50} values of 0.52 μM and 0.95 μM, respectively (Fig. 13). The same experiments were performed also at 24h (data not shown).



[µM] Lymphocytes + T-ALL cell lines	IC_{50} at 48 h			
	BGT226	Torin-2	MK2206	ZSTK474
UNSTIMULATED T-Lymphocytes	>2.0	>2.0	>2.0	>2.0
STIMULATED T-Lymphocytes	1.61	1.8	>2.0	>2.0
MOLT-4	0.06	0.1	1.58	0.52
JURKAT	0.08	0.1	>2.0	0.95

Figure 13. Cytotoxicity of BGT226, Torin-2, MK-2206 and ZSTK474 in primary T-lymphocytes, MOLT-4 and JURKAT cell lines.

MTT assay of primary T-lymphocytes and T-ALL cell lines treated with increasing concentrations of BGT226, Torin-2, MK2206 and ZSTK474 for 48 h. SD was less than 7%. The concentration of each drug is reported under the graph. One representative experiment of three is shown. IC_{50} values of all drugs at 48 h treatment in primary T-lymphocytes and T-ALL cell lines.

To further, assess the cytotoxicity of the PI3K/Akt/mTOR inhibitors mentioned above, we analyzed the changes in cell viability using the Muse™ Cell Analyzer. Cells were treated with IC_{50} concentration for each drug (BGT226, Torin-2, MK2006, and ZSTK474), after 48 h; staining with DNA binding dye was performed. The percentage of viable and dead cells were calculated according to the manufacturer protocol. BGT226 and Torin-2 documented higher cytotoxic effect on mitogenic stimulated T-Lymphocytes, MOLT-4, and JURKAT than MK2206 and ZSTK474. No drug affected the viability of Unstimulated T-Lymphocytes (Fig. 14).

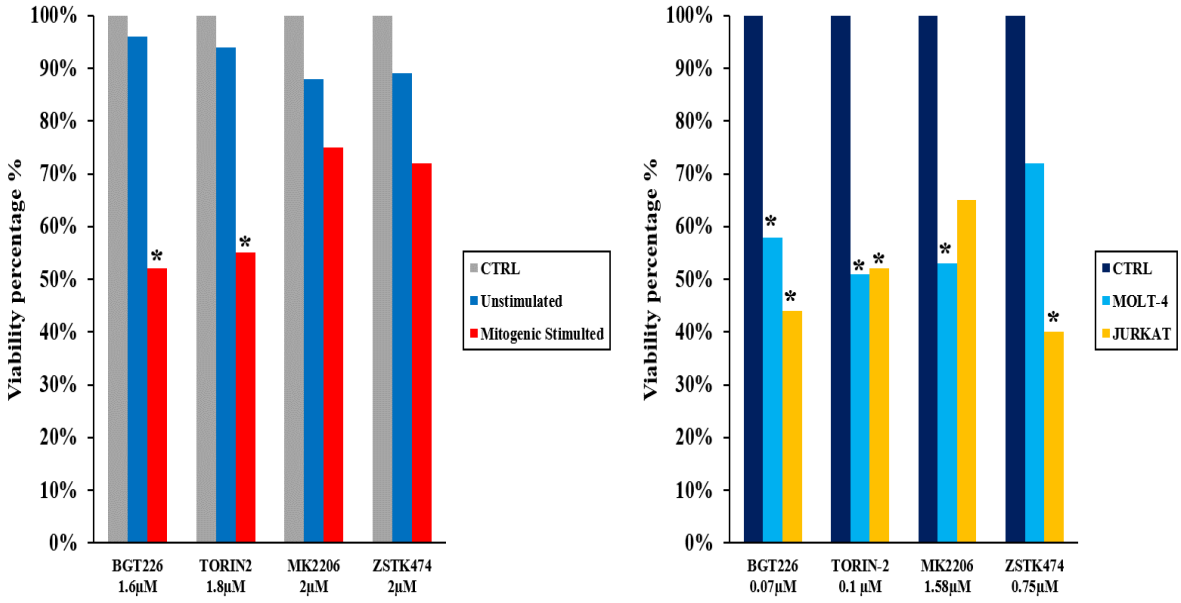


Figure 14. Effects on viability by BGT226, Torin-2, MK-2206 and ZSTK474 in primary T-lymphocytes, MOLT-4, and JURKAT cell lines.

Primary T-lymphocytes and T-ALL cell lines treated with the drugs at IC_{50} concentration for 48 h. SD was less than 10%. Asterisks indicate statistically significant differences with respect to untreated cells (*$p<0.05$).

4.2 BGT226 and Torin-2 induce G0/G1 phase of cell cycle on mitogenic stimulated T-lymphocytes and T-ALL cell lines

Given the importance of the PI3K/Akt/mTOR signaling pathway in the regulation of cell proliferation [214], we also investigated the effects of the drugs on the cell cycle progression of Unstimulated and mitogenic stimulated T-Lymphocytes, and in T-ALL cell lines (MOLT-4 & JURKAT). Cells were treated with BGT226 and with Torin-2 for 24 h, stained with Propidium Iodide (PI) for analysis by Muse™ Cell Analyzer. Of interest, BGT226 produced a shift of cells from G2/M and S-phase to the G0/G1 phase in mitogenic stimulated T-lymphocytes, MOLT4 and JURKAT cell lines in nanomolar concentration (100 nm) indicating dead/apoptotic cells, with a proportion of 90%, 68%, and 71% respectively. In contrast, Torin-2 also leads to accumulation of cells in the G0/G1 phase - with 87% in mitogen-stimulated T-lymphocytes, 60% MOLT4 and 45% in JURKAT cell lines. While in quiescent T-lymphocytes, no effect noticed by both drugs (Fig. 15).

Overall, these findings demonstrated that BGT226 and Torin-2 potently reduced the growth of mitogenic stimulated T-lymphocytes and T-ALL cell lines and this effect was due to G0/G1 cell cycle arrest.

