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Inhibition of the PI3K/Akt/mTOR signaling pathway as a therapeutic target for Acute Lymphoblastic Leukemia

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ABSTRACT

Acute Lymphoblastic Leukemia (ALL) is a malignant disorder characterized by the abnormal clonal proliferation of B-cell progenitors (B-ALL) or immature stage thymocytes (T-ALL).

Constitutive activation of the PI3K/Akt/mTOR network is a common feature of B- and T-ALL, influencing cell growth and survival. The PI3K/Akt/mTOR inhibitors are currently being developed for clinical use either as single agents or in combination with conventional chemotherapy for T-ALL patient treatment.

In this study it has been investigated the effects of a panel of PI3K/Akt/mTOR inhibitors on healthy human $CD4^+$ T-cells when compared with T-ALL cell lines. Then, it has been verified whether a multi-inhibition treatment against Akt protein could enhance the efficacy of individual drug administration and overcome drug resistance as well as to obtain a decrease in single drug concentration, by testing the effects of combined treatments with three Akt inhibitors with different mode of action, GSK690693, MK-2206 and Perifosine on T-ALL cell lines. Combined administration of the drugs displayed a significant synergistic cytotoxic effect and affected PI3K/Akt/mTOR pathway at much lower concentration than single drug use. Highest synergistic effect for full inhibition of Akt was also related to the timing of every drug administration. The results obtained suggested that targeting Akt as a key protein of PI3K/Akt/mTOR pathway with multiple drugs might represent a new and promising pharmacological strategy for treatment of T-ALL patients.

It has also been investigated the role of microRNAs (miRNAs), a class of small noncoding RNAs, which play a role in various biological processes, including proliferation, apoptosis and tumorigenesis. The dysregulation of miRNAs is implicated in invasion in several human cancer types and leukemia is not an exception. By using *in vitro* models, it has been done an analysis of the effect of PI3K signaling inhibitors on expression of miRNA level involved in ALL disease and PI3K activation. The results obtained have shown that these drugs could modulate miRNA expression. Therefore, the regulation of miRNA expression profiling in ALL by using PI3K signaling inhibitors could be used as a new therapeutic approach in the near future.

In addition, it has been analyzed the efficacy of PI3K signaling pathway inhibitors in B- and T-ALL cell lines harboring the Abl1 tyrosine kinase gene fusion that lead to an aberrant cell proliferation.

It has been studied the effects of anti Bcr-Abl1 drugs such as Imatinib, Nilotinib and GZD824 associated with PI3K signaling inhibitors.

Drugs against PI3K/Akt/mTOR cascade administered in combination with Imatinib, Nilotinib and GZD824 decreased cell viability, induced apoptosis and autophagy in a marked synergistic manner.

These findings suggested that selected PI3K/Akt/mTOR inhibitors used in combination with anti Bcr-Abl1 drugs may be an attractive novel therapeutic intervention in Ph^+ B- and T-ALL.

1. INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) (PI3K/Akt/mTOR) signaling pathway is hyperactivated or altered in many cancer types and regulates a broad range of cellular processes including survival, proliferation, growth, metabolism, angiogenesis and metastasis. The PI3K/Akt/mTOR cascade is regulated by a wide-range of upstream signaling proteins and it regulates many downstream effectors by collaborating with various compensatory signaling pathways, primarily with Raf/Mek/Erk pathway. Limited clinical success of the available targeted therapeutic agents and challenges mediated by tumour heterogeneity across different cancer types emphasize the importance of alterations in the PI3K/Akt/mTOR network in the design of effective personalized treatment strategies. The PI3K/Akt/mTOR represents the intricate crosstalk between compensatory pathways, which can be utilized to study the Akt signaling mechanism in detail and improve the personalized combinatorial therapeutic strategies [1].

Acute Lymphoblastic Leukemia (ALL) is the most common malignancy in childhood. Despite enormous improvement of prognosis during the last half century, ALL remains a major cause of childhood cancer-related mortality. During the past decade, whole genomic methods have enhanced the knowledge of disease biology. Stratification of therapy according to early treatment response measured by minimal residual disease (MRD) allowed risk group assignment into different treatment arms, ranging from reduction to intensification of treatment. Progress has been achieved in academic clinical trials by optimization of combined chemotherapy, which continues to be the mainstay of contemporary treatment [2]. New insights into the biology and genetics of ALL as well as novel clinical observations and new drugs are changing the way to diagnose, risk-stratify and treat adult patients with ALL. New genetic subtypes and alterations refine risk

stratification and uncover new actionable therapeutic targets. The incorporation of more intensive, pediatric and pediatric-inspired approaches for young adults seem to have a positive impact on survival in this population [3].

Due to the crucial role of PI3Ks in regulating cell cycle, metabolism, and survival, the PI3K signaling cascade in human leukemias including acute myeloid leukemia (AML), T-ALL and pre-B ALL [4] is one of the most often altered pathways [5, 6] and different compounds targeting members of the PI3K network have been developed and entered clinical trials [7]. In this study it has been studied the cytotoxic effects of a panel of PI3K/Akt/mTOR and PI3K isoform inhibitors on human $CD4^+$ T-cells, then it has been documented that multiple inhibition of Akt protein was cytotoxic against T-ALL cells and had synergistic effects, with more potent efficacy than single or double compound administration at the same concentration. It has been also explored their efficacy against T- and pre-B ALL cell lines harboring Nup214-Abl1 and Bcr-Abl1 fusion gene, respectively. This innovative research aimed to analyze the multiple Akt targeting by drugs with different mechanism of action as a new promising treatment for T-ALL patients with PI3K/Akt/mTOR pathway hyperactivation and the efficacy of anti-Nup214-Abl1 and Bcr-Abl1 mutation, alone and in combination with several PI3K signaling inhibitors, to provide a new therapeutic option to overcome the mechanism of resistance to tyrosine kinase inhibitor (TKI) treatment of Ph^+ ALL positive cells.

microRNAs (miRNAs), a new class of endogenous small noncoding RNAs, were originally identified as small non-coding RNAs that control the timing of larval development in Caenorhabditis elegans [8, 9]. They have subsequently been associated with several types of cancer [10] and involved in various cellular processes, including DNA methylation, cellular growth, differentiation and apoptosis [11-14]. Their abnormal levels in tumors have important consequences since certain miRNAs overexpressed in tumors contribute to oncogenesis by tumor suppressors downregulation [15]. The dysregulation of miRNAs has been reported in a variety of human cancers and hematological malignancies [16]. In a microarray-based study it has been demonstrated that miRNAs expression correlates with both the type of leukemia and the prognosis of patients [17, 18]. miRNAs expressed differentially in distinct stages of lymphopoiesis, can hybridize with target messenger RNAs, regulating their post transcriptional expression and influence the direction of lymphoid precursor maturation. In malignant lymphopoiesis there is an aberrant expression of miRNAs involved and those aberrations can be used as signatures of different ALL subtypes. In addition, changes in the expression of several miRNAs may have functional relevance with leukemogenesis or drug resistance. As a

result, reversal of the expression of these miRNAs may contrast the disease and improve clinical outcomes. However, among the studies of miRNAs, there are still some problems that need to be addressed to understand the function of miRNAs in ALL more thoroughly [19]. Recently, besides the immunophenotyping of ALL, an increasing number of studies showed that the miRNA expression profiles in acute leukemia have cooperative interactions in the development of leukemia. Therefore, the miRNA expression profile can be used as biomarkers in diagnosis, differential diagnosis, prognosis, and therapy of hematological cancers [20-22].

1.1 The PI3K/Akt/mTOR signaling pathway

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) signal transduction pathway is activated by several stimuli regulating cell proliferation and survival, translation, autophagy and metabolism. Several members of the PI3K/Akt/mTOR cascade play crucial roles in maintaining cell homeostasis under normal physiological conditions [23]. PI3K comprises a family of lipid kinases which phosphorylate the 3-OH group of inositol lipids. They are classified into class I, II, and III based on primary structure and regulation. Class I PI3Ks are heterodimeric enzymes, displaying a catalytic and a regulatory subunit. The catalytic subunits comprise p110α, p110β, p110γ, and p110δ, which associate in different ways with the regulatory subunits (p85α, p85β, and p55γ, p101, p84, and p87PIKAP) [24].

Akt requires phosphorylation of both Ser473 and Thr308 amino acidic residues to be fully activated and regulates several downstream, processes including positive activation/regulation of mTOR functions [25].

mTOR is a 289-kDa serine/threonine protein kinase, which regulates cell growth. mTOR is the catalytic subunit of two multi-protein complexes: complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is responsive towards growth factors, nutrients, energy, or oxidative stress and phosphorylates the eukaryotic initiation factor-4E (eIF4E)-binding protein 1 (4E-BP1) and S6K which are important for protein translation. mTORC2 phosphorylates Akt at the Ser473 residue for complete activation of the PI3K/Akt/mTOR pathway [26] (Fig. 1).

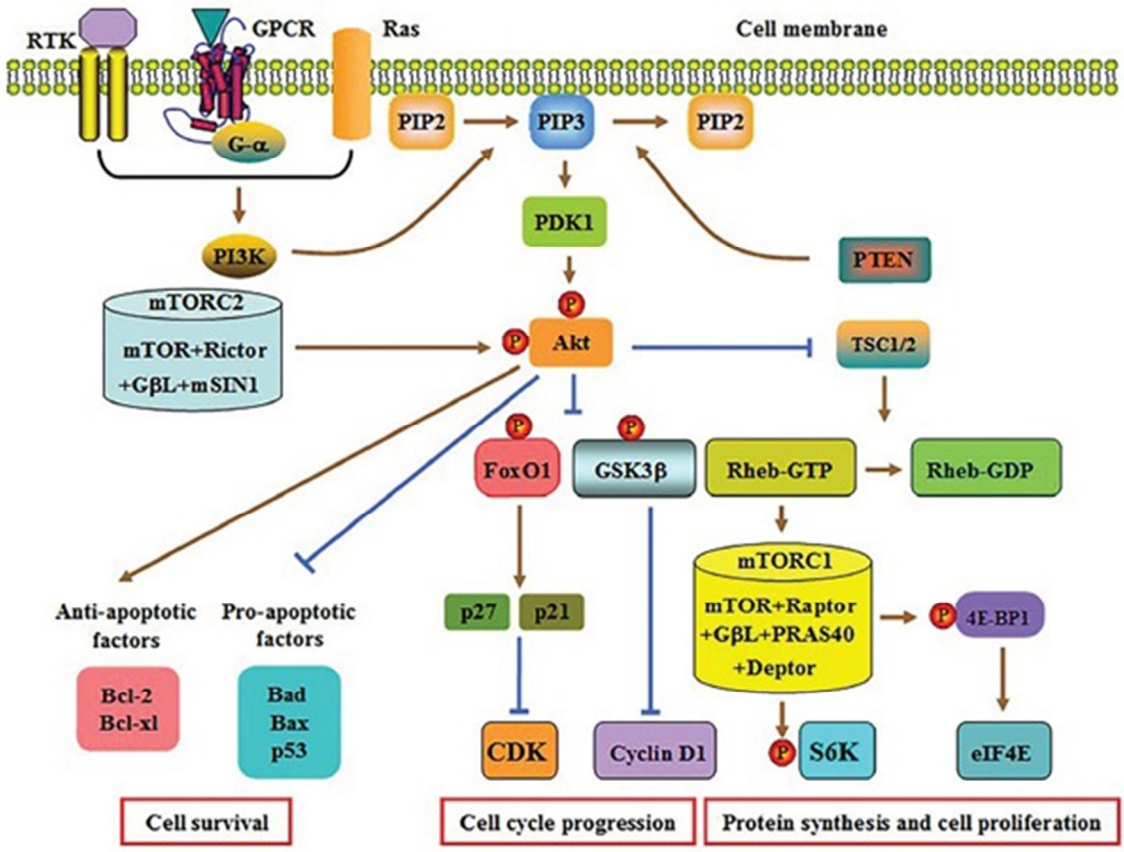


Figure 1: The PI3K/Akt/mTOR signaling pathway. PI3K is activated upon binding of an extracellular ligand to RTK, GPCR or Ras. Activated PI3K converts PIP2 to PIP3, which is able to be reversed by PTEN. PIP3 then recruits PDK1, which phosphorylates and partially activates Akt. The mTORC2 mediates the second phosphorylation to fully activate Akt. Subsequently, the formation of the TSC1/2 heterodimer is significantly decreased, which leads to a marked increase in Rheb-GTP content and mTORC1 activation. The mTORC1 induces S6K phosphorylation to release 4E-BP1, which stimulate protein synthesis and cell proliferation. Akt activation enhances cyclin D1 and CDK expression by inhibiting GSK3β and FoxO1, respectively. Both effects contribute to cell cycle progression. Activated Akt can also suppress apoptosis via regulating the expression of apoptosis-associated genes. Raptor, regulatory-associated protein of mTOR; GβL, mammalian LST8/G-protein β-subunit like protein; PRAS40, proline-rich Akt substrate 40; Deptor, DEP domain containing mTORinteracting protein; mSIN1, mammalian stress-activated protein kinase interacting protein 1.

1.2 PI3K isoforms

Human cells express three classes of PI3K enzymes. There are three class II PI3Ks (PI3K-C2α, β, γ) and a single class III PI3K (hVPS34). Mammals express four class I catalytic isoforms (p110α, β, γ, and δ encoded by PIK3Cα, PIK3Cβ, PIK3Cγ, and PIK3Cδ) that catalyze the phosphorylation of PtdIns-4,5-P2 to generate PtdIns-3,4,5-P3 (Fig. 2). This phospholipid acts as a second messenger to recruit cytoplasmic proteins to specific plasma membrane or endomembrane locations. The p110α and p110β proteins are expressed ubiquitously, whereas expression of p110γ and p110δ is enriched in immune cells. Each catalytic isoform forms a dimer with a regulatory subunit that modulates the activity and

subcellular localization of the complex (Fig. 2). In normal cells, PtdIns-3,4,5-P3 is induced transiently by growth factor stimulation and is rapidly metabolized by lipid phosphatases, including the tumor suppressor PTEN, which terminates PI3K signaling via removal of the 30-phosphate from PtdIns- 3,4,5-P3. Cancer cells frequently contain elevated amounts of PtdIns-3,4,5-P3 due to increased activity of oncogenic signaling proteins residing upstream of PI3K or to mutational activation of PI3K itself. Many cancers also exhibit loss of PTEN function, which elevates basal and stimulated PtdIns-3,4,5-P3 abundance by reducing the turnover rate of this second messenger. In a meta-analysis of cancer genome sequencing studies, PIK3Cα and PTEN were found to be the second and third most highly mutated genes in human cancers. Activation of class I PI3Ks occurs through multiple upstream pathways that couple a broad range of cell surface receptors to specific PI3K isoforms. Generally, PI3Ks are capable of being activated by receptor-coupled tyrosine kinase activities, small Ras-related GTPases, and heterotrimeric G proteins. Each class I isoform has a domain that interacts with members of the Ras GTPase superfamily (Fig. 2). For p110α, p110γ, and p110δ, this domain binds to Ras or R-ras subfamily members, whereas p110β interacts with the Rac/cdc42 subfamily. Three of the class I catalytic isoforms (for p110α, β, and δ; collectively known as the class IA subgroup) associate with regulatory subunits whose SH2 domains bind to phosphotyrosyl residues on growth factor receptors or adaptor proteins such as IRS1. The other catalytic isoform (p110γ; known as class IB) associates with regulatory subunits (p101, p87) that mediate binding to bg subunits of heterotrimeric G proteins following activation of G protein-coupled receptors (GPCRs). Adding to this complexity, the p110β isoform contains a Gbg-binding site that enables this isoform to be a coincidence detector for GPCR and tyrosine kinase signaling [27]. Through an unknown mechanism, p110δ in B- lymphocytes is activated by chemokine receptors, which are members of the GPCR family. In murine macrophages, p110γ can be activated downstream of tyrosine kinases as well as GPCRs [28]. In summary, GPCRs and RTKs exhibit considerable plasticity in terms of coupling to the various class I PI3Ks, determined in part by the cellular context. Structural and biophysical studies have clarified the mechanisms of activation of different class I isoforms [29]. The p110α isoform and its activation by physiological signals as well as by cancer-associated PIK3Cα mutations. p110α associates with one of five different regulatory subunits (p85α, p55α, p50α, encoded by PIK3R1; p85β, PIK3R2; p55γ, PIK3R3) and each of these subunits contains two SH2 domains (N-SH2, C-SH2) flanking a coiled-coil region known as the inter-SH2 (iSH2) domain (Fig. 2). The catalytic and regulatory subunits make additional contacts that maintain the enzyme in a low activity state under basal conditions. The helical, kinase and

C2 domains of the catalytic subunit contact the p85-N-SH2 domain; the C2 domain also contacts the p85-iSH2 domain. Binding of the regulatory subunit's SH2 domains to phosphotyrosines relieves these inhibitory contacts and positions the dimer near the membrane where it can access substrate and receive further inputs from Ras and other signaling components.

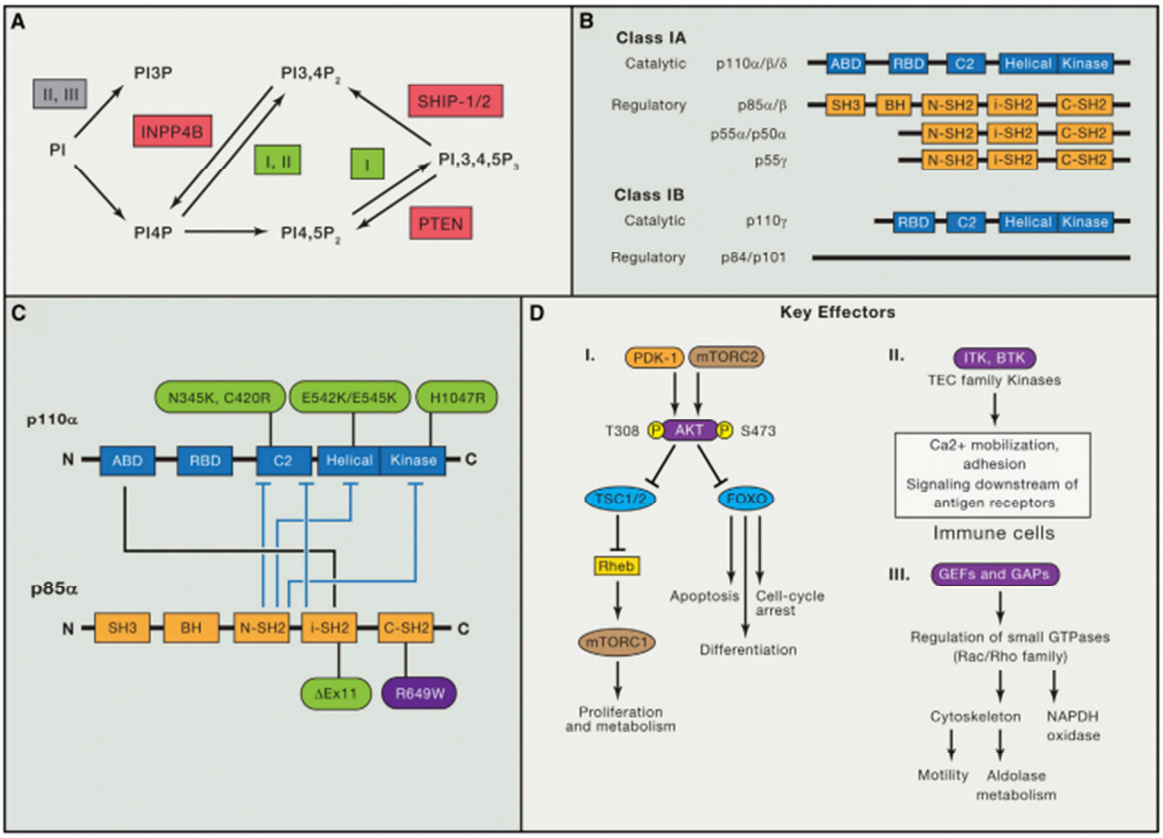


Figure 2: Overview of phosphoinositides, class I PI3K protein isoforms, p110α activity, regulation and PI3K downstream subunits effectors. (A) Schematic overview of the major synthesis and degradation pathways for PtdIns-3-P (PI3P), PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. The classes of PI3K (I, II, or III) that mediate reactions are indicated. Lipid phosphatases are in red. INPP4B, inositol polyphosphate-4-phosphatase, type II. (B) Domain structure of class I PI3K catalytic and regulatory subunits. ABD, adaptor-binding domain; RBD, Ras-binding domain; BH, breakpoint cluster region homology. (C) Diagram of the intramolecular interactions between class IA catalytic and regulatory subunits (p110α and p85α are displayed as well studied examples). Tight binding of the ABD to iSH2 confers stability to p110α. The other contacts shown in blue block arrows diminish basal activity and are relieved upon regulatory subunits binding to pTyr. Cancer-associated activating mutations are shown in green. SHORT syndrome mutation in p85α (R649W) is in purple. (D) Brief summary of key PI3K effectors: PDK-1, Akt, TEC family kinases, and GEFs/GAPs for small GTPases. Akt has many other important substrates not shown here [30]. The specific GEFs that mediate PI3K dependent Rac activation to promote motility and aldolase release are not known.