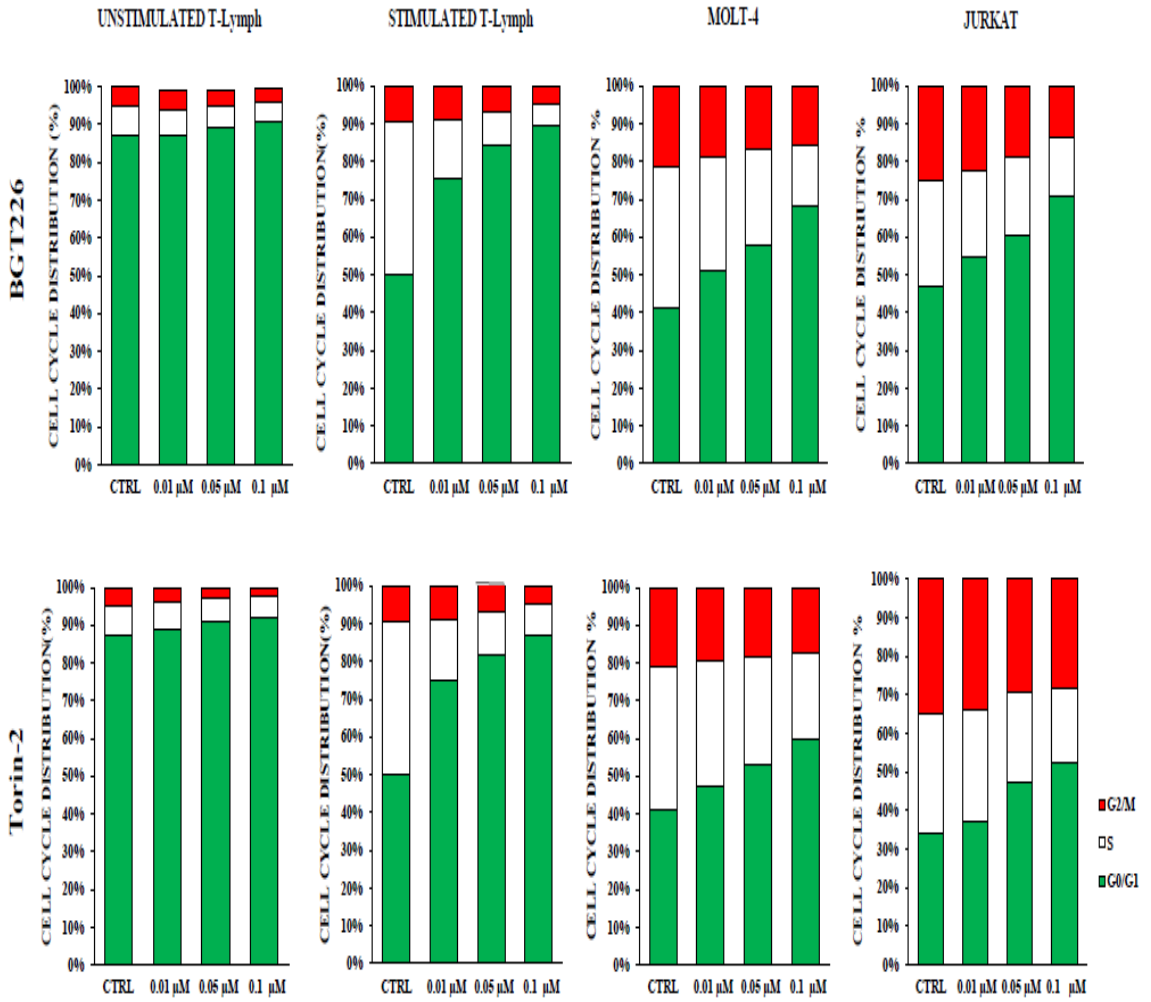


Figure 15. BGT226 and Torin-2 affect cell cycle in primary T-lymphocytes, MOLT-4, and JURKAT cell lines.

Unstimulated, mitogenic stimulated T-Lymphocytes, MOLT-4, and JURKAT cells were treated with increasing concentrations of BGT226 and Torin-2 for 24 h. Both drugs treatment resulted in an increase of cells in G_0/G_1 phase and in a decrease of cells in S and G_2/M phase in all cells. CTRL, control (untreated) cells. SD was less than 10%.

4.3 Activation status of PI3K/AKT/mTOR pathway in T-ALL cell lines and primary T-lymphocytes.

By Western blot analysis, we evaluated the baseline expression of some PI3K/AKT/mTOR pathway key proteins in both Unstimulated, mitogenic stimulated T-lymphocytes, and in T-ALL cell lines (MOLT-4 and JURKAT).

The mitogenic stimulated T-Lymphocytes, MOLT-4, and JURKAT displayed mTOR phosphorylation at Ser 2448 and Ser 2481 residues (readout for mTORC1 and mTORC2, respectively). The phosphorylation was not evident in Unstimulated T-Lymphocytes, whereas the total form of the protein was expressed in all cell types. The mitogenic stimulated T-Lymphocytes, MOLT-4, and JURKAT displayed a relevant phosphorylation on Ser 473 residue of Akt read out of mTORC2 and on Ser 235/236 residue of the ribosomal protein S6 kinase, readout of mTORC1 activity. Total form of Akt and S6 proteins were expressed in all cell types (Fig. 16).

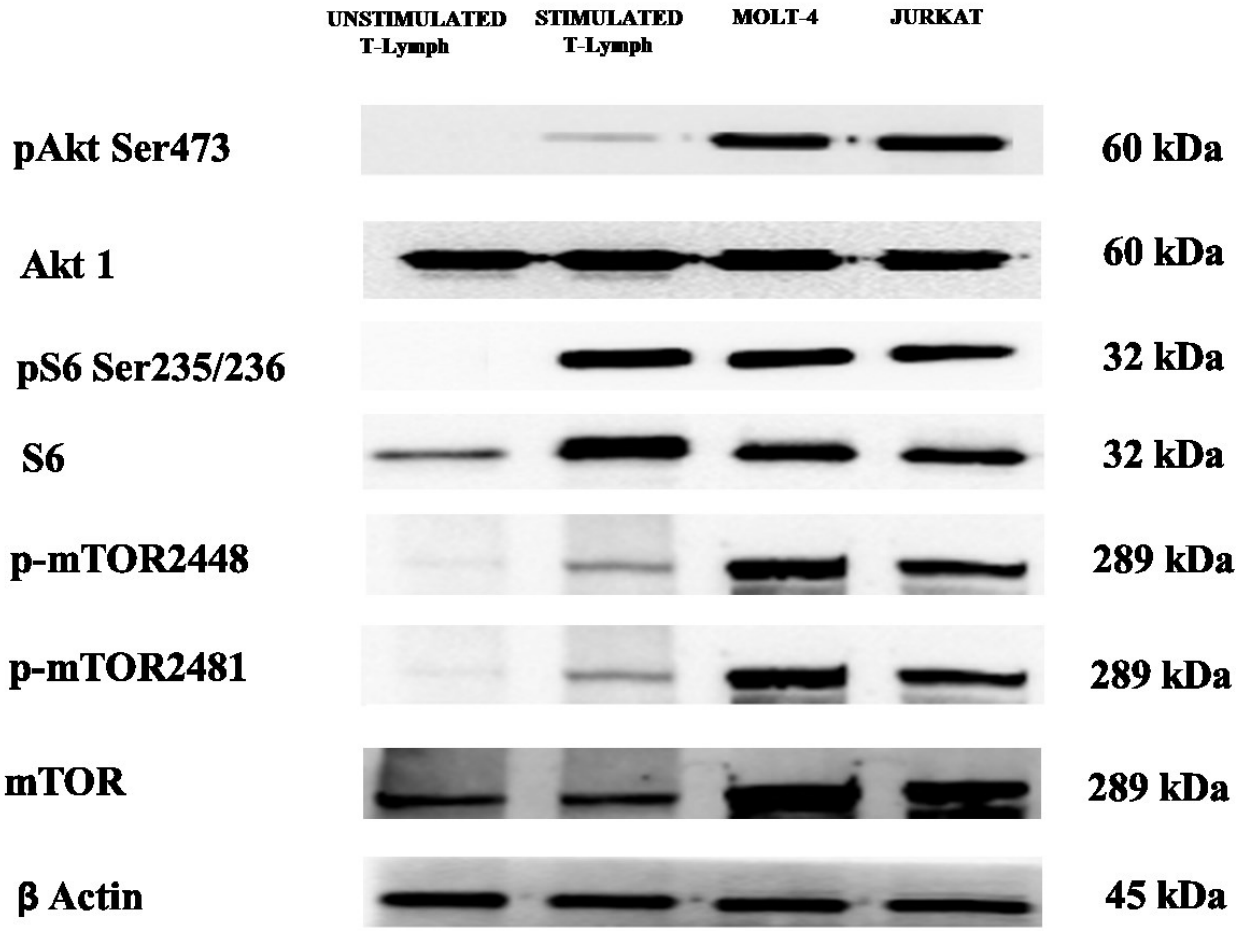


Figure 16. Expression and phosphorylation status of mTOR, Akt, and their downstream targets in primary T-lymphocytes and T-ALL cell lines.

Western blot analysis of primary T-lymphocytes and T-ALL cell lines to detect the expression and phosphorylation levels of Akt, mTOR, and its downstream substrates. Twenty-five μg of protein were blotted on each lane. Antibody to β-actin served as loading control.

4.4 BGT226 and TORIN-2 affect the PI3K/AKT/mTOR pathway in mitogenic stimulated T-lymphocytes and T-ALL cell lines but not in normal lymphocytes.

Different types of cellular stimuli are responsible for the activation of the PI3K cascade. One of them is tyrosine kinase, which plays an important role in PI3K activation [214]. We determined whether BGT226 and Torin-2 could affect the downstream signal transduction factors that promote PI3K/Akt/mTOR-mediated cell survival. Mitogenic stimulated T-lymphocytes, MOLT-4, and JURKAT cells were treated with increasing concentrations of BGT226 and Torin-2 for 2 h, then western blot analysis was performed (Fig. 17). Both drugs decreased the phosphorylation levels of mTOR on both Ser 248 and Ser 2481 residues. The phosphorylation of mTOR on Ser2481 is mTORC2-selective autophosphorylation site [215]. The mTORC1 substrate S6 was completely dephosphorylated on Ser235/236 residue, already at low concentrations of BGT226 and Torin-2. mTORC2 inhibition had a readout in Ser 473 Akt dephosphorylation and it was observable in all cell lines treated with BGT226 and Torin-2 from the lowest concentrations.