1.3 PI3K effectors

The most proximal outcome of PtdIns-3,4,5-P3 production by class I PI3Ks is the recruitment of specific proteins to membrane-signaling complexes. The shared property of these PI3K effectors is a pleckstrin homology (PH) domain selective for PtdIns-3,4,5-P3 and/or PtdIns-3,4-P2. Within the family of PI3K effectors are subsets with distinct enzymatic or signaling functions. These include serine/threonine kinases of the AGC kinase family, tyrosine kinases of the TEC (tyrosine kinase expressed in hepatocellular carcinoma) family and modulators of small GTPase activities, termed guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Fig. 2). In this way, multiple, diverging downstream pathways can be simultaneously triggered by PI3K activation. Compared to other effectors, members of the Akt sub-family of AGC serine/threonine kinases (Akt1, Akt2, Akt3) seem to be activated more universally downstream of receptor-mediated PI3K activation. In fact, Akt phosphorylation often serves as a surrogate readout of class I PI3K activation. This tight coupling of PI3K to Akt is likely the result of two factors. First, phosphorylation of the Akt activation loop (Thr 308 on Akt1) occurs through a relatively straightforward mechanism, involving dual recruitment to the plasma membrane of Akt and its upstream activating kinase, phosphoinositide-dependent kinase-1 (PDK-1). Membrane colocalization of the constitutively active PDK-1 with Akt facilitates PDK1-mediated phosphorylation of Akt. Second, the PH domains of both Akt and PDK-1 have affinity for both PtdIns- 3,4,5-P3 and PtdIns-3,4-P2. The latter lipid can be produced from PtdIns-3,4,5-P3 by SHIP-1 and SHIP-2 (Fig. 2), is often sustained after a transient peak of PtdIns-3,4,5-P3 and may promote Akt activation at endomembranes. Although Akt phosphorylation on Thr308 is both necessary and sufficient to mediate many downstream events, additional phosphorylation sites control substrate selectivity, stability and possibly subcellular localization [30]. mTOR complex-2 (mTORC2) phosphorylates Ser473 of the Akt hydrophobic motif [31]; this modification promotes maximal Akt activity and seems particularly important for a subset of substrates, including forkhead box, subgroup O (FOXO) transcription factors [32]. The mechanisms by which mTORC2 is activated to phosphorylate Akt have not been fully resolved [33, 34]. Akt phosphorylates many substrates involved in cell proliferation, metabolism, survival and motility [30]. Mutations in the PH domain that promote membrane localization occur frequently in cancer (e.g., Akt1-E17K in 4%–8% of breast cancer patients), supporting the idea that Akt is an important PI3K effector in oncogenic signaling. Notably, Akt plays an evolutionarily

conserved role in growth factor signaling downstream of PI3K. There is a strong link between PI3K and BTK function in B cells, first shown by knockout studies in mice where deletion of Pik3r1 or Pik3cd caused defects in B-cell development and survival similar to those in mice lacking BTK [35]. GEFs for Rho/Rac/cdc42 family GTPases are less widely appreciated, but nonetheless critical effectors of class I PI3K signaling. These small GTPases are regulated by many GEFs, of which only a subset bear PH domains with selectivity for PtdIns-3,4,5-P3. The last PI3K effector is mTOR. This serine-threonine kinase forms two cellular complexes known as mTORC1 and mTORC2, with distinct subunit composition and substrate selectivity (Fig. 3) [36]. Apart from Akt-Ser473, established substrates of mTORC2 include analogous sites in serum- and glucocorticoid-regulated kinases (SGKs) and protein kinase C (PKC) isoforms. mTORC1 phosphorylates numerous substrates that promote anabolic metabolism to support cell growth and proliferation. mTORC1 activity can be increased by mitogenic signals through PI3K/Akt, Ras/Erk and other pathways [37] but also requires coordinate signals delivered through nutrient-sensing pathways [36]. Thus, mTORC1 represents a key signaling node that coordinates anabolic metabolism and cell mass accumulation with growth factor receptor stimulation and nutrient availability. Rapamycin is a bacterially derived product that binds to intracellular FKBP12, thereby generating a complex that binds to mTORC1 at an allosteric site, termed the FKBP12-rapamycin binding (FRB) domain. Importantly, although rapamycin is exquisitely selective for mTORC1, this drug has differential effects on the phosphorylation of distinct mTORC1 substrates (Fig. 3). ATP-competitive mTOR kinase inhibitors (TORKi) fully suppress kinase activity of both mTORC1 and mTORC2 without affecting integrity of the complexes (Fig. 3). Comparisons of rapamycin and TORKi have provided valuable insights into the function of mTOR complexes and their substrates. The direct mTORC1 substrate S6 kinase-1 (S6K1) contributes to metabolic reprogramming by increasing glycolysis and protein, lipid, and nucleotide biosynthesis (Fig. 3) [38]. S6K1 is highly sensitive to inhibition by rapamycin and the disruption of S6K1-mediated negative feedback might contribute to limited efficacy of rapamycin and its derivatives (termed rapalogs) in cancer. mTORC1 also initiates powerful negative feedback regulation of growth factor receptor signaling, such that inhibition of mTORC1 or S6K1 leads to elevated activation of PI3K, Akt and the Erk pathway [36, 39]. The eukaryotic initiation factor-4E (eIF4E)-binding proteins (4EBPs) are key mTORC1 substrates that control cell proliferation and survival. Phosphorylation of 4E-BPs by mTORC1 inhibits their binding to eIF4E, enabling assembly of the latter with eIF4G and eIF4A to form an active, cap-binding translation initiation complex known as eIF4F.

Among cap-dependent mRNA transcripts, those that are more sensitive to decreased eIF4F activity are enriched in cell cycle and survival factors. Pharmacological and genetic studies have validated eIF4F as an oncogenic node and targetable vulnerability in cancer cells [40]. Importantly, 4E-BP phosphorylation is inhibited to a greater extent by TORKi than by rapamycin (Fig. 3), the more penetrating inhibition of translation initiation by TORKi contributes to the more profound inhibition of cell growth and proliferation by these agents.

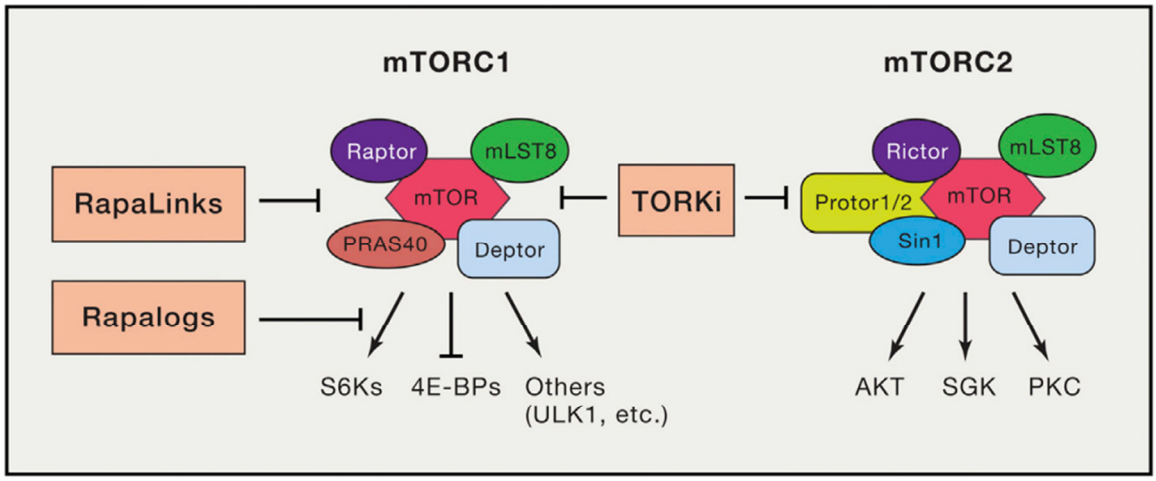


Figure 3: Overview of mTORC1 and mTORC2 complexes, key substrates and inhibitors. The processes inhibited by different classes of mTOR inhibitor are shown. First-generation rapalogs are partial inhibitors of mTORC1 that inhibit phosphorylation of S6Ks more than 4E-BPs. Second-generation TORKi fully inhibit mTORC1 and mTORC2. Third-generation RapaLinks fully but selectively inhibit mTORC1 and also overcome single resistance mutations to rapalogs and TORKi.

1.4 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukaemia (ALL) is a rare disease. In 2016, 6590 new cases were reported in the US. Registry data from North America and Europe report an overall incidence of 1-7 and 1-28 cases per 100.000 individuals per year, respectively [41]. Although most cases of ALL are diagnosed in the paediatric population (median age at diagnosis is 15 years), ALL is reported across all age groups and is associated with poor survival in older patients [42]. Significant advances have been made in recent years in diagnosis and treatment. ALL is increasingly recognized as a genetically heterogeneous disease and a flurry of new genetic subtypes have been recently characterized. This has refined risk-stratification with some of these genetic lesions representing attractive actionable therapeutic targets.

B-cell acute lymphoblastic leukemia (B-ALL) is the most common malignancy in children, originated in a single cell and characterized by the accumulation of blast cells that are phenotypically reminiscent of normal stages of B-cell differentiation accounting for 80% of pediatric ALL [43, 44]. Thank to progress in polychemotherapy protocols, the majority of B-ALL pediatric patients achieve complete remission. However, the prognosis of relapsed and chemoresistant patients remains poor [45]. Also, infants and young adults with B-ALL display a much worse prognosis [46, 47]. Therefore, the identification of novel targeted therapies to support conventional chemotherapy is urgently required to further improve the outcome of this disorder.

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy characterized by proliferation of thymocytes at various stages of development [48]. This disease is reported in 10–15% of children and 25% of adult ALL patients, with a significant percentage of resistance to chemotherapy and an extremely poor prognosis in case of relapse [49, 50].

1.5 Role of the PI3K/Akt/mTOR network in ALL

Since acute leukemias can still have an extremely poor outcome, at present great interest surrounds the development of novel and less toxic therapeutic strategies that may target aberrantly activated signaling networks involved in proliferation, survival, and drug resistance of leukemic cells [51].

One such pathway is represented by the PI3K/Akt/mTOR signaling network. Several lines of evidence, obtained in preclinical settings of acute leukemias, have documented how this network could be targeted by small molecule protein kinase inhibitors (SMI) [52-54]. Indeed, the PI3K/Akt/mTOR pathway is probably the most easily druggable signaling network in human neoplasias and an impressive array of inhibitors, targeting critical components of this cascade, have been designed by drug companies [55]. However, optimal therapeutic strategies have yet to be identified for a successful treatment of acute leukemias. Inhibition of critical signaling nodes such as PI3K or mTOR induced cell cycle arrest, apoptosis and lowered drug-resistance of leukemic cells [52, 53, 56, 57]. Several phase I/II clinical trials are now underway, in which PI3K or mTOR inhibitors are being tested in leukemic patients [58-60].

PI3K/Akt/mTOR signaling upregulation is very common in T-ALL, being detectable in 70-85% of the patients [61] and portends a poorer prognosis [62]. Similarly to AML, multiple mechanisms could lead to PI3K/Akt/mTOR increased activity in T-ALL cells.

Much attention has been devoted to PTEN, since the initial report by Ferrando and coworkers documenting that PTEN gene expression was inactivated in T-ALL cell lines and patients displaying Notch-1 activating mutations, through a repressive mechanism mediated by Hairy and Enhancer of Split homolog-1 (HES-1) [63, 64]. In T-ALL cell lines, PTEN loss correlated with resistance to Notch inhibitors, raising concerns that patients with PTEN-negative disease could not respond to Notch inhibitor therapy [64]. However, it has been subsequently demonstrated that PTEN loss did not relieve primary T-ALL cells of their "addiction" to Notch-1 signaling [65]. It has been reported that PTEN downregulation 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 [66]. Furthermore, in a zebrafish model of T-ALL, c-Myc, which is typically overexpressed downstream of activated Notch-1 in T-ALL [67] caused PTEN mRNA downregulation [68].

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 [61].

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 [69, 70].

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. 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 Leukemia-Initiating Cell (LIC) activity [71].

In T-ALL, cytokines produced by the thymic/bone marrow microenvironment could be involved in upregulation of PI3K/Akt/mTOR signaling. These include interleukin (IL) -4 [72], and IL-7 [73, 74]. 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 [75]. A source for IL-7 could be represented also by thymic epithelial cells [76]. 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 [77].

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) [78]. CXCL12 is produced by bone marrow stromal cells in T-ALL patients [79] and has been recently demonstrated to be involved in PI3K/Akt activation and drug-resistance in T-ALL cells [80].

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 [73]. Thus, the pathophysiological relevance of MEK/ERK activation in T-ALL needs to be further investigated. In any case, MEK/ERK upregulation is observed in about 38% of adult T-ALL patients [81].

1.6 Therapeutic strategies acting on PI3K/Akt/mTOR network in ALL

The PI3K/Akt/mTOR pathway is also involved in drug resistance, sensitivity to therapy and metastasis [57]. PIK3Cα mutations may act as driver mutations in certain cancers responsible for metastasis [82]. Novel PI3Kα inhibitors have been isolated and they inhibit metastasis. Most PI3K inhibitors are cytostatic rather than cytotoxic and it has been questioned whether treatment with a single PI3K inhibitor will be effective [83].

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 [84].

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 [85]. 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.

It is well established that aberrant activation of the PI3K/Akt/mTOR cascade is associated with the pathogenesis of several types of hematologic malignancies [60], including B- and

T- ALL [86, 87]. Therefore, this pathway is an attractive target to efficiently treat B-ALL patients. In particular, PI3K isoforms are now regarded as key targets for the development of innovative therapeutic strategies. This pathway is also responsible for T-ALL survival and drug-resistance and could be targeted by small molecules inhibitors (SMIs) [50]. Aberrant regulation of the PI3K/Akt/mTOR axis often confers a proliferative advantage to tumor cells and contributes to the development of drug resistance mechanisms. At present only class I PI3Ks have been shown to be associated with cancer: indeed PIK3Cα, the gene which encodes for p110α PI3K, is mutated in a variety of tumor types [88, 89]. Mutations of the p85 subunit have also been shown to be oncogenic [90, 91]. Thus, targeting the PI3K/Akt/mTOR axis represents an attractive novel therapeutic strategy for ALL.

1.6.1 PI3K isoform inhibitors

Class I phosphatidylinositol 3-kinases (PI3Ks) comprises members of a conserved family of heterodimeric intracellular lipid kinases capable to activate Akt which in turn phosphorylates target proteins thus affecting cell growth, cell cycle progression, and cell survival [92, 93]. Class IA PI3Ks comprises a catalytic subunit (p110α, p110β, p110γ, p110δ) and a tightly bound regulatory subunit (p85α, p55α, p50α, p85β, or p55γ). The regulatory subunits maintain the integrity of the catalytic one and direct the heterodimer to membrane associated signaling complexes [94]. Currently several PI3K inhibitors are under development: ZSTK474, a specific pan-PI3K inhibitor [95], displays potent antitumor efficacy on various solid tumors (phase 1/2 of clinical trials) [96]. ZSTK474 inhibits class I PI3K isoforms with IC50 of 37 nM in a cell-free assay, mostly PI3Kδ. ZSTK474 treatment at 0.5 μM significantly decreases the level of phosphorylated Akt and GSK-3β, as well as the cyclin D1 protein expression. ZSTK474 also inhibits the phosphorylation of other downstream signaling components that are involved in regulating cell proliferation including FKHRL1, FKHR, TSC-2, mTOR and p70S6K in a dose-dependent manner. ZSTK474 does not inhibit mTOR at 0.1 μM and even at a concentration of 100 μM, ZSTK474 inhibits mTOR activity less than 40%. BYL719 is a specific class-IA PI3K inhibitor, which acts by binding the ATP binding domain of the p110α subunit [97]. BYL719 is a potent and selective PI3Kα inhibitor with IC50 of 5 nM in a cell-free assay and minimal effect on PI3Kβ/γ/δ (phase 2 of clinical trials) [98, 99]. Pharmacological blockade of both p110γ and p110δ reduced T-ALL proliferation and survival, indicating these isoforms as therapeutic targets for T-ALL treatment [100]. TGX221, is a p110β-selective inhibitor. AS605240 has been previously described as an

isoform selective ATP-competitive inhibitor of PI3Kγ [101] while CAL-101 is an oral p110δ inhibitor currently under clinical evaluation in patients with B-cell malignancies [102]. IPI145, an oral p110δ and p110γ subunits inhibitor with K_i and IC50 of 23 pM/243 pM and 1 nM/50 nM in cell-free assays, is highly selective for PI3K δ/γ than other protein kinases. IPI145, is reported to be in phase 3 study.

1.6.2 Opportunities and challenges in cancer with isoform-selective PI3K inhibitors

Isoform-selective and isoform-sparing PI3K inhibitors that preferentially inhibit the activity of one or more PI3K isoforms might circumvent the intrinsic toxicity associated with pan-PI3K inhibition and might be more permissive for exploration of combination therapies. Preclinical studies have shown that cancer cells bearing mutant PIK3Cα or HER2 amplification are frequently sensitive to p110α inhibition [99], whereas PTEN mutant or null tumors are more sensitive to p110β inhibition. However, increased dependency on p110β is not an obligate outcome of loss-of-PTEN function. In a mouse model of ovarian endometrioid adenocarcinoma driven by PTEN deletion and expression of oncogenic Kras, inhibition of p110α, but not p110β, was sufficient to prevent tumor growth. Similarly, ovarian epithelial cells deficient for PTEN and p53 were either p110β or p110α dependent, depending on the absence or presence of an oncogenic KRAS allele. Moreover, endometrial tumor cells deficient for PTEN, either with or without KRAS mutation, required dual p110α and p110β inhibition to decrease phospho-Akt levels and cell viability [103]. Non small cell lung carcinoma (NSCLC) cell lines bearing either PIK3Cα or PTEN mutation also exhibit resistance to isoform-selective inhibitors but retain responsiveness to pan-PI3K inhibitors [104]. Therefore, both tissue of origin and genomic context influence PI3K isoform dependence in cancer cells, making the elucidation of robust, predictive biomarkers for PI3K/mTOR pathway inhibitor responsiveness a daunting challenge for translational oncologists. PI3K isoform switching represents a well-documented mechanism of resistance to isoform-selective PI3K inhibitors. The Engelman group [105] found that inhibition of PI3K signaling in HER2-amplified or PIK3Cα mutant breast cancer lines by the p110α-selective inhibitor, BYL719, was followed by a rebound in PtdIns-3,4,5-P3 levels after 24 h. Reversal of the drug effect was attributable to activation of p110β and was associated with increased p110β recruitment to HER3. Interestingly, in mutant PIK3Cα-expressing cells, the rebound in PtdIns-3,4,5-P3 level was not associated with a corresponding increase in phospho-Akt (pAkt), suggesting that

changes in PtdIns-3,4,5-P3 levels are not obligatorily linked to downstream pathway activation. In breast cancer xenografts bearing overexpressed and mutationally activated PIK3Cα, tumor regression was only observed after treatment with a combination of p110α- and p110β-selective inhibitors. Similar results were reported by Baselga and colleagues, who found that treatment with a selective p110β inhibitor led to transient suppression of, followed by a significant rebound in, pAkt levels, which were attributed to the upregulation of the IGFR1-IRS1-p110α signaling cascade. Similarly, combined treatment with p110α- and p110β-selective inhibitors caused greater tumor growth inhibition in both a PTEN null prostate cancer and ER^+ breast cancer models [106]. In PTEN-deficient, ER^+ breast cancer xenografts, treatment with the triple combination of fulvestrant (a selective estrogen receptor degrader) together with inhibitors of p110α and p110β was required to trigger maximal, sustained tumor regressions. In contrast, treatment with the fulvestrant and p110β inhibitor doublet resulted in transient inhibition followed by a striking rebound in pAkt, cyclin D1/3 and phospho (pRb) levels. Collectively, these studies and others provide compelling support for the conclusion that the clinical activity of p110β-selective inhibitors is limited by the development of resistance due to isoform switching to p110α-mediated signaling. Parenthetically, it is noteworthy that an activating mutation in the human PIK3Cβ gene (encoding p110β D1067Y) leads to broad resistance to PI3K inhibitors; however, in contrast to PIK3Cα mutations, this mutation is rarely observed in cancer patients. Despite the evidence for p110α/p110β redundancy in preclinical models, growing clinical evidence points to the potential of p110α-selective inhibitors in defined patient populations. This large study will go a long way toward establishing whether the PI3K isoform-selective inhibitors will deliver both efficacy and acceptable safety in patients with tumors expressing mutationally activated p110α.

1.6.3 Clinical use of isoform-selective PI3K inhibitors in leukemia

Over the last few years, several pan and isoform-selective class I PI3K p110 inhibitors have been disclosed by pharmaceutical companies. They display favorable drug properties and suppress tumor growth in different preclinical models of cancer. Therefore, some of them have recently entered clinical trials, also for hematological malignancies [7].

PI3K inhibitors are an important new therapeutic option for the treatment of relapsed and refractory B-cell lymphoid malignancies. CAL-101 is a PI3Kδ inhibitor that has been approved for the treatment of lymphoma and chronic lymphocytic leukemia (CLL) in the relapsed/refractory setting and several other PI3K inhibitors are being developed targeting

other isoforms of the PI3K enzyme, which results in distinct toxicities and variable efficacy in the clinical setting [107]. For instance, it is known that Akt activation underlies Glucocorticoid (GC)-resistance in T-ALL via a direct phosphorylation of Glucocorticoid receptor (GR) at the Ser134 residue [108] and PI3K p110 inhibitors sensitized resistant MLL-rearranged ALL cells to prednisolone [109]. Therefore, PI3K p110 inhibitors are regarded as innovative drugs for the treatment of these types of blood cancer.

Although in T-ALL it has been investigated whether either pan or isoform-selective PI3K p110 inhibitors are more cytotoxic to leukemic cells [100, 110], similar studies have not been performed at all in B-ALL. Evangelisti et al (2017) used preclinical models of B-ALL to test the cytotoxic effects of both pan and isoform-selective PI3K p110 inhibitors. Moreover, using dexamethasone (DEX), a GC frequently used in B-ALL therapy, they investigated the ability of PI3K p110 inhibitors to improve the therapeutic efficacy of GCs and/or their capability to decrease GC-resistance. Their findings strongly advocated the use of PI3K p110 inhibitors for improving the clinical outcome of pediatric B-ALL patients.