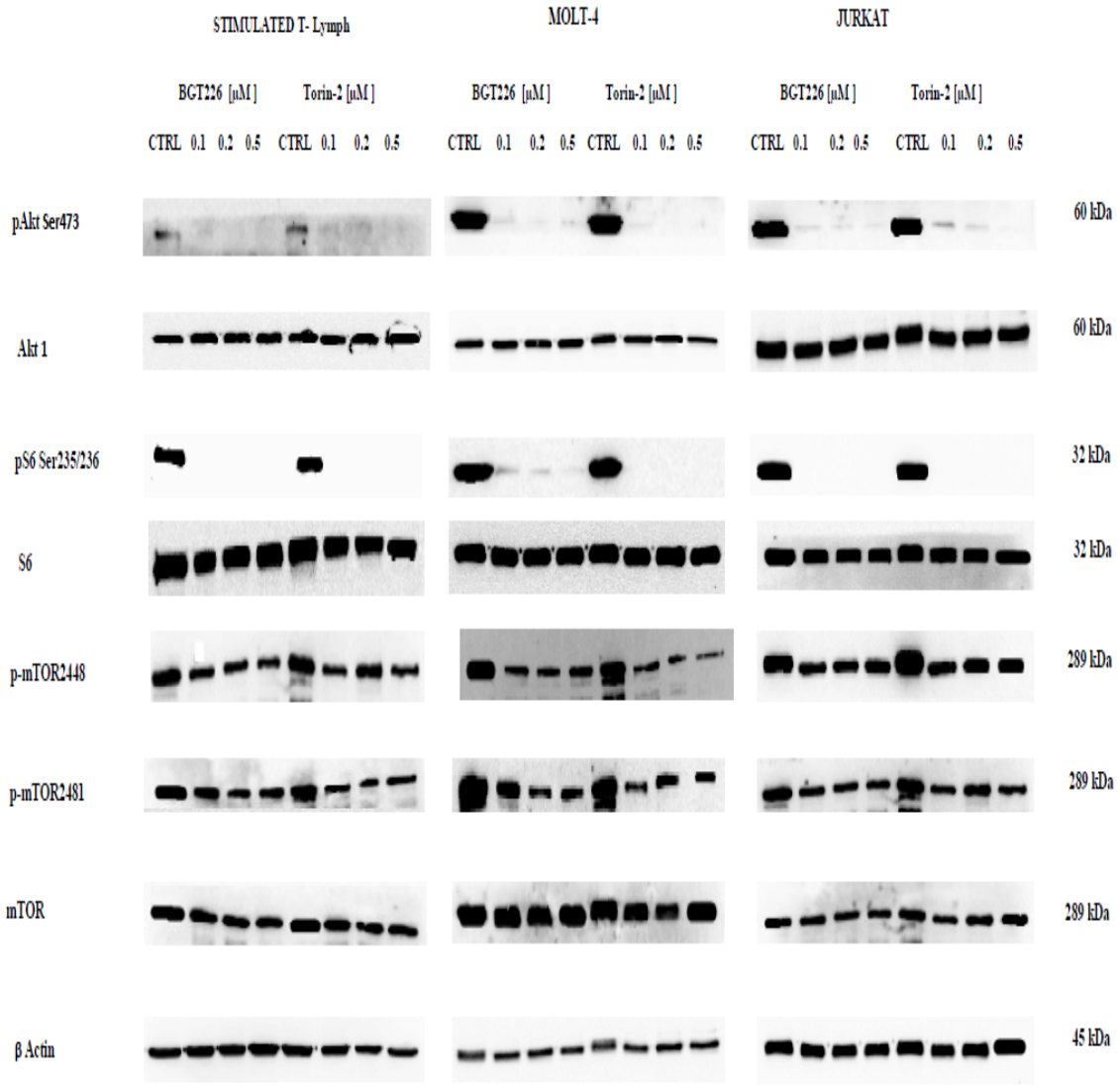


Figure 17. Expression and phosphorylation status of mTOR, Akt, and their downstream targets in primary T-lymphocytes and T-ALL cell lines treated with BGT226/Torin-2.

Western blot analysis for phosphorylated and total Akt, mTOR and its substrate S6 in mitogenic stimulated T-lymphocytes, MOLT-4 and JURKAT cells treated for 2 h with increasing concentrations of BGT226 and Torin-2. For all experiments, twenty-five μg of protein was blotted on each lane. β-actin served as loading control.

4.5 BGT226 and TORIN-2 induce autophagy.

Autophagy plays a very important role in cell cycle, either to be a form of programmed cell death or a protective mechanism against apoptosis [134, 216]. Therefore, a considerable attention is focused on the pharmacological approaches aimed to regulate autophagy, which represent a new area for the development of therapeutics protocols. Moreover, in the last few years, various works described the occurrence of autophagy in acute leukemia cells [217]. To find out if treatment with BGT226 and Torin-2 could induce autophagy, we analyzed by Western blot the expression of microtubule-associated protein 1 light chain 3 LC3A/B I (non-lipidated) and its conjugated form LC3A/B II (lipidated). After 24 h of treatment with increasing concentrations of BGT226 and Torin-2, the levels of lipidated form LC3A/B II increased gradually in a dose-dependent manner, for both drugs in all cells especially for MOLT-4, JURKAT, and mitogenically stimulated T-lymphocytes. The Unstimulated T-lymphocytes showed no expression of lipidated form LC3A/B II (Fig. 18).

To verify whether autophagy was either a cell survival or cell death mechanism we used the autophagy inhibitor 3-MA (3-Methyladenine), whose ability is to block autophagy with the inhibition of class III phosphoinositide 3-kinase (PI3K) [218]. We treated mitogenic stimulated T-lymphocytes and JURKAT cells with BGT226, Torin-2 or 3-MA alone and in combination for 24 h. Results showed that 3-MA alone did not affect cell growth, even at high concentrations (10μM). On the other hand, when 3-MA was administered with 0.25 μM BGT226 or Torin-2, the cell lines become more sensitive to the cytotoxic effect of both drugs (Fig. 18). These results clarify the protective role of autophagy from the cytotoxic effects induced by BGT226 and Torin-2 in mitogenic stimulated T-Lymphocytes and in JURKAT cells.