A pan PI3K p110 inhibitor displayed the most powerful cytotoxic effects and synergized with DEX. However, also a dual γ/δ PI3K p110 inhibitor was effective in combination with DEX, even when used on DEX-resistant cells. Therefore, IPI145 could have a clinical relevance not only for B-ALL patients that respond to GCs by decreasing the drug dosage needed and adverse effects, but also for patients who do not respond to GCs. Moreover, the use of a dual inhibitor could spare the patients the side effects elicited by the inhibition of all the four PI3K p110 catalytic subunits.

IPI145 a potent inhibitor of both the γ and δ isoform with 25 mg BID being a satisfactory dose for CLL and is potentially active in the presence of BTK mutations [111]. IPI145 achieved an overall response rate (ORR) of 58% in a group of 49 heavily pretreated relapsed and refractory CLL patients, irrespective of the doses (25–75 mg BID). Several pan and isoform-selective class I PI3K p110 inhibitors have been disclosed by pharmaceutical compagnies over the last few years. They display favorable drug properties and suppress tumor growth in different preclinical models of cancer. Therefore, some of them have recently entered clinical trials, also for hematological malignancies [93].

1.6.4 Akt inhibitors

Drugs targeting the PI3K/Akt/mTOR cascade can interfere with diverse biologic processes also in healthy cells, thus rising concerns about their use in therapeutics. In particular, it is essential to gain knowledge about their effect on immune cells, as it would be desirable to

preserve patient's immunity. Akt inhibition may represent a potential therapeutic strategy in ALL. 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.

NVP-BGT226, is a novel class I PI3K/mTOR inhibitor is an ATP-competitive dual PI3K/ mTORC1/2 inhibitor used for treatment of advanced solid tumors [112]. As previously reported by Neri's group, it was cytotoxic to a panel of hepatocarcinoma cell lines under both normoxia and hypoxia conditions [113].

MK-2206 is an allosteric Akt inhibitor which inhibits both Thr308 and Ser473 phosphorylation. It also inhibits the downstream effects of insulin on Glut-4 translocation and glucose transport. MK-2206 decreased T-ALL cell viability by the 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 [114]. 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.

GSK690693 is an ATP-competitive pan Akt 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 effects of GSK690693 were 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 than 1 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 [115].

Perifosine [octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate] (KRX-0401, Keryx / AOI Pharmaceuticals, Inc. and licensed to Aeterna Zentaris) is a synthetic novel alkylphospholipid which inhibits the translocation of Akt to the cell membrane, blocking the growth of several different human cancers [116]. So, via its interference with the turnover and synthesis of natural phospholipids, disrupts membrane-linked signaling

pathways at several sites including lipid rafts, thereby inhibiting the PI3K/Akt survival network. The effects of perifosine have been examined on many different tumor types. Perifosine induces caspase-dependent apoptosis and downregulates P-glycoprotein expression in multidrugresistant T-ALL cells by a JNK-dependent mechanism [117]. 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 renal cell carcinoma (RCC) and multiple myeloma (MM). In the USA it has orphan drug status for the treatment of MM and neuroblastoma.

1.6.5 mTOR inhibitors

Rapamycin (Rapamune, Pfizer) is a macrolide, produced by the microorganism *Streptomyces hygroscopius* and showed antifungal properties. Shortly after its discovery, immunosuppressive properties were detected, which later led to the establishment of rapamycin as an immunosuppressant. In the 1980s, rapamycin was also found to have anticancer activity although the exact mechanism of action remained unknown until many years later [118]. In the 1990s there was a dramatic change in this field due to studies on the mechanism of action of rapamycin and the identification of the drug target [119]. It was found that rapamycin inhibited cellular proliferation and cell cycle progression. It was approved by the U.S. Food and Drug Administration (FDA) in 1999 to prevent rejection in organ transplant patients. Rapamycin/rapalogs act as allosteric mTORC1 inhibitors and do not directly affect the mTOR catalytic site [59]. They associate with the FK506 binding protein 12 (FKBP-12) and by so doing, they induce disassembly of mTORC1, resulting in repression of its activity [120].

The rapalogs have been examined in clinical trials with patients having various cancers including: brain, breast, hepatocellular carcinoma (HCC), leukemia, lymphoma, MM, non-small cell lung cancer (NSCLC), pancreatic, prostate, and RCC [121]. Furthermore rapamycins are being considered as anti-aging and anti-obestity drugs as well as to prevent diabetic neuropathy [122].

The rapalogs includes temsirolimus (CCI-779) and everolimus (RAD001), which are being evaluated in cancer clinical trials [123].

Temsirolimus has higher water solubility than rapamycin and is ester analogue of sirolimus that is rapidly converted to the parent compound after intravenous administration therefore administrated by intravenous injection. The rapalogs everolimus (40-O-(2-hydroxyethyl)-

rapamycin) is the second novel rapamycin analog [118] approved by FDA for the treatment of advanced renal cell carcinoma, subependymal giant cell astrocytoma (SEGA) associated with Tuberous Sclerosis (TS) and Progressive neuroendocrine tumors of pancreatic origin (PNET), as single agent therapy and for the treatment of hormonereceptor positive breast cancer as combination therapy with exemestane [124].

It has been recently explored the therapeutic potential of RAD001, in preclinical models of pre-B ALL [4]. It has documented that RAD001 decreased cell viability, induced cell cycle arrest in G0/G1 phase and caused apoptosis in B-pre ALL cell lines. Autophagy was also induced, which was important for the RAD001 cytotoxic effect, as downregulation of Beclin-1 reduced drug cytotoxicity. RAD001, used in the micromolar range and administered 24 h before MK-2206 showed the capacity to synergize with MK-2206 in both cell lines and patient samples [4].

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

The mTORC1/mTORC2 dual inhibitors are the second generation of mTOR inhibitors designed to compete with ATP in the catalytic site of mTOR (ATP-competitive kinase inhibitors). They inhibit all of the kinase-dependent functions of mTORC1 and mTORC2 and therefore, block the feedback activation of PI3K/Akt signaling, unlike rapalogs that only target mTORC1 [125]. 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 [126]. These types of inhibitors have been developed and several of them are being tested in clinical trials. Like rapalogs, they decrease protein translation, attenuate cell cycle progression and inhibit angiogenesis in many cancer cell lines and also in human cancer.

In fact they have been proven to be more potent than rapalogs [125]. 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. It potently target mTORC1-dependent T389 phosphorylation on S6K. Torin-2 also exhibited potent biochemical and cellular activity against PIKK family kinases including ATM, ATR and DNA-PK, the inhibition of which sensitized cells to irradiation. Similar to the earlier generation compound Torin1 and in contrast to other

reported mTOR inhibitors, Torin2 inhibited mTOR kinase and mTORC1 signaling activities in a sustained manner suggestive of a slow dissociation from the kinase.

1.7 The effectiveness of ALL treatment by multiple targeting the PI3K/Akt/mTOR signaling pathway

The obvious goal of current inhibitor development is to improve the effectiveness of treatment of cancer patients with small molecule signal transduction inhibitors. However, this has proven to be difficult for multiple reasons: first, there is a distinct genetic susceptibility for the success of a signal transduction inhibitor in suppressing cellular growth and proliferation, second, many of the small molecule signal transduction inhibitors are cytostatic as opposed to being cytotoxic and therefore they will need to be combined with a therapeutic modality that induces cell death, and third, more than one signal transduction pathway may be activated in the cancer cells.

Rapalogs have shown objective responses in only a subset of patients and unfortunately the responses are frequently shortlived. Mechanisms of acquired resistance to rapalogs are unknown. These therapeutic failures have been attributed, in part, to KRAS or BRAF mutations. Since KRAS is frequently mutated in human cancer, many cancers will have constitutive mTOR activity, but may not be sensitive to rapamycin as they will have Raf/MEK/ERK pathway activation. Since rapalogs function by binding FKBP-12, mutations in FKBP12 or the FKB domain of mTOR can suppress binding affinity and lead to rapalog resistance [127]. Direct mTOR inhibitors will overcome this resistance. The presence of the IGF1R/PI3K-mediated feedback loop, which results in ERK activation, is another mechanism of resistance to rapamycin rapalogs [51, 59, 85, 128].

Resistance to rapamycin has been also associated with rapamycin-induced Akt activation, as a result of inhibition of the S6K/IRS-1 feedback loop. Rapamycin not only inhibits S6K phosphorylation but also induces Akt Ser473 phosphorylation, hence activating Akt. Therefore, there is a growing interest in multi-component target therapies: the combined delivery of multiple drugs is an attempt to overcome drug resistances and to improve clinical outcome. Approaches to prevent Akt activation, such as the use of specific inhibitors, are being pursued [129]. However, an alternate approach is to target this pathway with mTOR kinase inhibitors that potently inhibit mTORC1 as well as mTORC2, thus inhibiting Akt Ser473 phosphorylation, and thereby preventing or attenuating the feedback loop activation of Akt and potentially treating PI3K/Akt/mTOR dependent cancers more effectively [130]. Another approaches is targeting simultaneously both Akt

and mTOR protein, in order to obtain a more complete inhibition of the pathway, without the feedback loop activation of Akt.

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

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

1.8 Advances of clinical trials in ALL

In ALL disease, progress has been achieved in academic clinical trials by optimization of combined chemotherapy, which continues to be the mainstay of contemporary treatment. The improvement of prognosis in childhood ALL is one of the most successful stories of modern medicine [2].

Scientists are conducting research strategies and clinical trials that hold the promise of increasing remission and cure rates of ALL patients. Specific agents under study in clinical trials for ALL are:

Bortezomib (Velcade) (proteasome inhibitor), was approved to treat myeloma and some types of lymphoma (phase 1/2), is now being studied in combination with other drugs such as belinostat for the treatment of relapsed or refractory ALL. It is also being studied for treating newly diagnosed pediatric patients with T-ALL cell.

Clofarabine (Clolar) (antimetabolite), it is already approved to treat pediatric ALL (phase 2), clofarabine is now showing promising results in studies of adults with ALL. It is also being studied in combination with other drugs such as mitoxantrone in clinical trials for the treatment of children whose ALL is relapsed or refractory.

Nelarabine (Arranon) is a type of antimetabolite drug, is approved for patients who have relapsed T-ALL cell (phase 3). It is now being studied in clinical trials in combination with other agents for the treatment of relapsed or refractory T-ALL cell. It is also being evaluated in combination with other drugs as part of an induction regimen for untreated T-ALL cell.

Ruxolitinib (Jakafi) (Janus kinase (JAK) inhibitor), is already approved to treat myelofibrosis and polycythemia vera patients, it is being studied in clinical trials in the treatment of pediatric refractory and relapsed ALL (phase 3). It is also being studied in combination with several chemotherapy drugs in the treatment of children with Ph^- like ALL and cytokine receptor like factor 2 (CRLF2) and JAK mutations.

Augmented Hyper-CVAD (cyclophosphamide, vincristine, doxorubicin and dexamethasone) is a well-established regimen for ALL. The augmented hyper-CVAD formulation was designed in 2011 and it includes intensified doses of vincristine, dexamethasone and asparaginase. Researchers are studying the efficacy of this combination for ALL treatment.

In immunotherapy, there are under study monoclonal antibodies, such as: rituximab (Rituxan), alemtuzumab (Campath), ofatumumab (Arzerra). These drugs are already approved in the treatment of other blood cancers. They are currently being studied for their use in combination with chemotherapy in clinical trials for untreated and relapsed/ refractory ALL. Blinatumomab (Blincyto), this drug is a bispecific, anti-CD19, CD3 T-cell engager, approved for the treatment of relapsed or refractory Ph^- B-cell precursor ALL. It is being studied in current trials for the treatment of refractory and relapsed ALL and also as therapy for older patients with newly diagnosed disease. Inotuzumab ozogamicin, this drug is an anti-CD22 monoclonal antibody that is bound to a toxic drug called calicheamicin. It is being studied, as part of a regimen with combination chemotherapy, in the treatment of relapsed and refractory ALL. Chimeric antigen receptor (CAR) T-cell therapy, is a type of immunotherapy that consists of engineering patients' own immune cells first to recognize and then to attack cancerous tumors. This approach has shown very promising results in patients with blood cancers. The T cells are genetically engineered to produce receptors on their surface called "chimeric antigen receptors" (CARs). These receptors recognize and bind to a specific target found on the cancerous cells. Clinical trials are studying the use of CAR T-cell therapy in the treatment of chemotherapy-resistant or refractory ALL in both adults and children.

1.9 Abl1 fusion genes in ALL

The Philadelphia (Ph) chromosome harboring the t(9;22) (q34;q11) translocation and the ensuing fusion gene Bcr-Abl1 lead to an aberrant cell proliferation (Fig. 4). Bcr-Abl1 is the most common cytogenetic abnormality and the most unfavourable prognostic factor in ALL [131], where 20–30% of them express the Bcr-Abl1 oncogene [132].

In the Philadelphia chromosome, the breakpoint may occur within one of four sites on the Bcr gene to yield three proteins of different sizes: p190, p210, and p230. The p190 Bcr-Abl1 fusion protein occurs in about 90% of children and between 50% and 80% of adults with Ph^+ pre-B ALL. The p210 Bcr-Abl1 gene constitutes the rest of the Ph^+ pre-B ALL population, while p230 characterizes chronic myelogenous leukemia (CML) [132]. Until

recently, Ph^+ pre-B ALL patients treated with conventional chemotherapy carried a very poor prognosis irrespective of their age (approximately 10% survival at 5 years). However, the outcome for patients with Ph^+ pre-B ALL has improved substantially with the introduction of the tyrosine kinase inhibitor (TKI) Imatinib in combination with chemotherapy. Second generation TKIs (dasatinib, nilotinib) have displayed a promising activity in Ph^+ B-ALL cases that developed resistance to Imatinib due to Bcr-Abl1 mutations, although there are Bcr-Abl1 mutations, such as T315I, that are resistant to these novel TKIs [133].

In Ph^+ pre-B ALL, the Bcr-Abl1 tyrosine kinase is upstream of the PI3K/Akt/mTOR pathway [134]. Bcr-Abl1 associates with a number of proteins (c-Cbl, Shc, GRB-2, and GAB-2) that bind the p85α subunit of PI3K [135], resulting in its activation. Accordingly, the Bcr-Abl1 inhibitor Imatinib downregulated mTORC1 activity in Ph^+ CML cells while Ph^+ pre-B ALL cell lines were hypersensitive to rapamycin [136].

PI3Ks play a key role in Bcr-Abl1-dependent models of murine leukemogenesis. Indeed, it was possible to create mice that had Pik3r1(p85α/p55α/p50α) deleted specifically in the B-cell lineage and Pik3r2 (p85β) deleted in all cells. As a consequence, there was decreased p190 Bcr-Abl1-mediated *in vitro* colony transformation of both α- and α-/β- progenitor B-cells. Moreover, $p190^+$/α-/β- B-cells displayed a severe loss of leukemogenic potential *in vivo* [94]. However, it was found that either genetic or pharmacological (wortmannin, LY294002) inhibition of PI3K only partially reduced mTORC1 activity, as assessed by phosphorylation of S6RP in these cells. To explore the mechanism of PI3K/Akt-independent mTORC1 regulation, the authors investigated the role of two other potential mTORC1-controlling pathways: MEK/ERK and amino acid sensing. Basal ERK phosphorylation was consistently elevated in α-/β- leukemic colony forming cells (L-CFCs) and blocked by treatment with a MEK inhibitor [94]. Nevertheless, MEK inhibition did not affect mTORC1 activity, as judged by phosphorylation of 4E-BP1, while p-S6RP levels were modestly reduced in both control and α-/β- L-CFCs, most likely due to stimulatory effects of ERK on p70S6K. When the contribution of amino acid sensing by withdrawal of leucine from the culture media was assessed, mTORC1 activity was rapidly extinguished in α-/β- L-CFCs, as reported in other cell systems. Amino acid sensing by mTORC1 was promoted by class III PI3K (hVPS34), an enzyme whose activity is sensitive to wortmannin [78]. This might explain the partial inhibition of mTORC1 signaling by wortmannin in α-/β- L-CFCs that lack class IA PI3Ks. Therefore, residual mTORC1 activity in α-/β- L-CFCs was MEK/ERK-independent and sustained by amino acid sensing and, perhaps, other pathways that remain to be defined [94].

However, there are some Bcr-Abl1-independent mechanisms of PI3K activation that resulted in Imatinib resistance [137] but they have not been analyzed thoroughly. Another reason for enhanced PI3K/Akt/mTOR signaling in Ph^+ B-pre ALL is due to the fact PP2A is functionally inactivated during the blast crisis of chronic myelogenous leukemia through the inhibitory activity of SET protein, which is regulated by Bcr- Abl1 [138]. Reactivation of PP2A activity by FTY720 (fingolimod, a PP2A activator which has been approved as an immunomodulator for oral use in patients with multiple sclerosis [139], led to leukemic cell growth suppression, enhanced apoptosis, impaired clonogenicity, and decreased *in vivo* leukemogenesis of Imatinib- and dasatinib-sensitive and -resistant Ph^+ B-pre ALL cells, as well as Ph^+ pre-B ALL progenitors ($CD34^{+/}CD19^+$). Importantly, healthy $CD34^+$ and $CD34^+/CD19^+$ bone marrow cells were unaffacted by FTY720. Moreover, pharmacologic doses of FTY720 suppressed *in vivo* Bcr-Abl1-driven leukemogenesis (including leukemogenesis promoted by the T315I Bcr-Abl1 mutant which is resistant to Imatinib and second generation TKIs) without exerting any toxicity in mice [140]. In Ph^- pre-B ALL cases, the mechanisms for PI3K/Akt/mTOR upregulation are unclear, however, they could be dependent on activation of signaling downstream of cytokine receptors, through interactions of leukemic cells with bone marrow stromal cells [141]. Interestingly, pediatric pre-B ALL patients with high expression of VLA-4 displayed an adverse outcome, which might be related to activation of PI3K/Akt/mTOR signaling [141]. Moreover, gain-of-function mutations in IL-7R have been identified in pediatric Ph^- pre-B ALL cases [142] that could account for pathway activation. Very recently, it has been shown that ETV6/RUNX1 silencing abrogated PI3K/Akt/mTOR signaling in pediatric precursor B-ALL, however, no mechanistic explanation for this phenomenon was presented [86]. The selective Bcr-Abl1 tyrosine kinase inhibitor Imatinib deeply changed the pharmacological treatment of another form of Ph^+ cancer, i.e. chronic myeloid leukemia (CML), by inducing long lasting remissions. Unfortunately, Imatinib showed much less efficiency in treating Ph^+ ALL and although unrelated to the Bcr-Abl1 kinase domain alterations, the underlying mechanisms are largely unknown. Nilotinib (The second generation TKI) shows both a stronger potency than Imatinib and also acts against most Imatinib unresponsive Bcr-Abl1 mutation variants. A new, third generation orally bioavailable Bcr-Abl1 inhibitor, GZD824, has been recently developed, with potency against a wide range of Bcr-Abl1 mutants [143]. In general, Ph^+ B-ALL is less sensitive to TKIs that CML. Therefore, novel drugs are needed to improve response rates and to circumvent TKI-resistance in Ph^+ B-ALL. GZD824 strongly inhibited the proliferation of

human leukemia cells harboring Bcr-Abl1, including K562 and KU-812 CML cell lines as well as SUP-B15 B-ALL cells, with IC50 values in the nanomolar range [143].

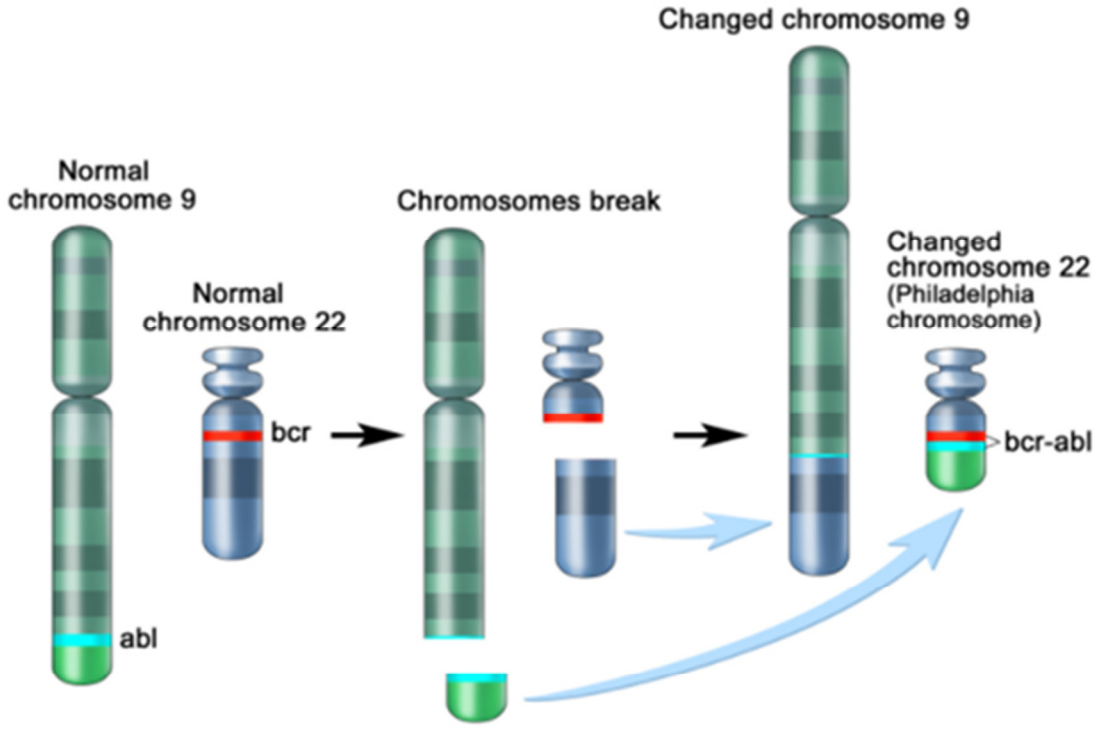


Figure 4: Bcr-Abl1 fusion gene formation. A piece of chromosome 9 and a piece of chromosome 22 break off and trade places. The bcr-abl1 gene is formed on chromosome 22 where the piece of chromosome 9 attaches. The changed chromosome 22 is called the Philadelphia chromosome.

1.10 miRNA features related to ALL development

miRNAs are small noncoding RNA molecules, ranging in length from 20 to 25 nucleotides, which primarily bind to the 3′ untranslated region (UTR) of messenger RNAs, resulting in a downregulation of target proteins through the degradation of this mRNA or through translational inhibition [144]. In cancer, miRNAs have been demonstrated to play critical roles by modifying or controlling all major hallmarks including cell division, self-renewal, invasion, and DNA damage among others [145].

The aberrations in the expression of miRNAs involved in malignant lymphopoiesis, can be used as signatures of different ALL subtypes. Moreover, changes in the expression of several miRNAs may have functional relevance with leukemogenesis or drug resistance. As a result, the reversal of the expression of these miRNAs may alleviate the disease to some extent and improve clinical outcomes [19]. Based on the relative expression of miR-148, miR-151, miR-424, miR-425-5p, miR-191, miR-146b, miR-128, miR-629 and miR-

126, the B and T lineages of ALL can be distinguished [146]. Analysis of over 430 miRNAs in 50 clinical T-ALL samples have revealed a common signature: miR-223, miR-19b, miR-20a, miR-92, miR-142-3p, miR-150, miR-93, miR-26a, miR-16 and miR-342. miR-19b,-20a,-26a,-92 and 223 have been shown to target T-ALL tumor suppressors such as IKAROS, PTEN, BIM, PHF6, NF1 and FBXW7 and lower expression of miR-223 has been reported in ALL cells isolated from pediatric patients and cell lines (Table 1). miR-612 and miR-499 have significant correlations with ALL susceptibility [147]. Target gene of MYC, TFs of CDX2 and lncRNA of XIST may play important roles in the development of B-ALL, serving as a potential therapeutic target [148]. Gain of function mutations in NOTCH1 are predominantly found in T-ALL, but recent evidence implicate NOTCH1 and NOTCH2 also in subsets of mature B-cell malignancies [149, 150]. Despite the fact that MYC is more prominently linked to B cell lymphoma biology, its relevance to T-ALL pathogenesis is well established, at least in part due to NOTCH1 ability to induce MYC expression [148, 151]. The miR-30 family represents a candidate for a putative MYC-dependent regulation of NOTCH1 and NOTCH2 expression. It has been confirmed that MYC negatively influences miR-30a expression and that this miRNA directly targets NOTCH1 and NOTCH2 [152] (Table 1). Using genetic and pharmacological models, it has been characterized a regulatory loop, where by the MYC-mediated inhibition of miR-30a de-represses NOTCH, eventually modulating its own expression [153].

miRNA	Function	Putative target	miRNA expression level in ALL compared to normal samples	References
miR-150	Transition from pro-B cell to pre-B cell	C-MYB	High	Zhang H. et al., 2009.
miR-155	Differentiation of T-cells into Th type 1 cells	PTEN	High	Zhang H. et al., 2009.
miR-181	B-cell differentiation T-cell development	BCL-2, MYC, CDX2, CD69, EGR1 and T-cell receptor	High	Zhang H. et al., 2009.
miR-17-92	Transition from pro-B cell to pre-B cell	CYLD, HOXA9, BIM, RUNX1 and MYC	High	Zhang H. et al., 2009.
miR-126	B-ALL development	MYC and CDX2	High	Zhang H. et al., 2009.
miR-223	Reduce cell growth and T-ALL development	IKAROS, PTEN, BIM, PHF6, NF1 and FBXW7	Low	Mavrakis KJ. et al., 2011; Schotte D. et al., 2011.
miR-19b	T-ALL development	IKAROS, PTEN, BIM, PHF6, NF1 and FBXW7	High	Mavrakis KJ. et al., 2011.
miR-20a	T-ALL development	IKAROS, PTEN, BIM, PHF6, NF1 and FBXW7	High	Mavrakis KJ. et al., 2011.
miR-92	T-ALL development	IKAROS, PTEN, BIM, PHF6, NF1 and FBXW7	High	Mavrakis KJ. et al., 2011;
miR-26a	T-ALL development	IKAROS, PTEN, BIM, PHF6, NF1 and FBXW7	High	Mavrakis KJ. et al., 2011;
miR-193a	Activation of PI3K cascade	PTEN	High	Bertacchini J. et al., 2015; Schotte D. et al., 2009.
miR-21	Regulation of PI3K/Akt/mTOR pathway	PTEN	High	Bertacchini J. et al., 2015.
miR-99a	Differentiation of human granulocyte and monocyte cells	mTOR, IGF-1 and MCL1	Low	Li Y. et al., 2012; Zhang H. et al., 2009; Li XJ. et al., 2013; Schotte D. et al., 2009.
miR-100	Differentiation of human granulocyte and monocyte cells	mTOR, IGF-1 and MCL1	Low	Zhang H. et al., 2009; Schotte D. et al., 2009; de Oliveira JC. et al., 2012.

miR-19	T-ALL development	PTEN, HOXA, CYLD, BIM and NOTCH1	High	Mavrakis KJ. et al., 2011;
miR-30a	B-ALL development	NOTCH1, NOTCH2 and MYC	High	Ortega M. et al., 2015; Schotte D. et al., 2009.
miR-29a	B-ALL development	MYC and CDX2	Low	Zaidi SK. et al., 2017; Zhang H. et al., 2009.

Table 1: miRNA involvement in ALL disease. Function, target genes and expression level of miRNAs implicated in B- and T-ALL development and differentiation process compared to normal samples.

1.11 miRNAs and their signature in PI3K signaling pathway in ALL

miRNAs are important regulators of key genes in the PI3K/Akt/mTOR pathway, suggesting miRNAs as a new therapeutic target also in leukemia (Fig. 5) [154]. It was shown in promyelocytic cell line NB4 that miR-223 is able to reduce cell growth by the inhibition of the insulin-like growth factor 1 receptor (IGF1-R), blocking PI3K signal [155]. Instead, miR-193-a is downregulated, determining the activation of the PI3K cascade, through the inhibition of the tumor suppressor PTEN [152]. miR-22 targets PTEN directly through a conserved site on the PTEN 3′UTR and it is upregulated by Akt, suggesting that miR-22 forms a feed-forward circuit in this pathway. A recent study revealed that miR-22 accelerated Akt activity upon growth factor stimulation, and attenuated its down regulation by serum withdrawal. Thus, in this regulatory network, miR-22 acts to enhance Akt signaling [156]. Also miR-21 is involved in the regulation of PI3K/Akt/mTOR pathway and the expression of this miRNA is correlated to drug resistance by downregulation of PTEN [157] (Fig. 5). The three members of the miR-29 family, -a,-b and-c, function as tumor suppressor in different tumors, including leukemia [146, 158]. The downregulation of miR-99-a and miR-110 is observed in ALL patients and their expression inhibit cell proliferation in ALL cell lines. This inhibition is due by two prosurvival effectors such as mTOR and IGF-1 [159] suggesting that these two miRNAs have different roles in myeloid cell and lymphocyte pathogenesis [160]. miR-100 and miR-99a overexpression inhibit IGF1R and mTOR and down-regulated MCL1 (Induced myeloid leukemia cell differentiation) [152] (Fig. 5). This evidence suggested that miR-100 and miR-99a act as tumor suppressors and their restoration might be a possible therapeutic strategy for patients with ALL [161, 162]. Finally, it has been also demonstrated that miR-221 negatively regulated PTEN by binding to its 3'UTR leading to inhibition of PTEN translation and activation of Akt pathway. Moreover, BCL-2, CCND1

and p27, downstream genes of pAkt, were regulated by miR-221 (Fig. 5). Therefore this miRNA is able to induce cell survival through targeting the PI3K/PTEN/Akt pathway and could be detected as a promising gene with its oncogene role [163].

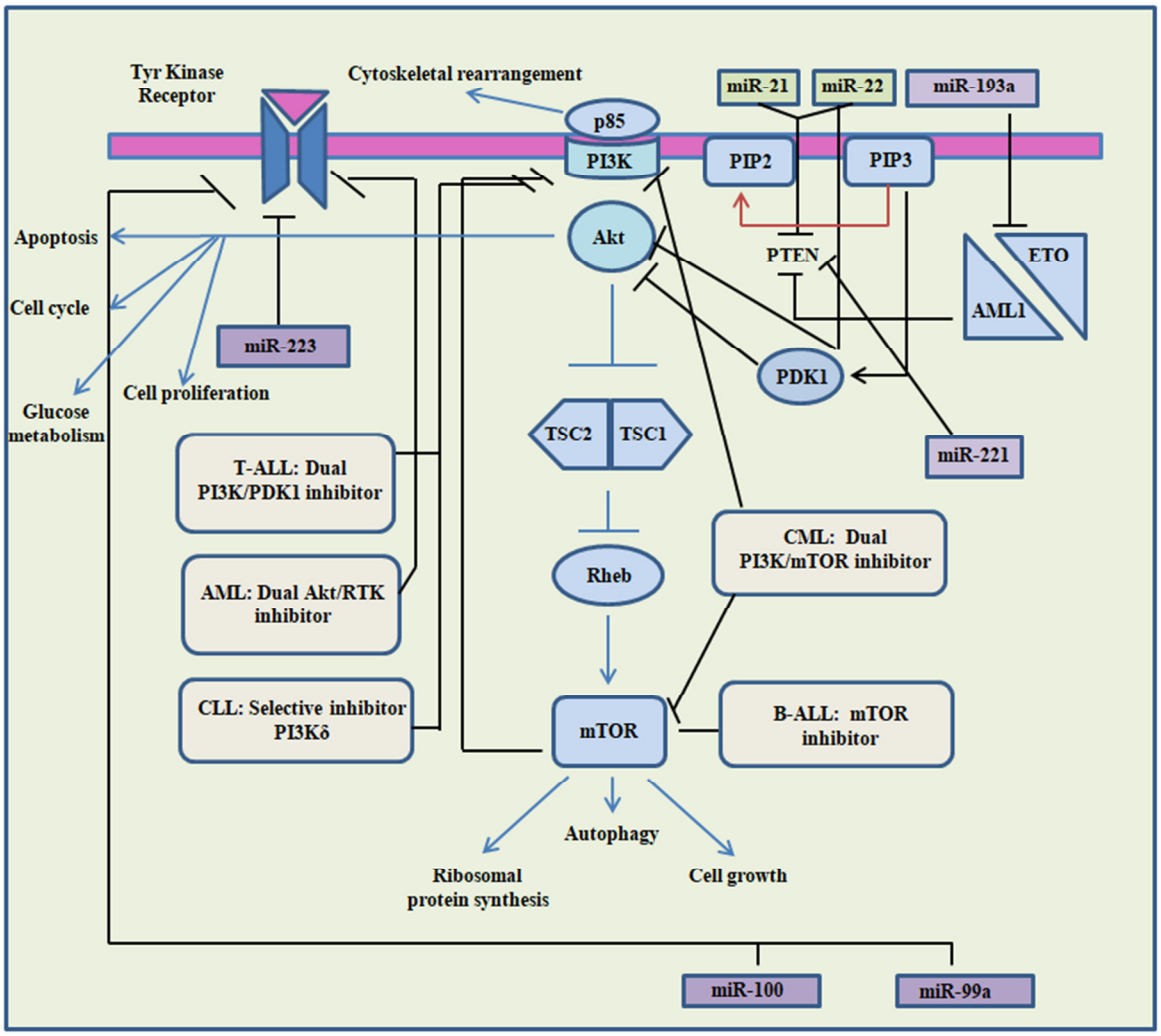


Figure 5: The effect of miRNA-mediated PI3K/Akt/mTOR signaling on leukemias. Scheme of the effector miRNAs and PI3K/Akt/mTOR pathway inhibitors in different leukemias. miR-21 and miR-22 are activator miRNA, and miR-99a, miR-100, miR-193a and miR-223 are inhibitor miRNA of the PI3K/Akt/mTOR pathway.

1.12 Role of miRNAs in ALL prognosis

miRNA signatures can be used not only in the diagnosis of the ALL, but also in their prognosis. Several miRNAs, involved in cell proliferation and apoptosis regulation, are implicated in leukemogenesis and they may interfere with either oncogenic or tumor-suppressor pathways influencing the prognosis of patients. Cellular miR-92a expression was significantly increased in a subset of ALL cells and ALL patients with overexpression

of miR-92a had poor prognoses (Table 1 and Table 2). Compared with peripheral blood mononuclear cells from healthy volunteers, the cell-to-plasma ratio of miR-92a expression was particularly higher in both ALL and AML cells (Table 1) [164].

It has also been suggested that expression level of miRNAs could be used as indicators of prognosis in children with ALL, such as higher expression of miR-128b at diagnosis predicted a better prognosis and prednisolon response [165]. A study of 147 patients with acute leukemia and 100 healthy individuals showed that acute leukemia (including both ALL and AML) patients with high miR-24 expression had shorter overall survival [166]. In B- and T-ALL, patients with high miR-16 had a significantly shorter disease-free survival (DFS) [167]. In another study the overall survival rate in miR-16 high-expression group decreased, thus showing a worst prognosis [168].

miRNA	**miRNA expression in normal BM (n=11) Median (range)**	**miRNA expression in ALL (n=128) Median (range)**	**P-Value (p<0.05) (calculated by the Mann-Whitney test)**
miR-92a	0.97 (0.45-3.91)	0.77 (0.08-5.04)	0.07
miR-100	1.04 (0.34-3.90)	0.21 (0.002-10.76)	< 0.01
miR-128a	1.02 (0.57-2.23)	4.32 (0.16-167.91)	< 0.01
miR-181b	1.41 (0.33-3.40)	5.06 (0.07-45.09)	< 0.01
miR-196b	1.07 (0.29-3.70)	0.03 (0.001-65.68)	< 0.01

Table 2: Clinical correlations related to miRNA expression in ALL samples. de Oliveira et al (2011) have shown that miRNAs studied presented a wide variability of expression in ALL patients compared to BM samples.

1.13 Therapeutic implications of miRNAs in ALL

The recent advances in understanding of the role of miRNAs in lymphoid malignancies demonstrate that miRNAs can effectively be used as tumor biomarkers with diagnostic, prognostic and predictive-of-response-to-therapy implications [169]. Glucocorticoids (GCs) are used in the therapy of ALL and related malignancies and they are able to induce apoptosis in lymphoid lineage cells. However, a proportion of patients with ALL are insensitive to prednisone. It has been shown that eight miRNAs (miR-18a, miR-532, miR-218, miR-625, miR-193a, miR-638, miR-550, and miR-633) can help to distinguish the patients sensitive from those insensitive to prednisone [170].

Both miR-128b and miR-221 are downregulated in MLL-rearranged ALL and it has been hypothesized they may reduce GCs sensitivity [171]. Increase of miR-128b downregulates target genes including MLL, AF4 and their fusion oncogenes, whereas the expression of miR-221 downregulates CDKN1B. Thus, the sensitivity of two cultured lines of MLL-AF4

ALL cells to GCs is strengthened. In a subsequent study, researchers illustrated that one novel mutation of miR-128b significantly reduced its processing, and the resultant downregulation of mature miR-128b gave rise to GCs resistance due to the failure to downregulate the fusion oncogenes. By regulating miRNAs, therapeutic effect of GCs may be improved [19]. Harada and colleagues in 2012 transiently overexpressed miR-17 in the pre-B ALL cell line (SUP-B15) and monitored the dexamethasone-induced levels of apoptosis. They found that overexpression of miR-17 reduced dexamethasone-induced cell death. On the contrary, inhibition of miR-17 through locked nucleic acid (LNA) inhibitor, increased dexamethasone sensitivity [172].

It has been found that miR-142-3p decreased NR3C1 protein expression and led to GC resistance by promoting T-leukemic cell proliferation but not apoptosis in ALL [173].

Liang et al (2017) firstly reported in ALL that miR-124 also targeting NR3C1 could enhance the resistance of prednisone sensitive CCRF-CEM cells to dexamethasone and inhibits cell apoptosis, thus showing a similar mechanism for GC resistance [174].

For ALL with Bcr-Abl1 fusion gene, the application of TKI may be a promising strategy, but the prognosis remains suboptimal [175]. Bcr-Abl1 and Abl1 are the direct targets of miR-203, which is silenced by genetic and epigenetic mechanisms in hematopoietic malignancies expressing either Abl1 or Bcr-Abl1 and the restoration of miR-203 expression reduces Abl1 and Bcr-Abl1 levels and inhibits cell proliferation [175, 176].

The inhibition of DNMT3A by forced expression of miR-217 may prevent drug resistance to TKI treatment in Philadelphia-chromosome-positive ALL patients. Hence, it may indicate another therapeutic strategy for Bcr-Abl1-positive ALL [177].

Demethylation could be a potential therapeutic strategy for ALL. In the MLL-AF4 ALL, miR-143 is epigenetically repressed by promoter hypermethylation in MLL-AF4-positive primary blasts and cell lines, but not in normal BM cells and MLL-AF4-negative primary blasts. Meanwhile, miR-143 was identified as a regulator of MLL-AF4 expression, and its restoration could induce apoptosis, negatively contributing to leukemia cell growth. Therefore, upregulation of miR-143 expression has therapeutic promise for MLL-AF4 B-cell ALL [178].

The α isoform of Protein kinase C (PKCα), has long been recognized as a regulator of tumor growth in a variety of cancers [179]. Increasing interest targeting this kinase isoform for cancer therapy has developed. Targeting PKCα-mediated signal transduction induces cell death in AML cells inhibiting BCL-2 phosphorylation and blocking ERK activation. Fang et al (2016) showed that overexpression of miR-150 significantly repressed

endogenous expression of PKCα and luciferase reporter/mutagenesis assays confirmed that PRKCα is a transcriptional target of miR-150.

2. AIM OF THE STUDY

The PI3K/Akt/mTOR signaling pathway is often activated in leukemias and plays a crucial role in leukemogenesis. The main goal of this research was to investigate the effect of PI3K signaling pathway inhibitors as a new therapeutic approach in ALL, with the following specific aims:

1) Analyze the cytotoxic effect of the PI3K signaling inhibitors on healthy human primary $CD4^+$ T-cells and T-ALL cell lines;

2) Verify the use of specific inhibitory compounds direct against key proteins of the PI3K pathway, such as Akt and mTOR proteins, not only as single agents, but also investigating strategies involving their combination for multiple hit of the signaling cascade at different levels in T-ALL cell lines;

3) Perform an analysis about the efficacy of these inhibitors on miRNA expression level implicated in ALL disease and PI3K activation;

4) Analyze the efficacy of Imatinib, Nilotinib and GZD824 alone and in combination with several PI3K/Akt/mTOR drugs in T-ALL cell lines harboring Nup214-Abl1 fusion gene;

5) Investigate the effects of a panel of PI3K isoform inhibitors in Ph^+ B-ALL cell lines by combining their anti-tumor activity with anti Bcr-Abl1 drugs.

3. MATERIALS AND METHODS

3.1 Materials

RPMI-1640 and McCoy's 5A medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Lonza Milano SRL (Milan, Italy). NVP-BGT226, MK2206, Torin-2, RAD001, Perifosine, GSK690693, XL-147, KU0063794, CCI-779, AZD8055, ZSTK474, BYL719, TGX221, AS605240, CAL101, IPI145, Imatinib, Nilotinib and GZD824 were obtained from Selleck Chemicals (Houston, TX, USA). For cell viability determination, CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Milan, Italy). Annexin V/7-ADD detection kit was from Merck-Millipore (Darmstadt, Germany). Western blot antibodies for total Akt-1, Ser473 p-Akt-1, Thr308 p-Akt-1, Tyr 202/204 p-ERK 1/2 and ERK 1/2 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. Bafilomycin A1, chloroquine,3-Methyladenine (3- MA), Z-VAD-fmk, Ficoll-Paque Plus and phytohemagglutinin and the mouse secondary antibody and the monoclonal β-Actin antibody were purchased from Sigma Aldrich (Milan, Italy). Dynabeads 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 culture

The B-ALL cell lines REH wt, REH shp53 and NALM-6 were obtained from Calin's Lab (The MD Anderson Cancer Center, Texas, USA). The T-ALL cell lines JURKAT, MOLT-4, CEM-S (drug sensitive), CEM-R (CEMVBL100, drug-resistant cells overexpressing 170-kDa P-glycoprotein), ALL-SIL, PEER, BE13 and the SUP-B15 Ph^+ B-ALL were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). BV173 and TOM-1 Ph^+ B-ALL cell lines were obtained from Dr. Fabrizio Pane's laboratory at University of Naples, Italy. The cells were grown in RPMI 1640 medium supplemented with 20% heat-inactivated FBS, 100 units/ ml penicillin and 100 mg/ml streptomycin. SUP-B15 was maintained in McCoy's 5A medium supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin. REH wt, REH shp53, NALM-6, JURKAT, CEM-S, CEM-R 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 The cells were grown at a density of 0.5 to 2 x 10^6 cells/ml and were incubated at 37°C with 5% CO_2.