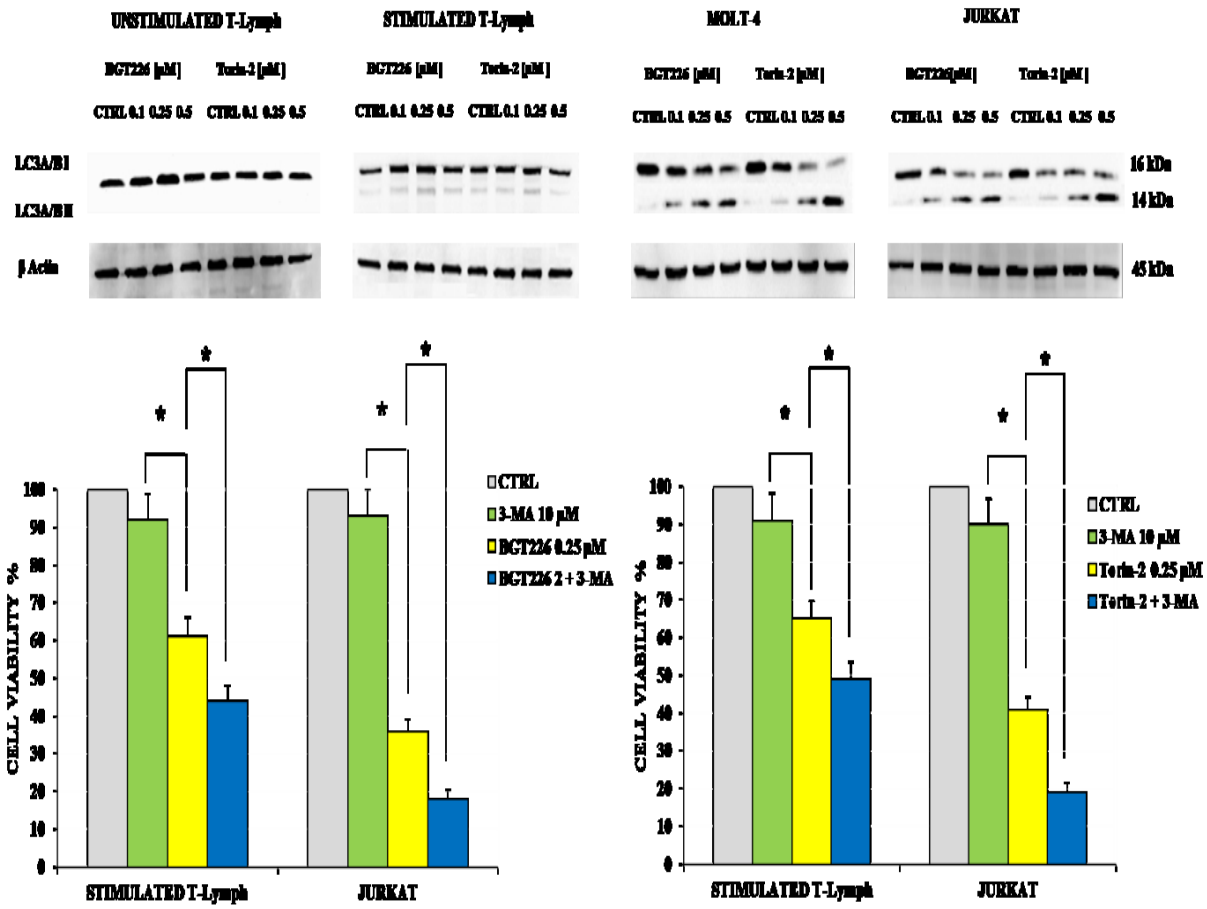


Figure 18. BGT226/Torin-2 induced autophagy in mitogenic stimulated T-lymphocytes and T-ALL cell lines.

On the upper panel, western blot analysis of primary T-Lymphocytes and T-ALL cell lines treated for 24 h with increasing concentrations of BGT226 and Torin-2. An increase of expression of fast-migrating (lipidated) LC3A/B in mitogenic stimulated T-Lymphocytes, MOLT-4, and JURKAT cells is shown. Twenty-five μg of protein were blotted on each lane. β-actin documented equal lane loading. On the lower panel, MTT assay documenting the effect of autophagy inhibitor 3-MA (3-Methyladenine) on the viability of mitogenic stimulated T-lymphocytes and JURKAT cells treated for 24 h with BGT226 and Torin-2. Results are the mean of three different experiments ± SD. Asterisks indicate significant differences with respect to untreated cells (*p< 0.05).

4.6 BGT2206 and Torin-2 induce caspase dependent apoptosis.

Previous studies mentioned that in T-ALL BGT226 could induce apoptosis [219]. In addition, previous works reported that Torin-2 is effective in Pre-B precursor-ALL and on a different panel of cancer cells [220]. In order to establish whether decreased viability was related to apoptosis, the Unstimulated and mitogenic stimulated T-Lymphocytes, MOLT4 and JURKAT cells were treated with increasing concentrations of BGT226 and Torin-2 for 24h, and then Western blot was performed to analyze the expression levels of poly(ADP-ribose)polymerase (PARP). The cleavage of PARP is significant in mitogenic stimulated T-Lymphocytes, MOLT-4, and JURKAT cells. The Unstimulated T-lymphocytes documented no evidence for apoptosis (Fig. 19).

To investigate whether activated caspases were involved in the apoptotic activity of BGT226 and Torin-2, we analyzed the effect of the broad-spectrum caspase inhibitor Z-VAD-fmk whose activity had been check already in different cancer cell lines [221, 222]. We administrated Z-VAD-fmk alone and in combination with BGT226 or Torin-2 for 24 h in mitogenic stimulated T-Lymphocytes and MOLT-4 cell line, then cells were examined by Annexin-V FITC binding. Results showed that Z-VAD-fmk 25 μM alone had no relevant effect on the cell viability but in combination with BGT226 and Torin-2 it significantly inhibited apoptotic cell death mediated by both drugs, in both mitogenic stimulated T- lymphocytes and MOLT-4 cells. Therefore, these results indicated that BGT226 and Torin-2 induced caspases-dependent cell death (Fig. 19).