3.3 Primary samples

Peripheral Blood $CD4^+$ T lymphocytes from healthy donors were obtained with informed consent according to institutional guidelines and isolated with Ficoll-Paque and magnetic beads labelling protocols (Dynabeads, Monza MI, Italy). Whole blood or buffy coat were diluted with PBS containing 0.1% BSA and 0.6% Na-citrate or 2 mM EDTA (without Ca_2^+ and Mg_2^+) in ratio 1:1. Thirty-five ml of the diluted sample were layered over 15 ml of Ficoll Paque medium and centrifuged at 600 g for 40 min at 20°C. The peripheral blood mononuclear cell (PMNC) layer was transferred to centrifuge tube containing three

volumes of PBS and centrifuged at 100 g for 10 min at 20°C. This step was repeated twice, supernatant was discarded and cells were suspended in complete RPMI-1640 medium. 500 µl PMNC were transferred into a test tube, at a density of 5 x 10^7 cells/ml supplemented with 100 µl of heat inactivated FBS and antibody mix and incubated at 4°C for 20 min. Followed by addition of 4 ml isolation buffer and centrifuged at 350 g at 4°C for 8 min. The supernatant discarded and the pelleted cells were suspended in 500 µl of isolation buffer, added with 500 µl of pre-warmed dynabeads and incubated for 15 min at 20°C. The cells bound to beads were resuspended using 4 ml isolation buffer. The supernatant containing the human $CD4^+$ T lymphocytes was obtained by placing the resuspended cells in magnet for 2 minutes. The human $CD4^+$ T lymphocytes were grown in complete RPMI-1640 medium with 10µg/ml phytohemagglutinin at a density of 1 x 10^6 cells/ml, in a CO_2 incubator at 37°C for 24 h [180, 181].

3.4 Western blot

The cells were homogenized for 30 min in cold lysis 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 Basel, Switzerland). Lysate was purified by centrifugation for 10 min at 4°C and 20–50 µg of solubilized proteins were resolved on 10% or 12% SDS-PAGE [182].

3.5 Cell viability analysis MTS

T lymphocytes, T-ALL, Nup214-Abl1 T-ALL and B-ALL Ph^+ cell lines were plated at 5 × 10^4 or 2 × 10^4 cells per well, in 96-well plates with RPMI-1640 medium supplemented with 10% or 20% FBS. The inhibitors were included in media at increasing concentrations. MTS (3-[4,5-Dimethylthythiazol-2-yl]-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay was performed to assess the sensitivity of cells to drugs, as previously described [183].

3.6 Cell cycle and PI/Annexin V assay

Cell cycle analysis was performed using the Muse™ Cell Analyzer (Merck Millipore, Milan, Italy). In brief, after 24 h of treatment, cells were harvested, centrifuged at 300 x g

for 5 min and washed once with 1X PBS. After fixing them with 70% ethanol for at least 3h at -20°C, cells were centrifuged at 300 x g for 5 min, washed once with 1X PBS and then 200 µl of Muse™ Cell Cycle reagent was added to each tube with an incubation of 30 min at room temperature in the dark. Samples were then analyzed according to the instrument protocol.

Apoptosis analysis was performed by staining with Annexin V/7-AAD, using the Muse™ Cell Analyzer (Merck Millipore, Milan, Italy) in according to the manufacturer's instructions. In brief, a 100 µl treated cell suspension was labeled for 20 min in the dark with the same volume of the Muse™ Annexin-V & Dead Cell reagent (Merck Millipore). Subsequently, quantitative detection of Annexin-V/7-AAD positive cells was performed using the Muse™ Cell Analyzer.

3.7 Autophagy analysis and detection of endogenous LC3

Autophagy analysis was performed using the Muse™ Cell Analyzer (Merck Millipore, Milan, Italy). In brief, 8×10^4 cells were plated in 96 well plates and treated with the different drugs for 24 h. Then, cells were harvested, treated with Autophagy Reagent A for 2–6 h, washed with Assay Buffer, incubated for 30 min in the dark with Anti-LC3 Alexa Fluor®555 Antibody and acquired by Muse. Samples were then analyzed according to the instrument protocol.

3.8 Caspase 3/7 activity assay

Caspase activity was measured with the ApoOne Homogeneous Caspase 3/7 assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. The induction of apoptosis and associated activation of caspases 3 and 7 were measured by enzymatic cleavage of the profluorescent substrate rhodamine 110, bis-N-CBZ-L-aspartyl-Lglutaml- L-valyl-L-aspartic acid amide (Z-DEVD-R110), which releases the intensely fluorescent rhodamine 110-cleaving group. Cells were seeded at a density of 1×10^5/ml and incubated in a 96-well plate in the presence or absence of drug for 48 h. 100 µl of the homogeneous caspase-3/-7 reagent was added to each well and the reaction mixture was incubated for 2 h at room temperature. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Results are expressed as relative fluorescence units (RFU), as previously descrive [184].

3.9 Combined drug effect analysis

The effects and the potential synergy of drug combinations were evaluated from quantitative analysis of dose-effect relationship, as described previously [4]. For each experiment, a combination index (CI) number was calculated using the Biosoft CalcuSyn software (Biosoft, Cambridge, UK). This method of analysis generally defines CI values from 0.9 to 1.1 as additive, from 0.3 to 0.9 as synergistic and below 0.3 as strongly synergistic, whereas values over 1.1 are considered as antagonistic.

3.10 Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Total RNA isolation from ALL cell lines was performed with Trizol (Invitrogen) according to the manufacturer's instructions.

A real-time quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) was used to validate microarray data for three miRNAs, namely miR-150-5p, miR-210-3p and miR-221-5p. TaqMan MicroRNA Assays Kits were purchased from Applied Biosystems (Foster City, CA). U6 RNA (Thermo Fisher) was used as a normalization reference. Kits were used in accordance with the manufacturer's instructions. RNAs were diluted at 2 ng/l in DEPC-water and 10 ng were used as a template in each reverse-transcription reaction. The subsequent quantitative PCR was carried out in duplicate for each sample for both the U6 control and each miRNA. Reactions were performed on the Applied biosystems 7500 real time thermal cycler, using the following conditions: 10 minutes at 95°C for enzyme activation, followed by 40 cycles, consisting of 15 seconds at 95°C and 1 minute at 60°C. miRNA expression level was measured using the $\Delta\ \Delta$ Ct method, where the Ct (threshold cycle) is the fractional cycle number when the fluorescence of each sample passes a fixed threshold and $\Delta\ \Delta$ Ct = (Ct miR - Ct U6) sample - (Ct miR - Ct U6) control. The miRNA relative fold change was determined using the $2^{\Delta\Delta Ct}$ method.

3.11 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 [185].

4. RESULTS

4.1 Activation status of PI3K/Akt/mTOR pathway in healthy CD4$^+$ T lymphocytes and T-ALL cell lines

A limited number of studies have analyzed the impact of PI3K/Akt/mTOR network inhibitors on human T-cells. The few published data have been mainly focused only on PI3K inhibitors, such as wortmannin and LY294002 or pan class I PI3K inhibitors [186] or drugs targeting selectively p110δ [187] or p110α [188].

First of all, by Western blot analysis, it has been evaluated the baseline levels of some key proteins involved in the PI3K/Akt/mTOR axis in both unstimulated and stimulated healthy CD4$^+$ T lymphocytes and in T-ALL cells (MOLT4 and JURKAT cell lines). It has been decided to study CD4$^+$ cells as they are helper cells that play important roles for regulating immunological responses [189]. Stimulated T lymphocytes, MOLT-4 and JURKAT cells showed a relevant phosphorylation at Ser473 and Thr308 of Akt and at Ser235/236 of ribosomal protein S6 kinase, a readout of mTORC1 activity. The same cell types also displayed mTOR phosphorylation at Ser2448 and Ser2481 residues, readout for mTORC1 and mTORC2, respectively. The phosphorylation was not evident in unstimulated CD4$^+$ T lymphocytes (Fig. 6).

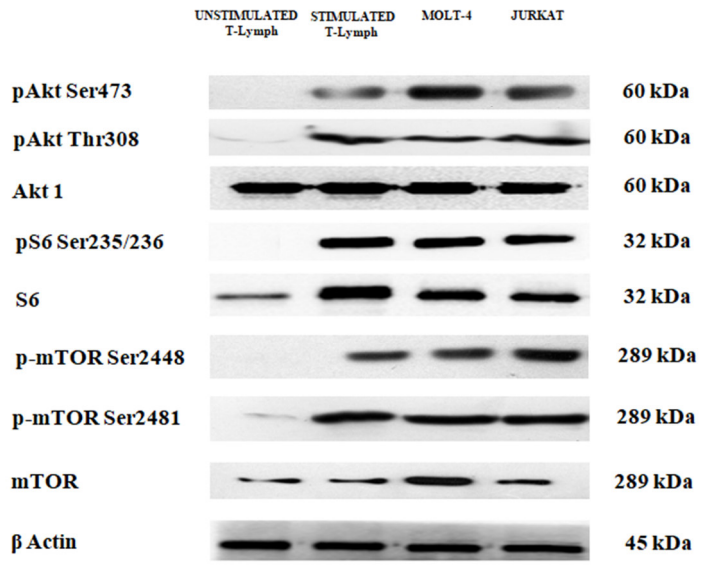


Figure 6: Expression and phosphorylation status of Akt, mTOR and the S6 downstream target in primary (unstimulated and stimulated) CD4$^+$ T lymphocytes and T-ALL cell lines. Western blot analysis of primary T lymphocytes and T-ALL cell lines to detect the expression and phosphorylation levels of Akt, mTOR, and S6 protein. Twenty-five μg of protein were blotted on each lane. β-actin was revealed as loading control.

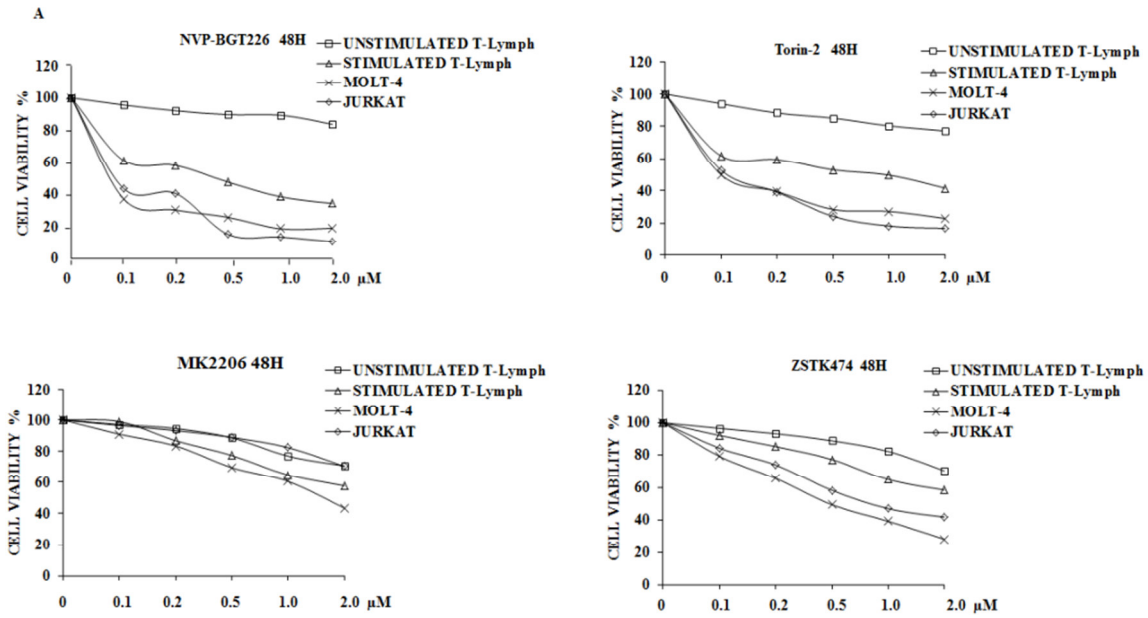
4.2 NVP-BGT226, Torin-2, MK-2206 and ZSTK474 are cytostatic and cytotoxic to stimulated T lymphocytes and T-ALL cell lines

Drugs targeting the PI3K/Akt/mTOR cascade can interfere with diverse biologic processes also in healthy cells, thus rising concerns about their use in therapeutics. In particular, it is essential to gain knowledge about their effect on immune cells, as it would be desirable to preserve patient's immunity. To achieve this aim it has been analyzed the effect of the four above mentioned drugs in human primary $CD4^+$ T-cells. Most of these inhibitors are currently being tested in clinical trials. It has been assessed the anti-tumor activity of NVP-BGT226, Torin-2, MK-2206 and ZSTK474 in human T-ALL cells and studied their effects on healthy $CD4^+$ T-cell, induced or not to proliferate.

NVP-BGT226 is a novel class I PI3K/mTOR inhibitor, as described above. Torin-2 potently targets mTORC1/2, and is an effective inhibitor of ATM, ATR and DNA-PK. The efficacy of MK-2206 as well as ZSTK474 have been tested in various preclinical models of human cancers, including leukemia [190].

To determine how this inhibitors could affect the viability of the primary $CD4^+$ T lymphocytes (both unstimulated and stimulated) and T-ALL cells, it has been performed MTS assays. Cells were incubated for 48 h with the inhibitors and then cell survival was analyzed (Fig. 7A and Table 3). Except for the unstimulated T lymphocytes, NVP-BGT226 and the mTORC1/2 inhibitor Torin-2 turned out to be the most powerful drugs in these cells. For unstimulated T lymphocytes, there was no relevant inhibition for both drugs (IC50 > 2 μM). For NVP-BGT226, cell viability impairment was more evident in T-ALL cell lines, with IC50 values of 0.08 μM for JURKAT, 0.06 μM for MOLT-4 and 1.61 μM for stimulated T lymphocytes. Similar results were obtained with Torin-2, with IC50 values of 0.1 μM for JURKAT and MOLT-4 and 1.8 μM for stimulated T-cells. Regarding MK-2206, the cells displayed higher values of IC50 (>2 μM) except for MOLT-4 cells with an IC50 of 1.58 μM. For ZSTK474 the same resistance for the primary T lymphocytes was observed, while in MOLT-4 and JURKAT cell lines the sensitivity of the drug was evident, with IC50 values of 0.52 μM and 0.95 μM, respectively. The IC50 of stimulated T lymphocytes was >2 μM (Table 3). To further assess the cytotoxicity of inhibitors targeting PI3K/Akt/mTOR, it has been analyzed the changes in cell viability using flow cytometry after treatment with 1μM of each drug for 48 h. Compared to MK-2206 and ZSTK474, NVP-BGT226 and Torin-2 displayed higher cytotoxic effect on stimulated T lymphocytes, MOLT-4 and JURKAT cells. None of the drugs affected the viability of unstimulated $CD4^+$ T lymphocytes (Fig. 7B). For all these reasons, for the subsequent experiments it has

been decided to test only BGT226 and Torin-2. Considering the important role of the PI3K/Akt/mTOR signaling cascade in regulating cell proliferation [87], it has been investigated the effect of the drugs on cell cycle progression. Cells were treated with the two most effective drugs (BGT226 and Torin-2) for 24 h and stained with Propidium Iodide (PI) for flow cytometric analysis. A concentration dependent increase of cells in the G0/G1 phase of the cell cycle and a concomitant decrease in cells of both S and G2/M phase were observed (Fig. 8). The increase was highly significant in stimulated T lymphocytes, MOLT-4 and JURKAT cells, whereas did not occur in unstimulated T lymphocytes.



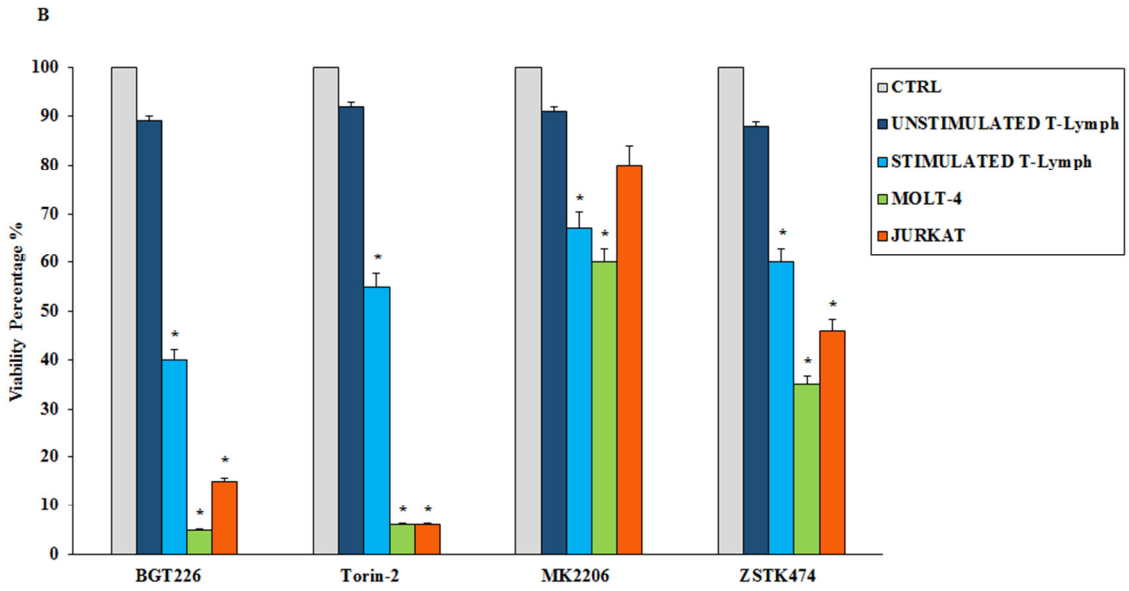


Figure 7: Cytotoxicity of NVP-BGT226, Torin-2, MK-2206 and ZSTK474 in primary T lymphocytes, MOLT-4 and JURKAT cell lines. (A) MTS assays of primary T lymphocytes and T-ALL cell lines treated with increasing concentrations of NVP-BGT226, Torin-2, MK-2206 and ZSTK474 for 48 h. SD was less than 7%. One representative experiments of three is shown. (B) Viability of primary T lymphocytes, MOLT-4 and JURKAT cell lines treated for 48 h with 1 μM NVP-BGT226, Torin-2, MK-2206 and ZSTK474. Asterisks indicate statistically significant differences with respect to untreated cells (*p<0.05).

	NVP-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

Table 3. IC50 values of cells treated for 48 hours with different drugs. Values are expressed in μM.

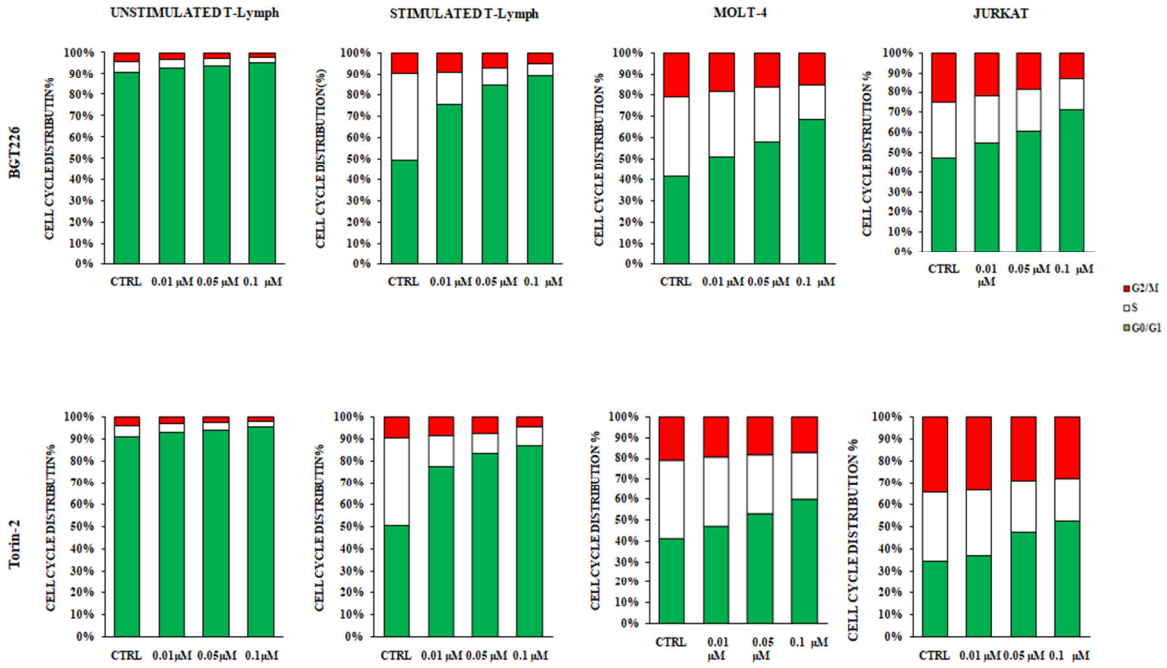


Figure 8: NVP-BGT226 and Torin-2 affect cell cycle in primary T lymphocytes, MOLT-4 and JURKAT cell lines. Unstimulated and stimulated T lymphocytes, MOLT-4 and JURKAT cells were treated with increasing concentrations of NVP-BGT226 and Torin-2 for 24 h. CTRL, control (untreated) cells. SD was less than 10%.

4.3 BGT226 and TORIN-2 down-regulate the PI3K/Akt/mTOR pathway in stimulated CD4+ T lymphocytes and T-ALL cells

To determine whether NVP-BGT226 and Torin-2 could affect factors that promote cell survival, stimulated T lymphocytes, MOLT-4 and JURKAT cells were treated with increasing concentrations of BGT226 and Torin-2 for 2 h and then analyzed by Western blot (Fig. 9). The inhibition of mTORC2 had a readout in Ser473 Akt dephosphorylation and it was observed in all cell types treated with BGT226 and Torin-2 starting from the lowest concentrations. Thr308 Akt was dephosphorylated by both drugs, as was GSK3β Ser21/9, an Akt substrate. The mTORC1 substrate S6 was completely dephosphorylated on Ser235/236 residue, already at the lowest concentrations of BGT226 and Torin-2. Both drugs downregulated the phosphorylation levels of mTOR at both Ser2448 and Ser2481 residues [191].

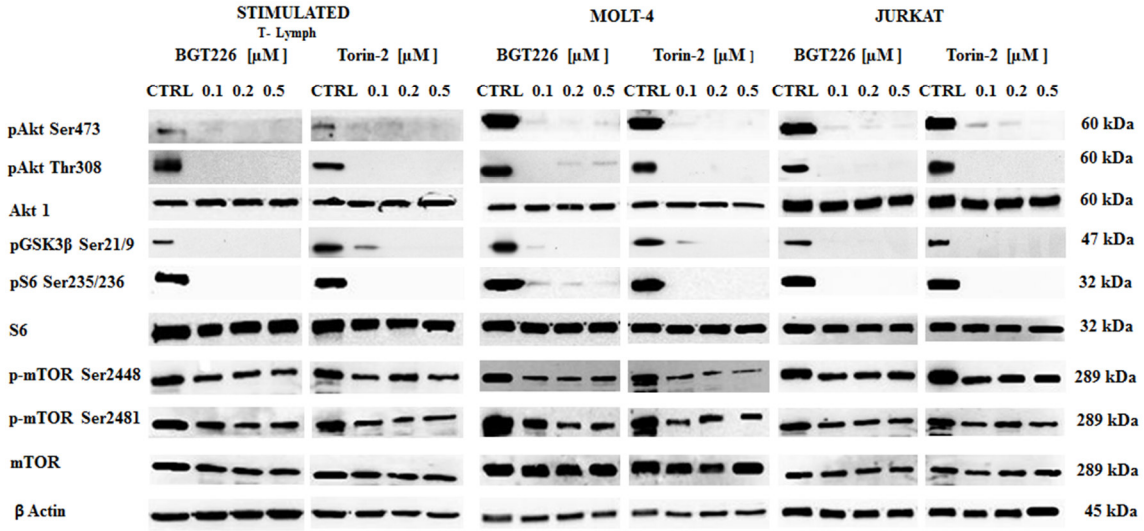


Figure 9: Expression and phosphorylation status of mTOR, Akt and their downstream targets in primary T lymphocytes and T-ALL cell lines. Western blot analysis of phosphorylated and total Akt, mTOR and their substrates GSK3β and S6 in stimulated T lymphocytes, MOLT-4 and JURKAT cells treated for 2 h with 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.

4.4 BGT226 and TORIN-2 induce autophagy

Autophagy plays a very important role in cell physiology, either as a form of cell death or as a protective mechanism against apoptosis [192]. Therefore, there is a growing interest on the pharmacological approaches aimed to regulate autophagy, which represent a new area for the development of therapeutics protocols. Moreover, over the last few years various papers described the occurrence of autophagy in acute leukemia cells (reviewed in [193]). To find out if BGT226 and Torin-2 could induce autophagy, it has been analyzed the expression levels of microtubule-associated protein 1 light chain 3 LC3A/B I (non-lipidated form) and of its conjugated form LC3A/B II (lipidated). After 24 h of treatment with BGT226 and Torin-2, the unstimulated T lymphocytes showed no expression of LC3A/B II which, in contrast, increased gradually in a concentration dependent manner, for both drugs, especially in MOLT4 and JURKAT cells (Fig. 10A). It has also been analyzed the expression of p62, another marker of autophagy. p62 levels decreased in response to drug treatments in stimulated $CD4^+$ lymphocytes and leukemic cell lines, but remained unchanged in quiescent T-cells (Fig. 10A). To establish whether autophagy was either a cell death or survival mechanism, it has been employed the autophagy inhibitor 3-Methyladenine (3-MA), which inhibits autophagy by blocking class III PI3K. It has been also used Bafilomycin A1, another autophagy inhibitor, to further assess the mechanism of

drug cytotoxicity. It has been treated stimulated T lymphocytes and JURKAT cells with BGT226, Torin-2, 3-MA or Bafilomycin A1 alone and in combination for 24 h. Results showed that 3-MA or Bafilomycin A1 alone did not affect cell viability, even at high concentrations (10 µM or 4 µM, respectively). On the other hand, when 3-MA or Bafilomycin A1 were administered with 0.25 µM BGT226 or Torin-2, the cells became more sensitive to the cytotoxic effect of both drugs (Fig. 10B and 10C). These findings highlighted a protective role of autophagy from cytotoxicity induced by BGT226 and Torin-2 in stimulated T-lymphocytes and in JURKAT cell line.

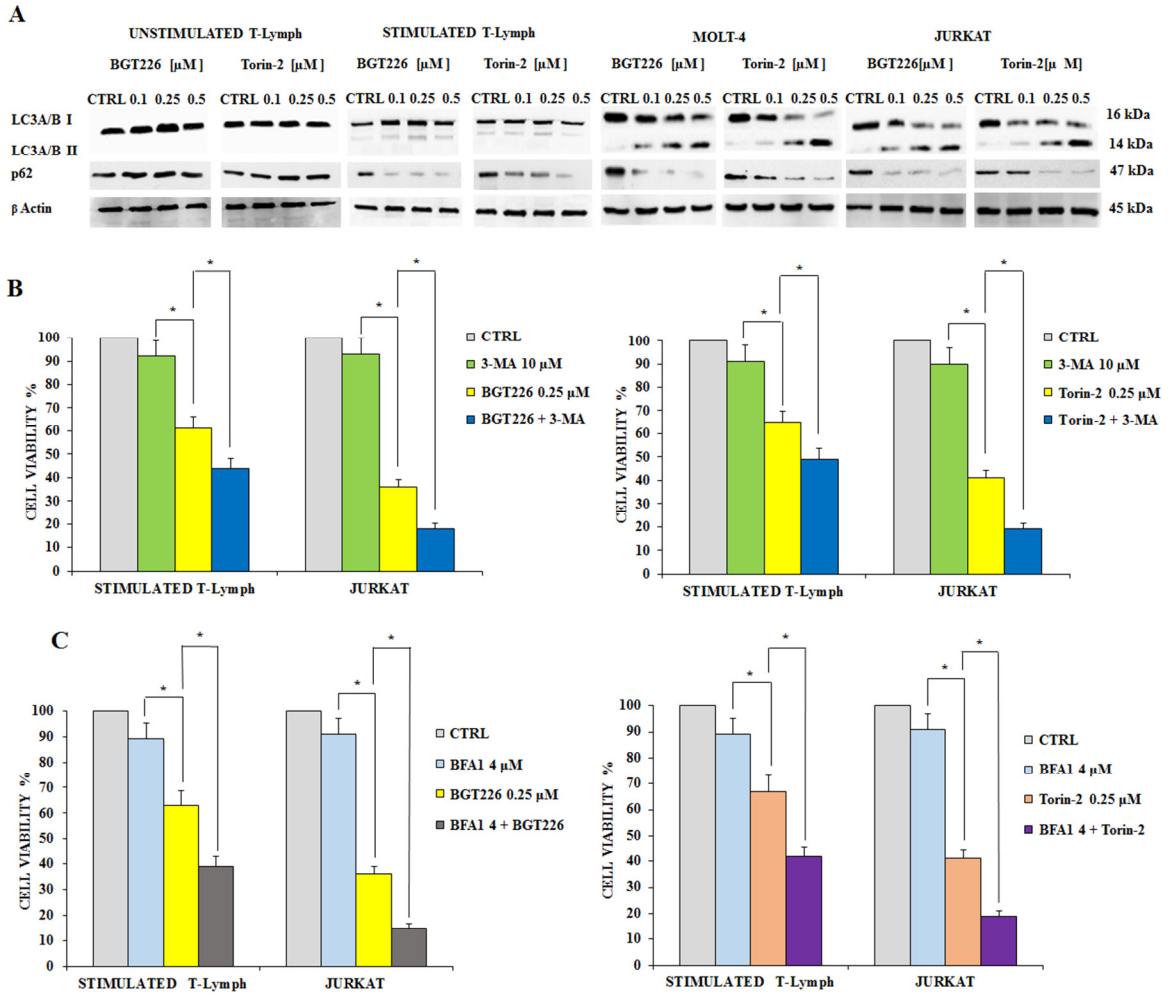


Figure 10: NVP-BGT226/Torin-2 induced autophagy in unstimulated, stimulated T lymphocytes and T-ALL cell lines. (A) Western blot analysis of primary T lymphocytes and T-ALL cell lines treated for 24 h with increasing concentrations of NVP-BGT226 and Torin-2. An increase of expression of fast-migrating (lipidated) LC3A/B and a reduction of p62 in 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. (B) MTS assay documenting the effect of the autophagy inhibitor 3-MA (3-Methyladenine) on the viability of stimulated T lymphocytes and JURKAT cells treated for 24 h with NVP-BGT226 and Torin-2. (C) MTS assay documenting the effect of the autophagy inhibitor Bafilomycin A1 on the viability of stimulated T lymphocytes and JURKAT cells treated for 24 h with NVP-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).

4.5 Apoptosis is required for mediating BGT226 and Torin-2 cytotoxicity

Previous studies documented that in T-ALL cells NVP-BGT226 could induce apoptosis [193]. In addition, recent results demonstrated that Torin-2 is effective in Pre-B precursor-ALL cells. In order to establish whether decreased viability was due to apoptosis, unstimulated and stimulated T lymphocytes as well as MOLT4 and JURKAT cell lines, were incubated with increasing concentrations of BGT226 and Torin-2 for 24 h, then Western blot was performed for analyzing the expression levels of poly (ADP-ribose) polymerase (PARP). The unstimulated T lymphocytes displayed no evidence for apoptosis. In contrast, significant cleavage of PARP was observed, especially in stimulated T lymphocytes, but also in MOLT-4 and JURKAT cells (Fig. 11A). To elucidate whether caspases were involved in the apoptotic activity of NVP-BGT226 and Torin-2, it has been analyzed the effect of z-VAD-fmk, a broad-spectrum caspase inhibitor whose activity had been checked already in different cancer cells [194]. It has been administrated z-VAD-fmk alone and in combination with NVP-BGT226 or Torin-2 for 24 h in stimulated T lymphocytes and MOLT-4 cells, then cells were analyzed by MTS assays. Results showed that z-VAD-fmk (25 μM) alone had no relevant effect on cell viability, however when combined with NVP-BGT226 and Torin-2, it significantly inhibited apoptosis mediated by both drugs, in stimulated T lymphocytes and MOLT-4 cells. Thus, these findings indicated that NVP-BGT226 and Torin-2 induced a caspase-dependent apoptosis (Fig. 11B). It has been also measured caspase 3/7 activation by enzymatic cleavage of the profluorescent substrate rhodamine 110, bis-N-CBZ-L-aspartyl-Lglutaml- L-valylL-aspartic acid amide (Z-DEVD-R110), with release of the intensely fluorescent rhodamine 110-cleaving group [195]. The activity of caspase 3/7 was increased after drug treatment and was down-modulated by z-VAD-fmk (Fig. 11C). Furthermore, it has been studied drug-induced apoptosis using Annexin-V staining in stimulated T lymphocytes, MOLT-4 and JURKAT cell lines treated with NVP-BGT226 or Torin-2 for 24 h. NVP-BGT226 effect was more relevant in MOLT-4 and stimulated T lymphocytes than in JURKAT cells, while the effect of Torin-2 was stronger in MOLT4 and JURKAT cells than in stimulated T lymphocytes (Fig. 12A). The percentages of live, early and late apoptotic cells in response to treatment with NVP-BGT226 and Torin-2 (0.1, 0.25 and 0.5 μM) are shown in Fig. 12B.

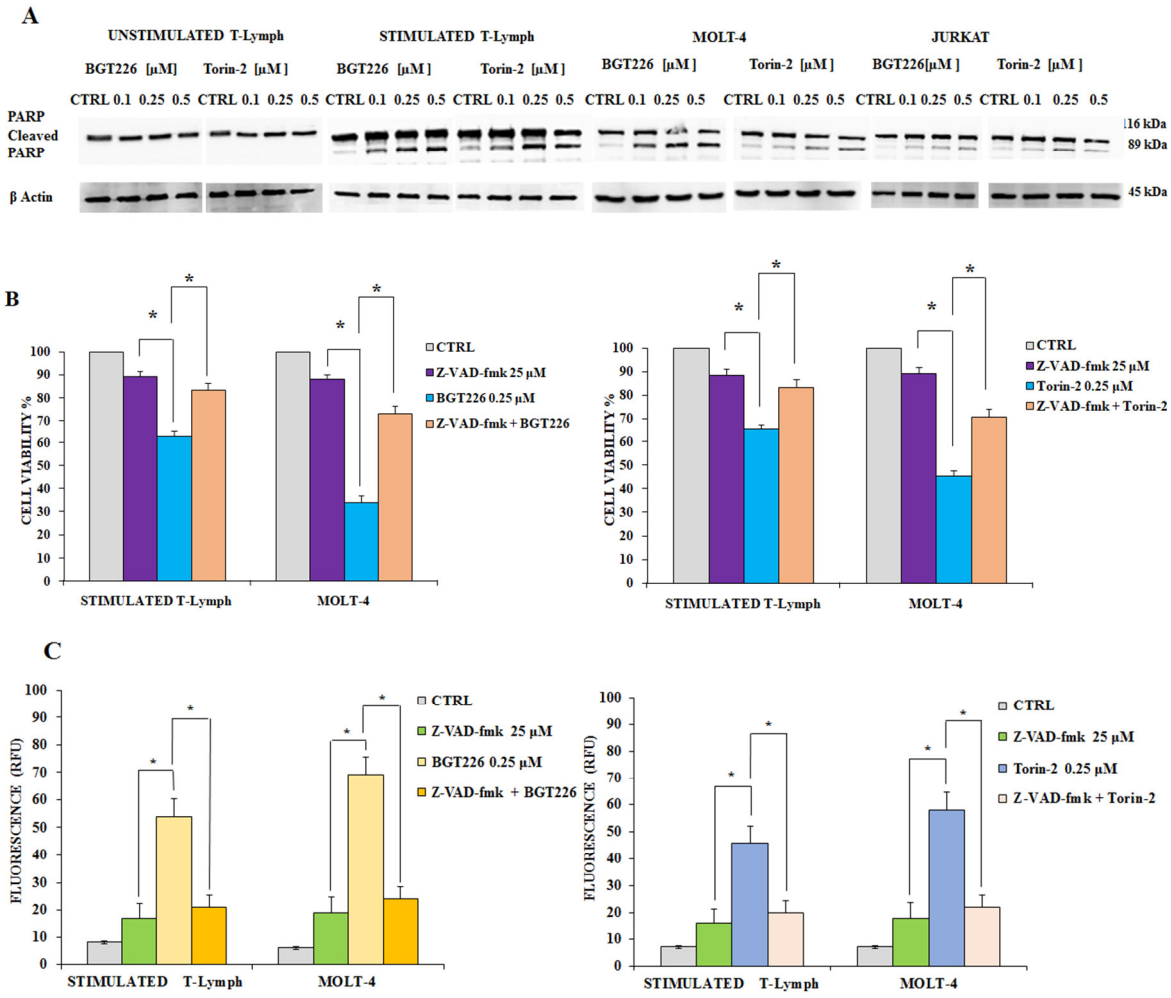


Figure 11: NVP-BGT226/Torin-2 induced autophagy in unstimulated, stimulated CD4+ T lymphocytes and T-ALL cell lines. (A) Western blot analysis documenting the increase of PARP cleavage in stimulated T lymphocytes and T-ALL cell lines treated for 24 h with increasing concentrations of NVP-BGT226 and Torin-2. Twenty-five µg of protein was blotted on each lane. β-actin served as loading control. (B) MTS assays after NVP-BGT226 and Torin-2 treatment, alone and in combination with the pan caspase inhibitor z-VAD-fmk, in stimulated T lymphocytes and MOLT-4 cells. The analysis was performed after 24 h of treatment with BGT226 and Torin-2 at 0.25 µM and z-VAD-fmk at 25 µM. (C) enzymatic cleavage of the profluorescent substrate Z-DEVD-R110, with release of the intensely fluorescent rhodamine 110-cleaving group, after NVP-BGT226 and Torin-2 treatment, alone and in combination with the pan caspase inhibitor z-VAD-fmk, in stimulated T lymphocytes and MOLT-4 cells. The analysis was performed after 24 h 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).

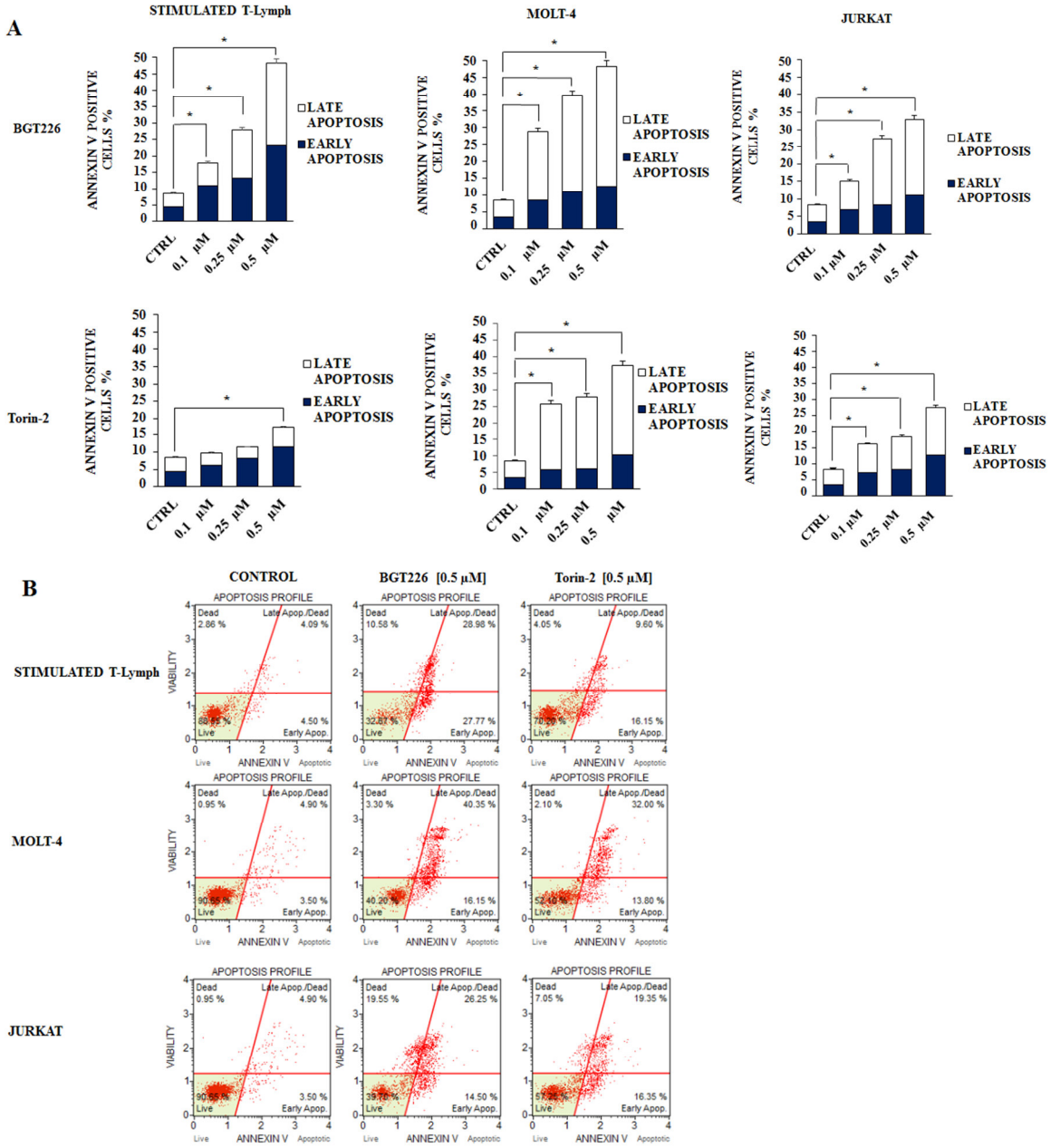


Figure 12: Flow cytometric analysis of drug-induced apoptosis. (A) Analysis of Annexin-V positive cells after NVP-BGT226 and Torin-2 treatment using the Muse™ Cell Analyzer in stimulated $CD4^+$ T lymphocytes, MOLT-4 and JURKAT cells. The analysis was performed after 24 h of treatment with increasing concentrations of the drugs. Results are the mean of three different experiments ± SD. (B) Flow cytometric plots of Annexin V-FITC/PI stimulated T lymphocytes, MOLT-4 and JURKAT cells, treated with 0.5 µM NVP-BGT226 and Torin-2. One representative of three different experiments that yielded similar results is shown.

4.6 Multiple Akt targeting showed a cytotoxic effect in T-ALL cell lines

No cytotoxic effects were displayed by the drugs on unstimulated $CD4^+$ T lymphocytes, as shown in Fig. 7, whereas all the inhibitors and particularly NVP-BGT226 and Torin-2 reached IC50 values in the nanomolar range in T-ALL cells.

There is a growing interest in multi-component chemotherapy: the combined delivery of multiple drugs is an attempt to overcome drug resistances and to improve clinical outcome. Therefore it has been determined whether the combination of drugs with the same target of action, i.e. Akt, will result in a more significant biological effect, as an antiproliferative therapy in order to overcome the risk of cell growth escape phenomena. To this aim it has been employed three drugs directed against Akt but with a totally different mode of action in T-ALL cell lines.

It has been examined by MTS assay the IC50 values of each drug on the four T-ALL cell lines. After 24 h of treatment, cell lines displayed different sensitivity to the single drugs. GSK690693 ranged from 0.31 or 0.21 μM, in MOLT-4 and JURKAT, to 7 or 5 μM in CEM-R and -S, respectively. MK-2206 IC50 ranged from 1.7 to 6.9 μM. Perifosine required higher concentration to obtain IC50 and ranged between 9.35 and 14.65 μM and this phenomenon is well known and has already been described [117, 196]. Next it has been studied if the simultaneous administration of GSK690693, MK-2206 and Perifosine could lead to a similar cytotoxic effect on the T-ALL cell lines with a significant decrease of the concentration of every single drug due to the synergy of the three compound combination. Therefore it has been treated cells with drugs administered together for 24 h, using the IC50 value as the highest one and decreasing progressively up to 1/20 of the IC50 value. MTS assays were then performed. As shown in Fig. 13, all the four more responsive cell lines showed the synergistic cytotoxicity of the triple drug combination, very significant in MOLT-4 and JURKAT cells. It has been calculated the CI with Calcusyn Software, to quantify the combined effects of the drugs, such as synergism or interference. It has not been maintained a constant ratio since it was necessary to fine tune each drug concentration to better understand the synergistic or interfering effect and to avoid a too high cytotoxicity depending on a single drug administered at a fixed constant ratio. The data analysis (not shown) indicated strong synergisms in all cell lines, even more evident in MOLT-4 cells, with the best CI value of 0.101 corresponding to the combination of 0.05 μM GSK690693, 0.2 μM MK-2206 and 2 μM Perifosine.