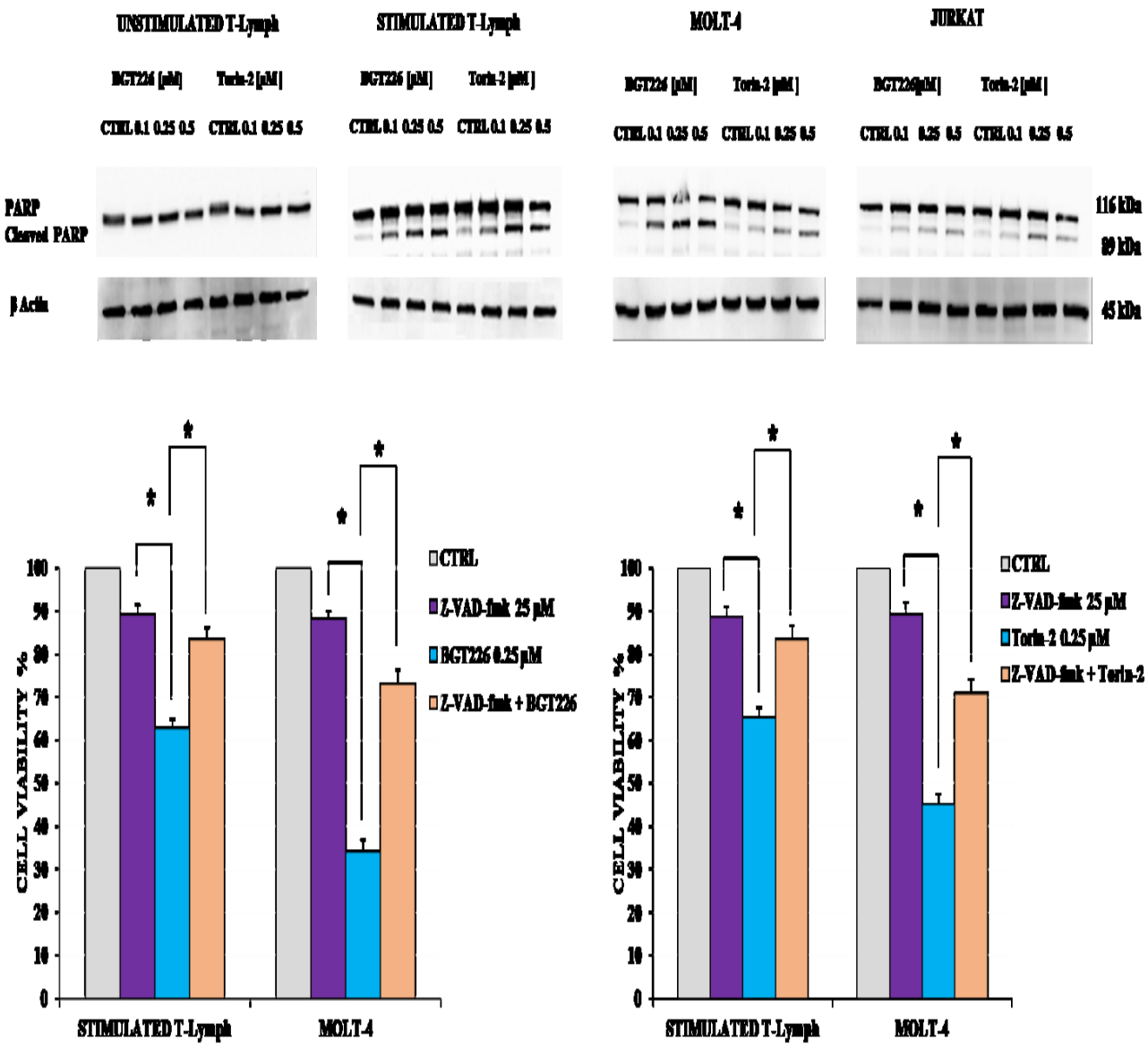
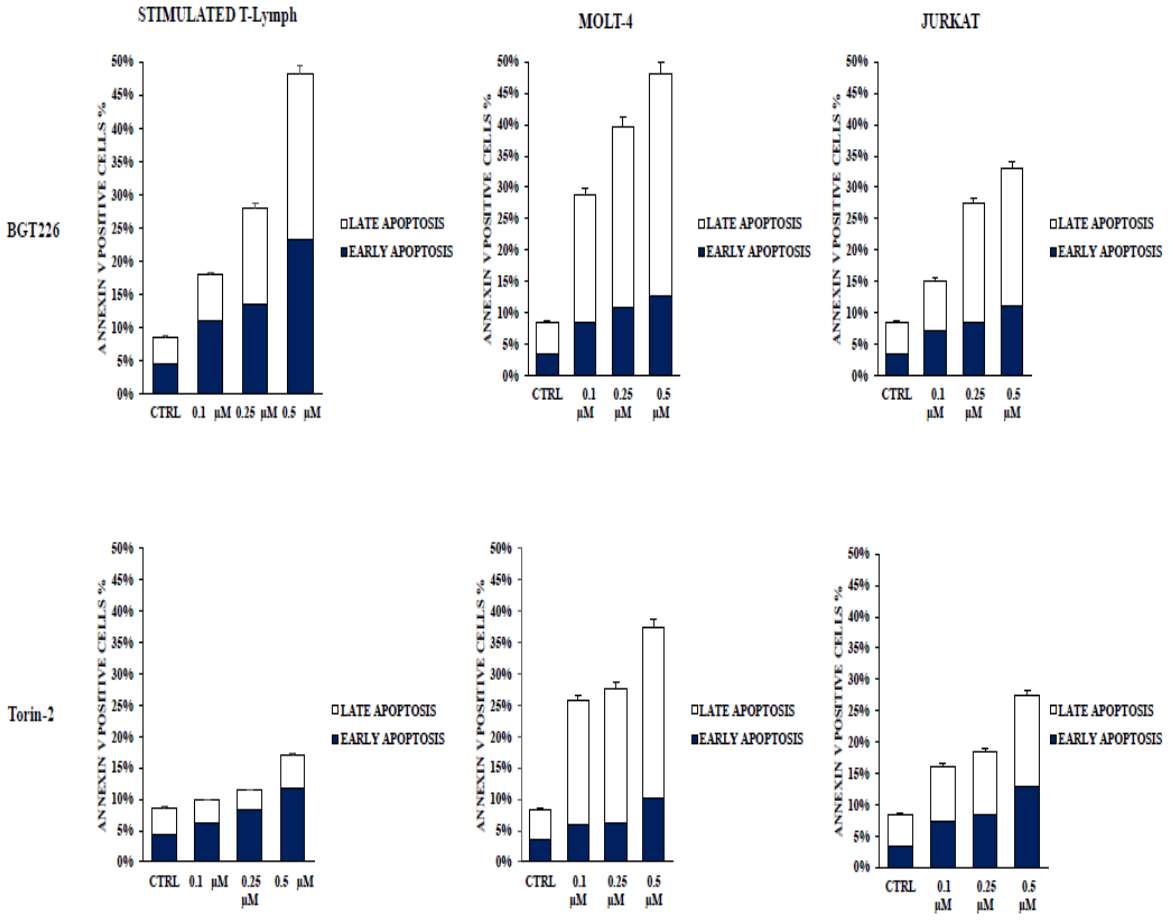


Figure 19.BGT226/Torin-2 induced apoptosis in mitogenic stimulated T-lymphocytes and T-ALL cell lines.

On the upper panel, Western blot analysis documenting the increase of PARP cleavage in mitogenic stimulated T-lymphocytes and T-ALL cell lines treated for 24 h with increasing concentrations of BGT226 and Torin-2. Twenty-five μg of protein was blotted on each lane. β-actin served as loading control. On the lower panel, Annexin-V analysis after BGT226 and Torin-2 treatment, alone and in combination with the pan-caspase inhibitor Z-VAD-fmk, in mitogenic stimulated T-Lymphocytes and MOLT-4 cells. The analysis was performed after 24h of treatment with BGT226 and Torin-2 at 0.25 μM and Z-VAD-fmk at 25 μM. Results are the mean of three different experiments ± SD. Asterisks indicate significant differences with respect to untreated cells (*p< 0.05).

Finally, we analyzed apoptosis induced by both drugs using Annexin-V staining in mitogenic stimulated T-lymphocytes, MOLT-4 and JURKAT cells treated with increasing concentrations of BGT226 or Torin-2 for 24 h. The analysis was performed using the Muse™ Cell Analyzer. The drugs induced a concentration-dependent apoptosis in all cell lines, with a more relevant effect in MOLT-4 and JURKAT cells. In particular, the BGT226 effect was more relevant in MOLT-4; and mitogenic stimulated T-Lymphocytes than JURKAT cells, while the effect of Torin-2 was sounder in MOLT-4 and JURKAT cells than in mitogenic stimulated T-lymphocytes (Fig. 20a). The population of live, early and late apoptosis and dead cells treated with BGT226 and Torin-2 0.1, 0.25 and 0.5 μM was well shown in (Fig. 20b). Overall, these findings demonstrated that BGT226 and Torin-2 potently reduced the growth of mitogenic stimulated T-lymphocytes and T-ALL cell lines and this effect was due to apoptosis and G0/G1 cell cycle arrest.