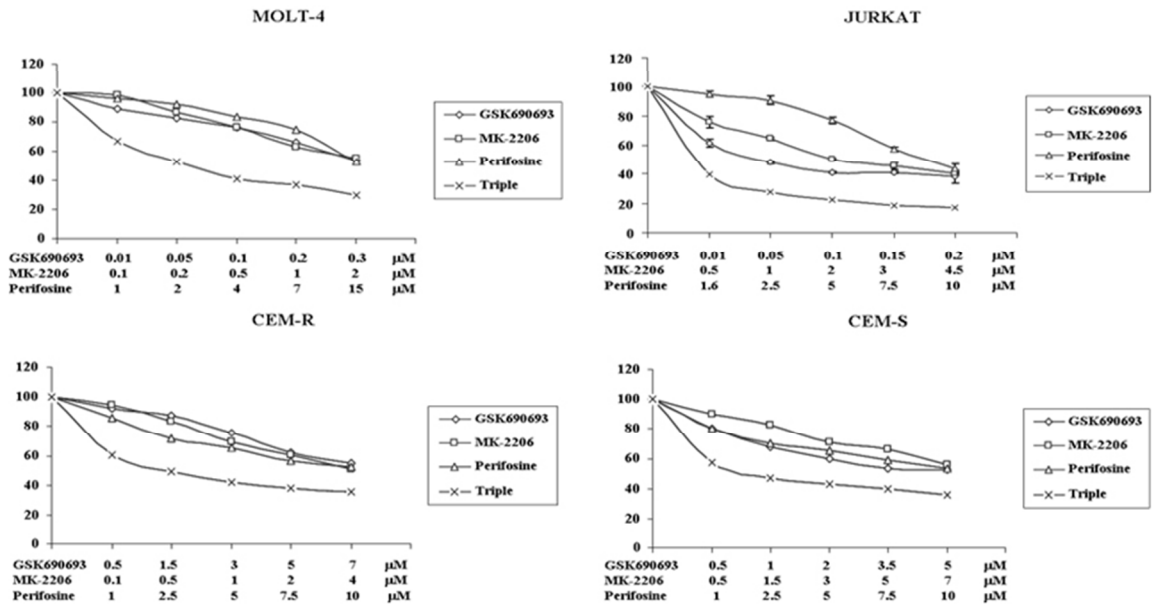


Figure 13: Cytotoxicity of Perifosine, MK-2206 and GSK690693 in T-ALL cell lines. MTS assay of T-ALL cell lines treated with Perifosine, MK-2206 and GSK690693, either alone and in triple combination for 24 h. Concentration of each drug is reported under the graph. One representative experiment of three is shown.

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

To verify if the multiple and simultaneous *in vitro* treatment with MK-2206, GSK690693 and Perifosine could lead to a modulation of PI3K/Akt/mTOR pathway, it has been checked the phosphorylation status of key components of this signaling cascade in this panel of more responsive cell lines. In particular it has been analyzed p-Akt, its downstream target, GSK3 α/β and the ribosomal protein S6 kinase, readout of mTORC1 activity, after 30 min of drugs exposure. GSK690693 and Perifosine were used at 1/2 of the IC50 concentration, whereas MK-2206 was used at 1/5 of IC50, since half of MK-2206 IC50 concentration was enough to completely abolish the Ser473 Akt phosphorylation already at 30 minutes. Akt phosphorylation was affected in different ways by single drug administration: in all cell lines MK-2206 very significantly reduced p-Akt, Perifosine only slightly reduced it and GSK690693 on the contrary increased the protein phosphorylation. The latter one is an already described phenomenon. This increase of Akt phosphorylation diminished the observable effect of double or triple compound combination, since p-Akt was not significantly reduced, unless when using MK-2206 in double exposure (Fig. 14). On the contrary, even after such a short time of treatment, in all of the four cell lines it was very evident the efficacy of the multiple hit on Akt. The triple administration of the drugs

completely abolished the phosphorylation on the downstream targets, Ser21/9 p-GSK3 α/β and Ser235/236 p-S6, with a much superior efficacy of the triple exposure when compared with the single or with the different double combinations (Fig. 14). The total amount of the proteins was unchanged in all the treatments (Fig. 14).

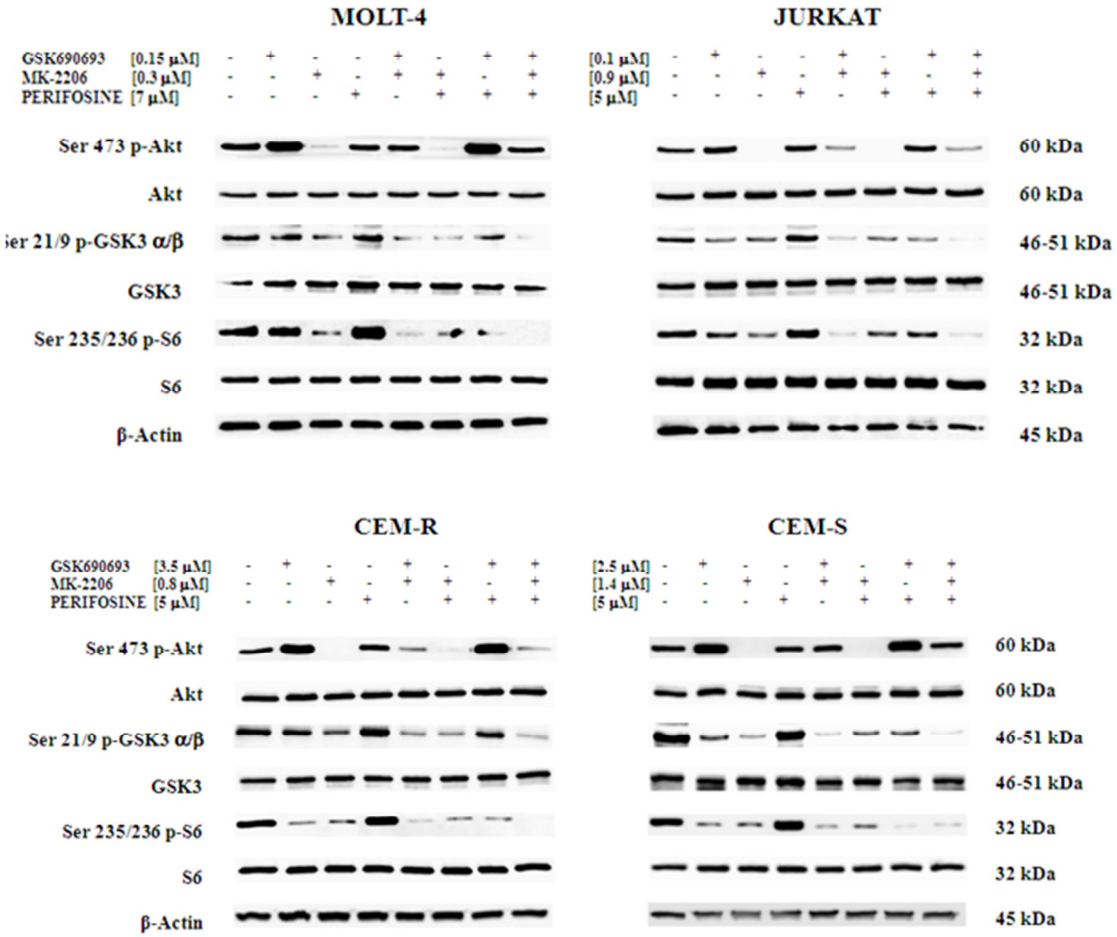


Figure 14: Multiple Akt inhibition affects PI3K/Akt/mTOR pathway and the Akt inhibition is time-dependent. Western blot analysis of Akt drug sensitive T-ALL cell lines for total and phosphorylated form of Akt and of its downstream substrate GSK3 α/β and of mTOR downstream target S6. Samples were treated for 30 minutes with GSK690693, MK-2206 and Perifosine, alone, or in double or triple combinations.

4.8 Pre-treatment with Perifosine enhances synergistic effect

Given that the GSK690693 drug alone led to Ser473 p-Akt increase, whereas MK-2206 alone almost turn off the signal, it has been explored if it is possible to find a compound combination capable of synergistically dephosphorylate Akt. First, it has been tested if there is any concentration capable to modulate Akt phosphorylation in JURKAT and MOLT- 4 cells. Therefore GSK690693 was administered at 1/2 of the IC50 value (0.1 µM

for JURKAT and 0.15 μM for MOLT-4 cells) and MK-2206 was contemporary given at increasing concentrations (0.3–0.5–1 μM). After 30 minutes of exposure, Western blot was performed. The best drug combination to observe p-Akt modulation resulted to be 1 μM MK-2206 for JURKAT and 0.5 μM for MOLT-4 cells (Fig. 15A). Then, it has been analyzed by Western blot the phosphorylation levels of Akt after treatment with 7 μM Perifosine at different time points. In both cell lines the drug affected in a time-dependent manner the Ser473 Akt phosphorylation (Fig. 15B). Finally, it has been merged the two previous assays pretreating cells for 6 h with Perifosine before at 30 min administration of GSK690693 and MK-2206. As shown in (Fig. 15C), in 6 h Perifosine pre-treated cells, the administration of GSK690693 reduced Ser473 p-Akt hyperphosphorylation. The combination of all three drugs allowed to obtain a full Akt dephosphorylation in both MOLT-4 and JURKAT cells, thus showing that full Akt inhibition with low drug doses is not only concentration but also time and drug sequence dependent.

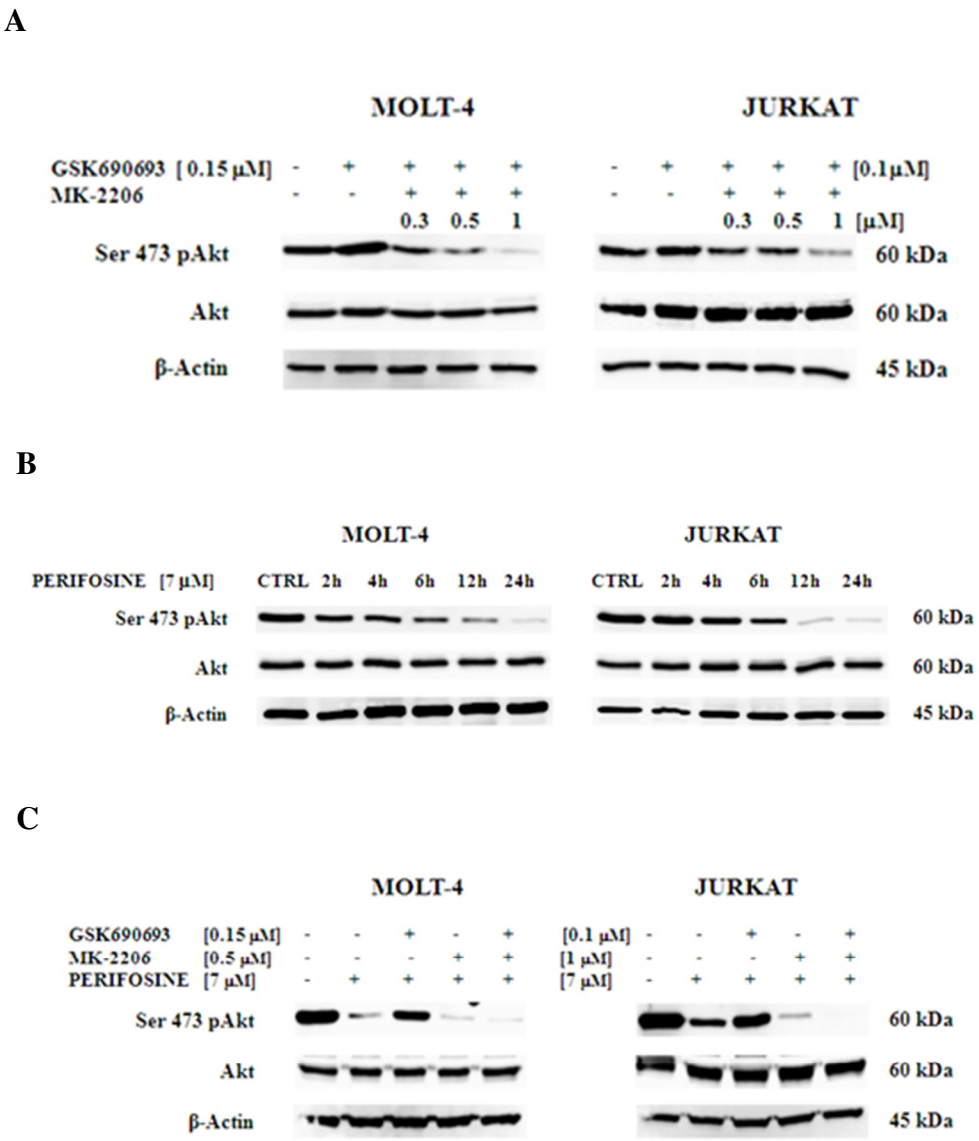


Figure 15: Synergistic effect enhanced by pre-treatment by perifosine. (A) Akt protein inhibition as detected by its phosphorylation status. MOLT-4 and JURKAT cells were treated for 30 minutes with a combination of a fixed concentration of GSK690693 and three different concentrations of MK-2206. (B) Akt phosphorylation levels in cells treated with 7 µM Perifosine at different time points. (C) p-Akt status in MOLT-4 and JURKAT cells pre-treated for 6 h with 7 µM Perifosine followed by GSK690693 and MK-2206 administered for 30 minutes. Twenty-five µg of protein was blotted to each lane. β-actin served as a loading control. For all panel one representative experiment of three is shown as well as cell lines are representative also of the others if not shown.

4.9 The multiple treatment has the capability to inhibit ERK pathway

Finally, it has been focused the attention on the MEK/ERK signaling pathway, which plays an important role in cell proliferation and growth [197] and potentially mediates resistance to drug-induced growth inhibition [198]. For this reason, it has been examined the p-ERK 1/2 phosphorylation status in JURKAT, MOLT-4 and CEM-S cells, after 24 h of treatment with the three drugs. Interestingly, the multiple treatment was capable to downregulate the

phosphorylation state of Tyr 202/204p-ERK 1/2 with a superior efficacy than single drugs, whereas the amount of total protein remains unchanged (Fig. 16).

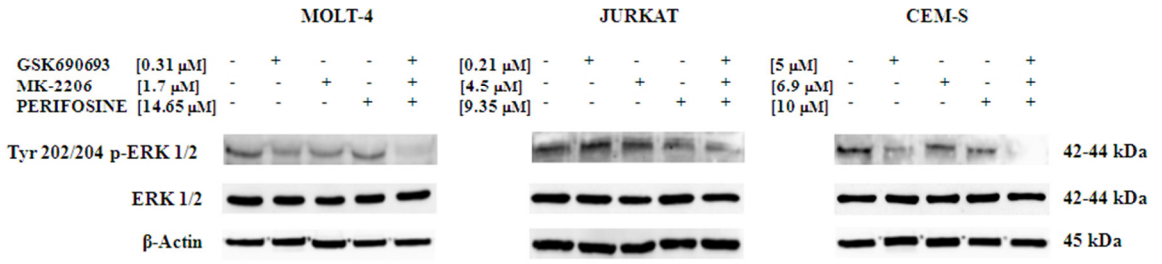


Figure 16: Multiple anti Akt drug treatment affects also MEK/ERK pathway. Western blot analysis for Tyr 202/204 p-ERK 1/2 phosphorylation status in MOLT-4, JURKAT and CEM-S cells, after 24 h of treatment with the three drugs alone and in combination. The triple treatment almost abolished p-ERK staining. Twenty-five μg of protein was blotted to each lane. β-actin served as a loading control. One representative experiment of three is shown as well as cell lines are representative also of the others not shown.

4.10 Analysis of miR-150, miR-210 and miR-221 expression level in ALL cell lines after treatment with PI3K signaling inhibitors

Several data have shown that the deregulated expression of specific miRNAs, which modulate the expression of oncogenes and tumor suppressors, is associated with the development of malignancies and the expression of miRNA signature could be used to classify human tumors [199]. For this reason, it has been analyzed whether drugs against PI3K pathway, such as ZSTK474, GSK690693 and NVP-BGT226, could affect miRNA expression involved in ALL progression. It has been performed a cell viability test (MTS assay) in three B-ALL cell lines (REH wild type (wt) and REH silenced for p53 (shp53) and NALM-6) and in T-ALL cell line (JURKAT) with the above mentioned inhibitors (data not shown). The IC50 values obtained in REH wt and REH shp53 cell lines at 48 h were reported in table 4, whereas the IC50 values obtained in NALM-6 and JURKAT cell lines at 72 h were reported in table 5. Then, it has been performed a screening of several miRNAs (including miR-21, miR-24-1, mir-23-b, miR-146, miR-148-a, miR-150, miR-155, miR-181-a, miR-195, miR-210 and miR-221) by qRT-PCR experiment. Several miRNAs have shown a downregulation compared to the control (such as miR-210 and miR-221) (Fig. 17B and Fig. 17C), whereas others have shown an upregulation (such as miR-150) (Fig. 17A). These data will encourage to continue exploring the field related to the miRNA modulation by the drugs in ALL *in vitro* models.

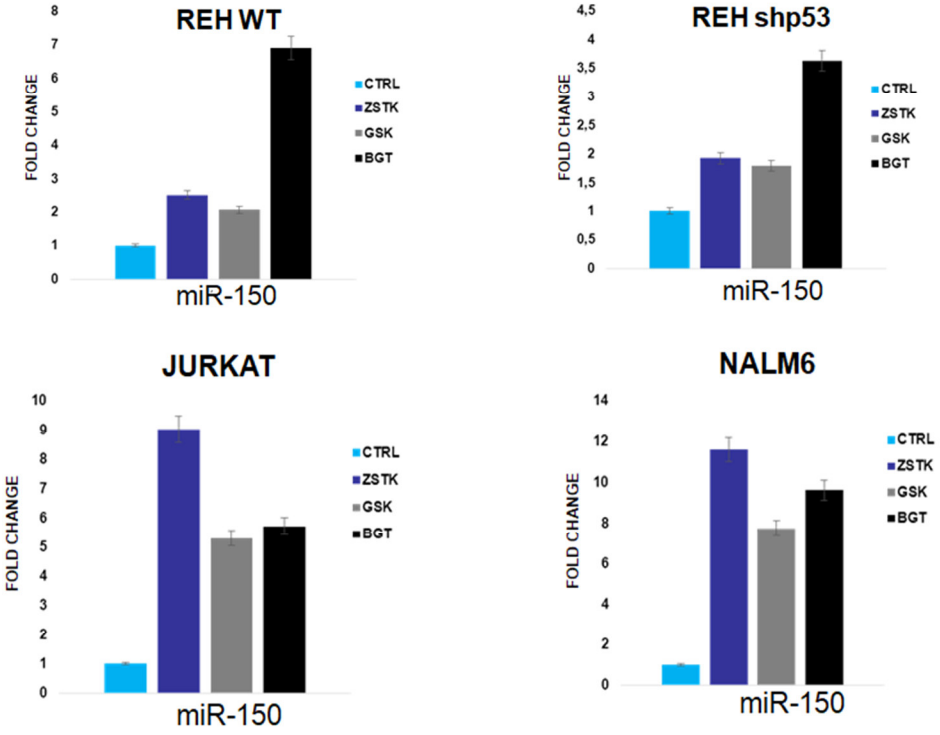
IC50 48h	ZSTK474	GSK690693	NVP-BGT226
REH wt 48h	5	0.1	0.1
REH shp53 48h	0.5	10	0.1

Table 4. IC50 values of REH wt and REH shp53 cells treated for 48 hours with different drugs. Values are expressed in µM.

IC50 72h	ZSTK474	GSK690693	NVP-BGT226
NALM-6	10	10	0.1
JURKAT	10	1	0.1

Table 5. IC50 values of NALM-6 and JURKAT cells treated for 72 hours with different drugs. Values are expressed in µM.