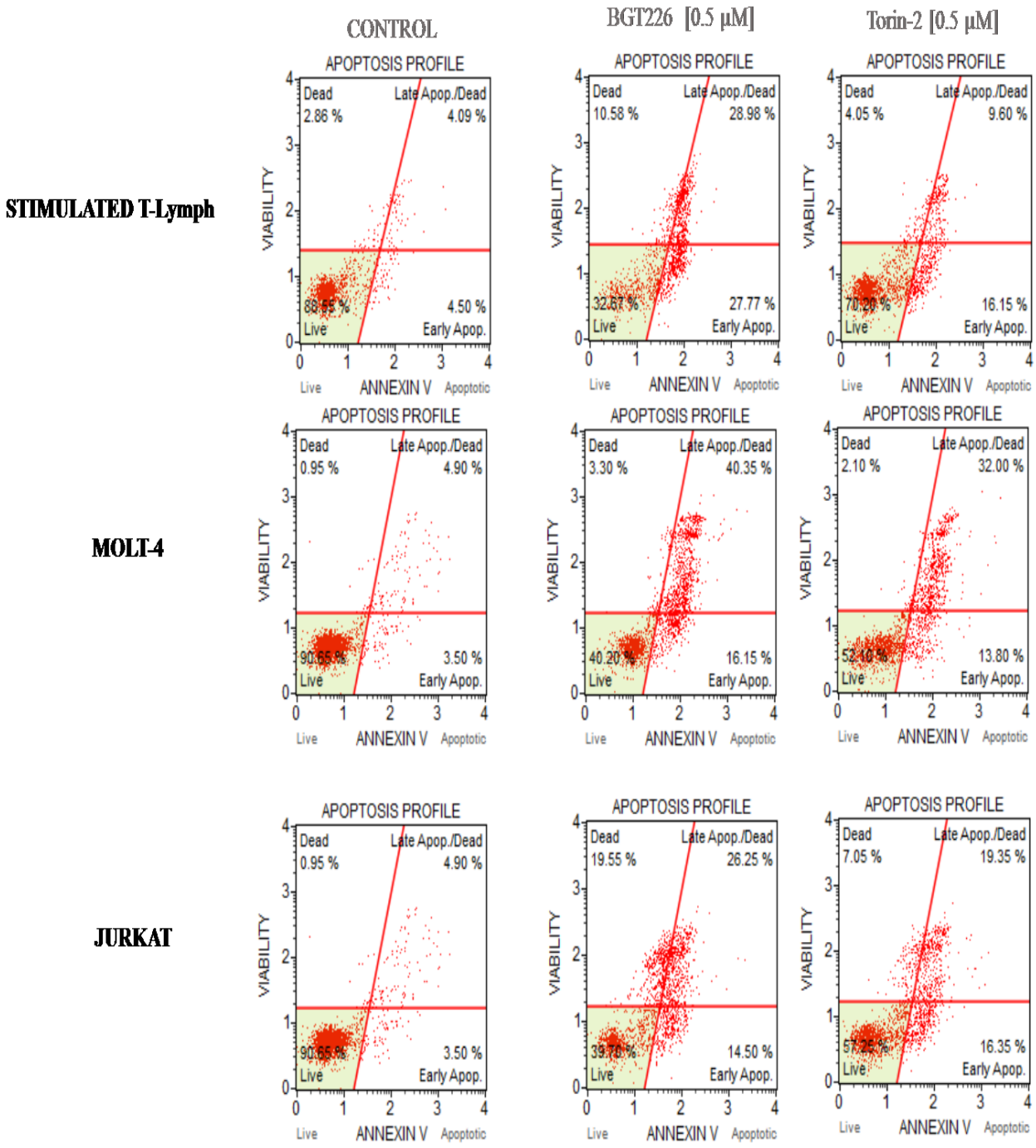


Figure 20. BGT226/Torin-2 induced concentration dependent apoptosis in mitogenic stimulated T-lymphocytes and T-ALL cell lines.

A: Analysis of Annexin-V positive cells after BGT226 and Torin-2 treatment using the Muse™ Cell Analyzer in mitogenic stimulated T-Lymphocytes, MOLT-4, and JURKAT cells. The analysis was performed after 24h of treatment with increasing concentrations of the drugs. Results are the mean of three different experiments ± SD. Asterisks indicate significant differences compared with CTRL (*p<0.05). B: Flow cytometric analysis of Annexin V-FITC/PI mitogenic stimulated T-Lymphocytes, MOLT-4 and JURKAT cells, treated with BGT226 and Torin-2 at 0.5 µM. One representative of three different experiments that yielded similar results is shown.

5. Discussion

Normally in the human body, the peripheral blood lymphocytes (PBL) pool is maintained at a steady level through different mechanisms. Unless the body is challenged by environmental stress, in which case the PBL pool is augmented to counteract inflammation or another stress and is then returned to a normal level [223, 224]. Majorly, the critical regulator of cellular viability, including insulin metabolism, protein synthesis, proliferation and apoptosis is the PI3K/Akt/mTOR signaling cascade [24]. Hence, it is not surprising that PI3K is activated by diverse stimuli in lymphocytes, which is required for the maintenance of proper adaptive immunity and self-tolerance [225, 226]. Therefore, dysregulation of this pathway is involved in pathogenesis 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 that is essential for cell cycle progression, differentiation, and growth [227-229]. Different studies showed that activation of this pathway is a common feature of a wide range of human cancers [230], including hematological malignancies, and act as an indicator of poor prognosis [231-235]. In acute leukemia, activating mutations in the PI3K/AKT signaling cascade of been reported frequently, leading to the activation of this pathway. Mutations event can occur at any stage of the disease and negatively influences the response to therapeutic treatments because it leads to therapy resistance [236, 237]. The outcome for patients in second or later relapse of acute lymphocytic leukemia (ALL) is dismal. It is common to draw the conclusion that ALL is a solved problem in pediatric oncology because 85% or more of pediatric ALL patients do very well. However, leukemia is still the most common cause of pediatric cancer mortality, and adult patients do not achieve the cure rates that pediatric patients do. Furthermore, as outcomes have improved with initial treatment, results for those who do not respond to first-line treatment are getting worse. Patients who relapse are harder to get back into remission, harder to get to transplant, and much harder to cure. Consequently, novel therapies absolutely are still needed in ALL for adults and for those pediatric patients who relapse.

Although the introduction of intensified, less toxic, and selective polychemotherapy protocols has improved the prognosis of this disorder, and achieve good survival rates of younger patients with acute leukemia. Therefore, the fundamental role of PI3K/Akt/mTOR pathway in tumor development and progression gives a significant interest and focus on developing inhibitors against components of this pathway, and now many compounds are 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 inducing apoptosis and/or autophagy [238-240]. Several studies highlighted that both PI3K and mTOR inhibitors are currently in use for treating acute leukemias [241, 242]. Consequently, specific inhibitors sustainably suppressing PI3K/AKT/mTOR signaling pathways may provide an improved antitumor response. Especially dual targeting inhibitors of PI3K/Akt/mTOR pathway at various points of the signaling.

Globally, targeting the PI3K/AKT/mTOR signaling pathways may be a promising approach to treat acute leukemia. It was previously noted, that the predominant antitumor effect of inhibitors of PI3K/AKT/mTOR signaling cascades is mediated via inhibition of cellular proliferation rather than induction of apoptosis [192, 243].

We herein evaluated the antileukemic efficacy of the novel dual PI3K/mTOR inhibitor NVP-BGT226, a pan-PI3Kinase inhibitor that also targets the rapamycin-sensitive mTOR complex 1 as well as the rapamycin-insensitive mTOR complex 2. A second generation ATP-competitive dual mTORC1/2, ATM, ATR and DNA-PK inhibitor Torin-2 in (T-ALL) cell line models, normal quiescent primary T-lymphocytes and mitogenic activated T- lymphocytes. We studied the distinct effects of both drugs on cellular proliferation, cell cycle progression, induction of apoptosis and autophagy. BGT226 proved to potently inhibit cellular proliferation in the low nanomolar range in the T-ALL cell lines with a higher concentration in mitogenic activated T-lymphocytes and non-relevant effect on quiescent T-lymphocytes. The sensitivity profile is thereby in the same range compared to the additionally tested dual mTORC1/2 inhibitor Torin-2. Therefore, both drugs were cytotoxic and cytostatic in a nanomolar range.

Moreover, cell cycle arrest in cancer cells is a major indicator of anticancer activity and has been implicated in different cancers [244]. We treated the selected cancer cell lines, mitogenic stimulated T-lymphocytes and quiescent T-lymphocytes with BGT226 and Torin-2 for 24 h and set up concentration-dependent cell cycle analysis by PI-stain flow cytometry. We documented a concentration-dependent increase in cells in the G0/G1 phase of the cell cycle and a concomitant decrease in cells in both S and G2/M phase (Fig. 15). This increase was more pronounced in mitogenic stimulated T- Lymphocytes, MOLT-4 and JURKAT cells than in quiescent T- Lymphocytes, where a significant G0/G1 phase was not appreciable. This observation argues for a potent and sustained cell cycle arrest caused by BGT226 and Torin-2. For normal T-lymphocytes when induced into the cell cycle, becomes more sensitive to drug effects, while the resistance to drugs observed in quiescent T-lymphocytes implies that if cells are not entering the cell cycle do not initiate any features of apoptosis, a property referred to here as ‘no cycle, no apoptosis’.
We also documented the increasing of cleaved PARP, a well-known marker of apoptosis, thus showing this as a mechanism for the cytotoxicity of both 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.

BGT226 and Torin-2 induced apoptosis in T-ALL cell lines and mitogen-stimulated T-lymphocytes as demonstrated by Annexin V and western blotting. Moreover, in the presence of the pan caspases, inhibitor Z-VAD-fmk apoptosis is blocked. Apoptosis resulted in playing a determinant role in the killing mechanism since the treatment with a pan-caspase inhibitor protected the cells from BGT226/Torin-2 cytotoxic effect. These results are in agreement with those observed with other drugs in acute myeloid leukemia, for Torin-2 in B-pre ALL and BGT226 in solid tumors [210, 220, 245, 246]

Autophagy is a response to growth limiting conditions, such as nutrient depletion and the presence of cytotoxic drugs [247] and it may trigger increased induction of apoptosis in cells [248]. The correlation between autophagy and tumorigenesis has been explored extensively, but whether autophagy acts as a pro-tumorigenic or

anti-tumor tool in tumor development and cancer therapy, still has to be elucidated in the different cases [249, 250]. We documented that, BGT226/ Torin-2 also induced autophagy in T-ALL cell lines and mitogenic stimulated T-lymphocytes and its inhibition by 3-MA further sensitized T-ALL cell lines and mitogenic T-lymphocytes to the cytotoxic effects of a 24 h treatment by both drugs. These findings suggest that in T-ALL cell lines and mitogenic T-lymphocytes, autophagy could have a tumor-protecting role when neoplastic cells are treated with PI3K/Akt/mTOR inhibitors. Due to these properties, autophagy inhibitors had been also studied as potential agents in cancer therapy, since autophagy could act as a cell-survival pathway in cancer, in agreement with our data [217, 251, 252].

The phosphorylation status of the key elements of the PI3K/Akt/mTOR pathway, assessed by Western blot, either was equally sensitive to BGT226 and Torin-2 inhibition in T-ALL cell lines or in mitogen-stimulated T-lymphocytes.

In conclusion, dual PI3K/mTOR inhibition is highly effective against T-Acute lymphoblastic leukemia cells, both in vitro as well as ex vivo. Notably, the novel dual PI3K/mTOR inhibitor NVP-BGT226 reveals extraordinary potency to inhibit proliferation as well as to induce apoptosis in the nanomolar range against a broad range of cell lines and ex vivo (mitogen-stimulated) samples tested. This also was conducted by using dual mTOR1/2 inhibitor Torin-2. Furthermore, these significant results making dual inhibition a highly promising agent for clinical testing in acute leukemia since these drugs do not have any effect on normal lymphocytes. This may include combination approaches as well as targeted therapy of TKI-resistant leukemias. Based on our studies, clinical evaluation of this agent for targeted treatment of acute leukemia subtypes is strongly indicated.

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