A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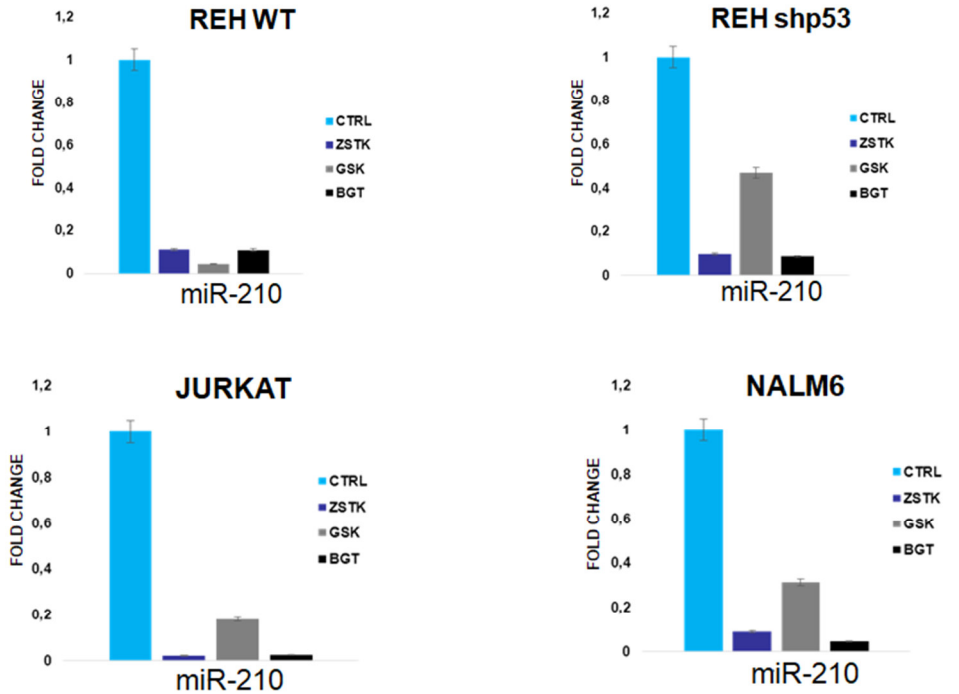
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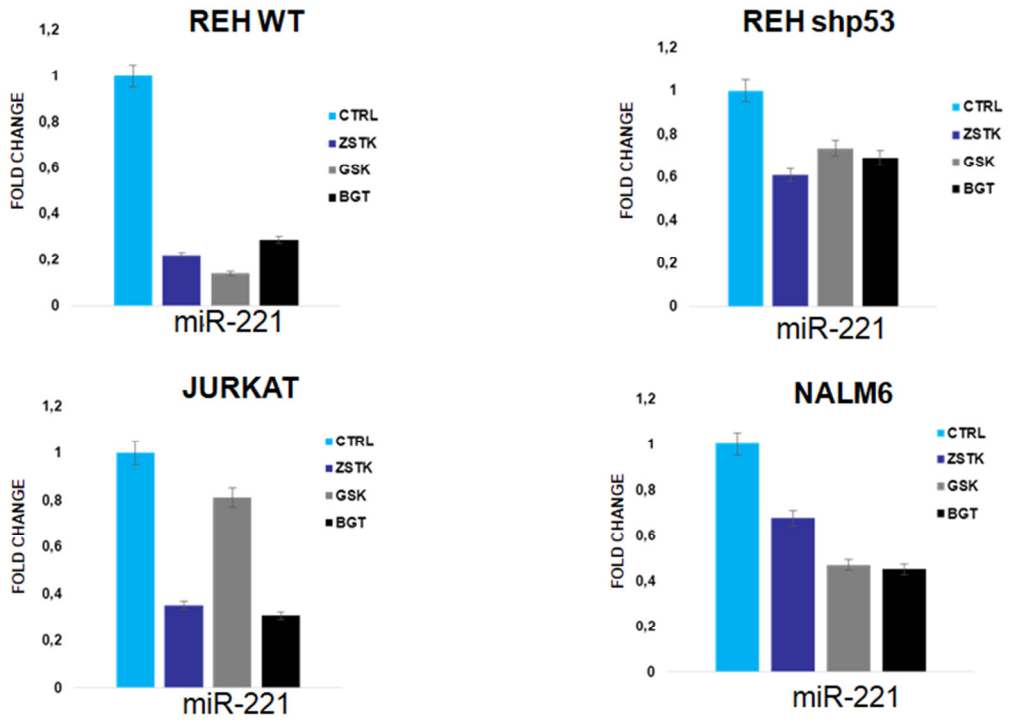


Figure 17: Expression level of miRNAs in REH, JURKAT and NALM-6 cell lines. (A) Expression level of miR-150 in REH wt, REH shp53, NALM-6 and JURKAT cell lines by qRT-PCR. These cells were treated at 48 h and 72 h with the ZSTK474, GSK690693 and NVP-BGT226 based on the IC50 calculated by MTS assay. ZSTK474, GSK690693 and NVP-BGT226 were abbreviated in ZSTK, GSK and BGT, respectively. (B) Expression level of miR-210 in REH wt, REH shp53, NALM-6 and JURKAT cell lines by qRT-PCR. These cells were treated at 48 h and 72 h with the ZSTK474, GSK690693 and NVP-BGT226 based on the IC50 calculated by MTS assay. ZSTK474, GSK690693 and NVP-BGT226 were abbreviated in ZSTK, GSK and BGT, respectively. (C) Expression level of miR-221 in REH wt, REH shp53, NALM-6 and JURKAT cell lines by qRT-PCR. These cells were treated at 48 h and 72 h with the ZSTK474, GSK690693 and NVP-BGT226 based on the IC50 calculated by MTS assay. ZSTK474, GSK690693 and NVP-BGT226 were abbreviated in ZSTK, GSK and BGT, respectively.

4.11 PI3K/Akt/mTOR network activation in Nup214-Abl1 positive cells

Nup214-Abl1 oncogene is the most frequent and highly specific in T-ALL and is associated with early relapse and poor outcome.

By Western blot analysis, it has been verified Nup214-Abl1 presence in three T-ALL cells (ALL-SIL, PEER and BE-13) that reportedly display this fusion protein [200] (Fig. 18). Then, it has been evaluated the phosphorylation level of two key proteins of the PI3K/Akt/mTOR signaling pathway in the same cell lines. All the T-ALL cells lines displayed phosphorylated Akt on both residues 473 and 308, however the expression was lower in BE-13 cells (Fig. 18). Moreover, BE-13 cells showed a weaker expression of the ribosomal S6 protein phosphorylated at Ser 235/236, which is a readout of mTORC1

activity, while in ALL-SIL and PEER cells basal expression of phosphorylated S6 protein was stronger. Total Akt and S6 proteins were expressed in all cell types (Fig. 18).

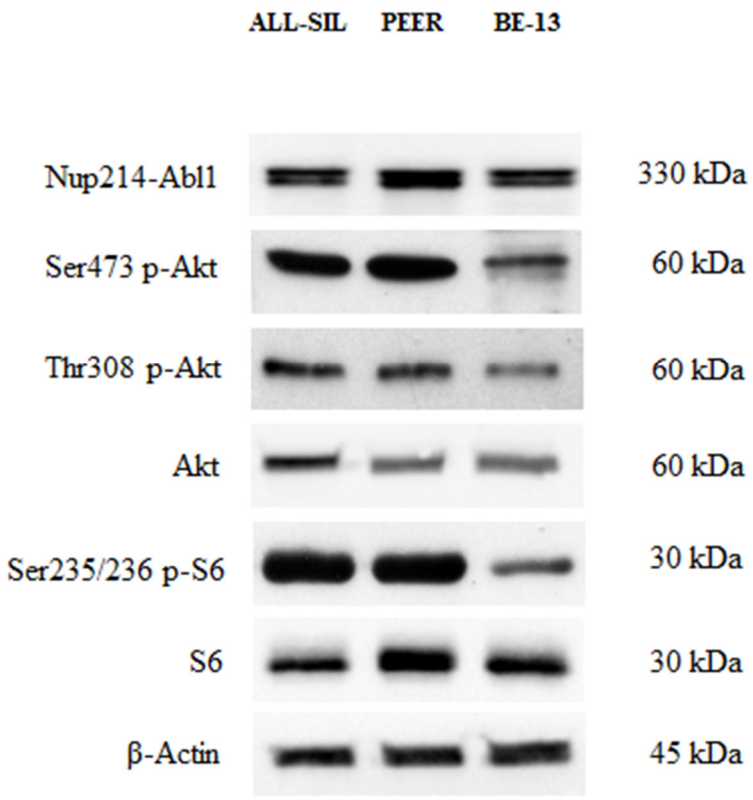


Figure 18: Expression and phosphorylation status of Akt and of the mTORC1 downstream target S6 in Nup214-Abl1 positive T-ALL cell lines. Western blot analysis of ALL-SIL, PEER and BE-13 cell lines to detect the expression and phosphorylation levels of Nup214-Abl1, Akt and S6 proteins. Twenty-five μg of protein were blotted on each lane. Antibody to β-Actin served as loading control.

4.12 Drugs targeting PI3K/Akt/mTOR display cytotoxic effects in Nup214-Abl1 positive cells

It has been analyzed by MTS assay the IC50 values of 5 different inhibitors, some of which targeted both mTOR complexes and 5 drugs with direct inhibition of PI3K/Akt pathway in ALL-SIL, PEER and BE-13 cell lines. A different sensitivity of the cells to each drugs was observed after 48 h of treatment. In ALL-SIL, the IC50 values of mTOR inhibitors ranged from 0.5 to 10 μM, while PEER and BE-13 cells showed less sensitivity to the drugs, with IC50 values ≥ 10 μM. (Fig. 19). Similar results were obtained with MTS assay of drugs against PI3K/Akt/mTOR. In ALL-SIL cells, the PI3K/mTOR inhibitor NVP-BGT226 appeared to be the most effective drug, with an IC50 value of 0.12 μM. The same drug was less potent in PEER and BE-13 cells, being the IC50 value in the range

between 1.5 and 2.0 μM. All the other drugs targeting PI3K or Akt displayed an IC50 ranging from 3.2 to ≥ 10 μM for ALL-SIL and > 10 μM in PEER and BE-13 cells, respectively (Fig. 19).

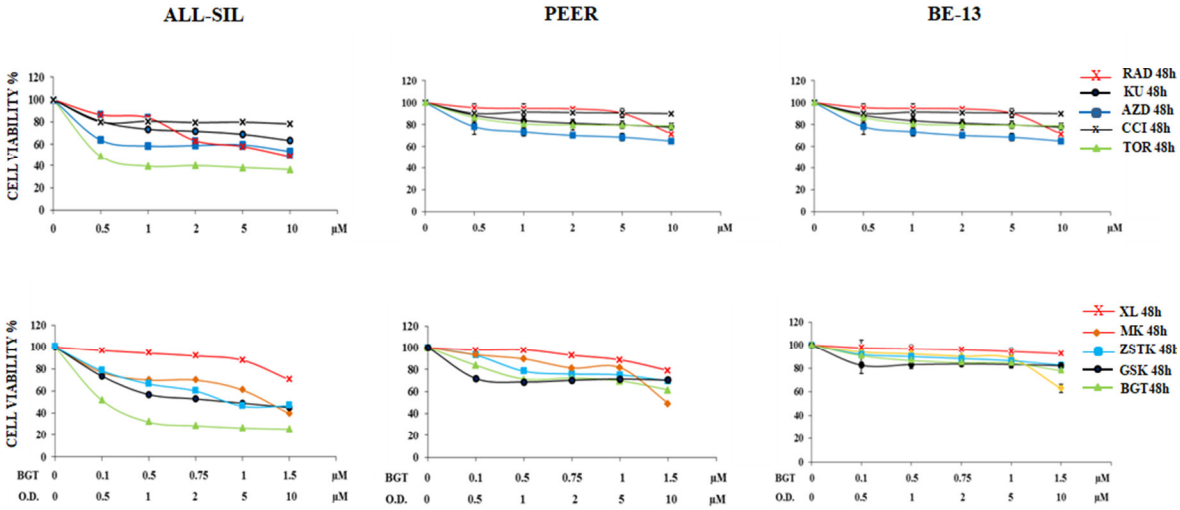


Figure 19: Cytotoxic activity of PI3K/Akt/mTOR inhibitors in ALL-SIL, PEER and BE-13 cell lines. MTS assays of ALL-SIL, PEER and BE-13 cell lines treated with increasing concentrations of PI3K/Akt/mTOR inhibitors for 48 h. Torin-2 and BGT226 appeared to be the most effective drugs for both cell lines. SD was less than 10%. Concentration of drugs is indicated in the X axis. O.D. is the abbreviation of Other Drugs. One representative experiments of three is shown. Abbreviations: RAD001, KU0063794, AZD8055, CCI-779, Torin-2, XL-147, MK-2206, ZSTK474, GSK690693 and BGT226 were abbreviated respectively as follows: RAD, KU, AZD, CCI, TOR, XL, MK, ZSTK, GSK and BGT.

4.13 Cytotoxic effects of Imatinib, Nilotinib and GZD824 in cell lines harboring Nup214-Abl1

Given that TKIs suppress Abl1 activity, they may potentially be used in the treatment of patients with Nup214-Abl1 positive T-ALL [201]. For this reason, ALL-SIL, PEER and BE-13 cells were treated for 48 h with increasing doses of three different TKIs, Imatinib, Nilotinib and GZD824, analyzed by MTS assay. Results showed that in ALL-SIL cells Imatinib, Nilotinib and GZD824 decreased cell viability and showed evident efficacy, with IC50 values ranging from 0.02 μM for GZD824 to 0.5 μM for Imatinib and Nilotinib. In PEER and BE-13 cells, Imatinib and Nilotinib showed less potency, while the effectiveness of GZD824 was confirmed, with an IC50 ≥ 0.05 μM (Fig. 20A). To further confirm the efficacy of Bcr-Abl1 and PI3K/Akt/mTOR inhibitors, the expression of Ser473 p-Akt, Ser235/236 p-S6 and of Tyr207 p-CrkL, a downstream substrate of Nup214-Abl1 kinase [202], were evaluated in ALL-SIL, PEER and BE-13 cells. 4 h treatment with 2 μM Imatinib or Nilotinib, or 0.1 μM GZD824 showed that the phosphorylation status of Akt on

Ser473 and S6 on Ser235/236 remained almost unaffected, while the phosphorylation of CrkL was nearly abolished. This finding demonstrated the selectivity of Imatinib, Nilotinib and GZD824 (Fig. 20B). At the same time, treatment for 4 h in ALL-SIL, PEER and BE-13 cells with 0.5 µM of NVP-BGT226, Torin-2, ZSTK474, and GSK690693 showed an almost complete shutdown of Ser473 Akt and S6 protein phosphorylation, while the phosphorylation status of CrkL was unchanged (Fig. 20C).

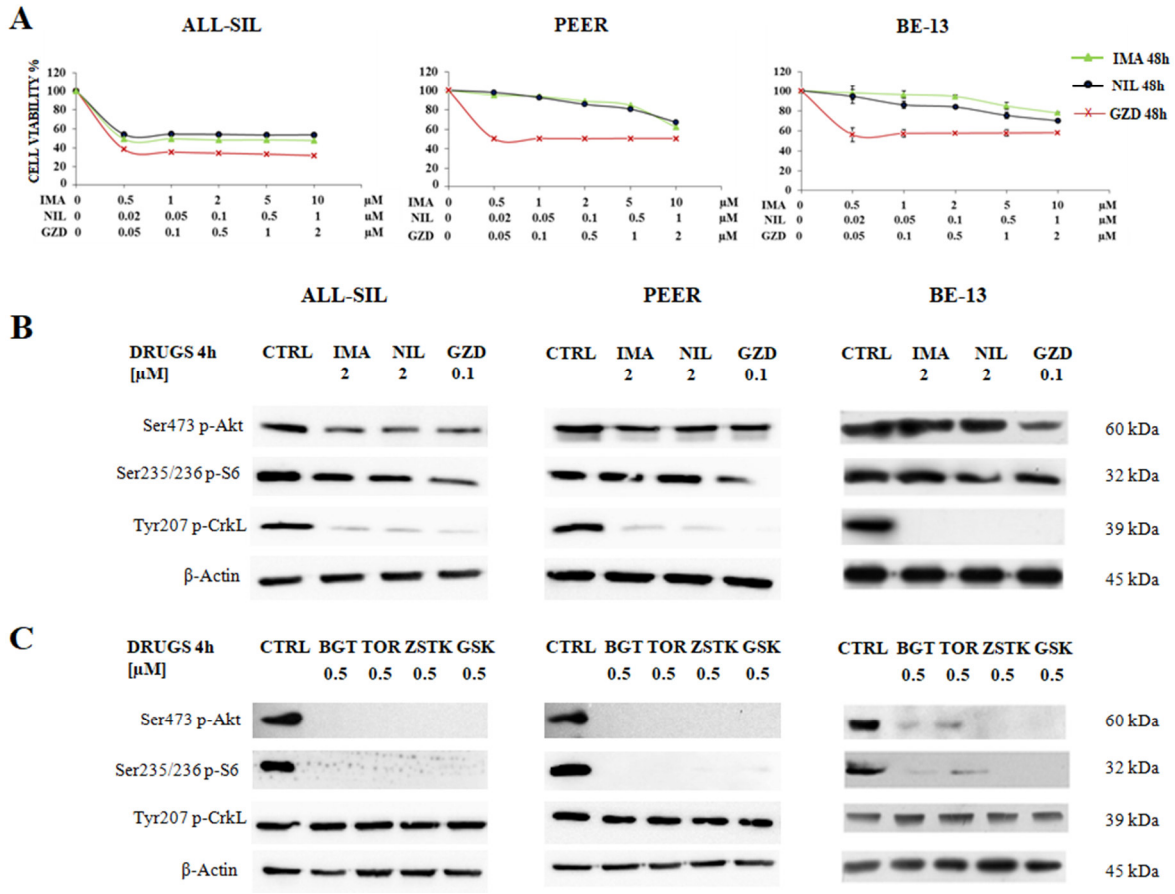
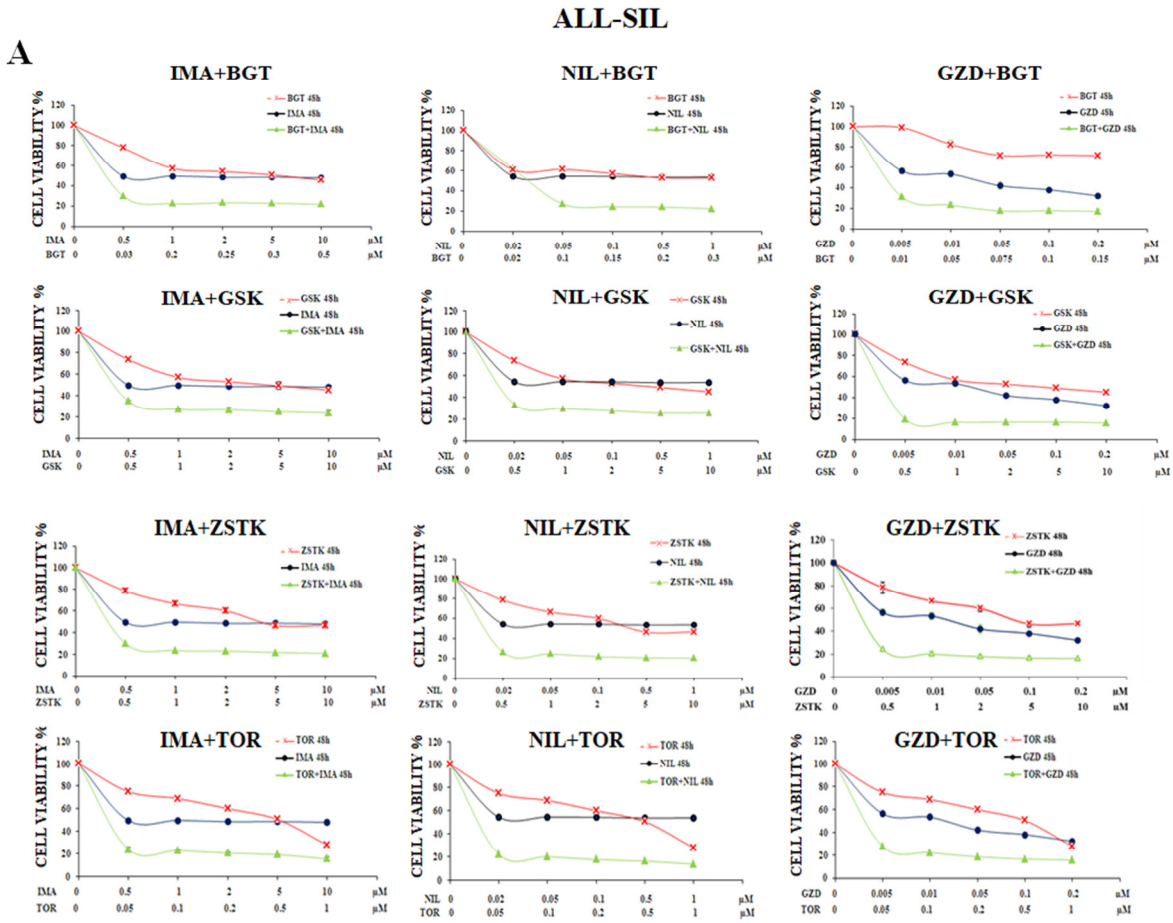


Figure 20: Effectiveness of Imatinib, Nilotinib and GZD824 in ALL-SIL, PEER and BE-13 cell lines. (A) T-ALL cell lines were treated with increasing concentrations of Imatinib, Nilotinib and GZD824 for 48 h. In PEER and BE-13 cells Imatinib and Nilotinib showed less potency, while the effectiveness of GZD824 was confirmed. SD was less than 10%. (B) Western blot analysis of phosphorylated Akt, S6 and CrkL in T-ALL cell lines treated for 4 h with 2 µM Imatinib or Nilotinib or 0.1 µM GZD824. Twenty-five µg of protein were blotted to each lane. β-Actin documented equal lane loading. Imatinib, Nilotinib and GZD824 were abbreviated in IMA, NIL and GZD. (C) Western blot analysis of phosphorylated Akt, S6 and CrkL in T-ALL cell lines treated for 4 h with 0.5 µM of NVP-BGT226, Torin-2, ZSTK474 and GSK690693. β-Actin served as loading control. NVP-BGT226, Torin-2, ZSTK474 and GSK690693 were abbreviated in BGT, TOR, ZSTK and GSK, respectively.

4.14 Synergism of Imatinib, Nilotinib and GZD824 with the PI3K/Akt/mTOR inhibitors NVP-BGT226, GSK690693, ZSTK474 and Torin-2 in ALL-SIL and PEER cells

To better assess the effects of the simultaneous *in vitro* treatment with Bcr-Abl1 and PI3K/Akt/mTOR inhibitors, it has been examined by MTS assay the efficacy of Imatinib, Nilotinib and GZD824 in combination with NVP-BGT226, GSK690693, ZSTK474 and Torin-2 for 48 h in ALL-SIL and PEER cells. Analysis of the results on graphs documented the existence of a significant synergism between Bcr-Abl1 and PI3K/Akt/mTOR inhibitors in ALL-SIL and PEER cells as shown in Fig. 21A and 21B. In PEER cells it has been repeated the experiments only with NVP-BGT226 and Torin-2, since these two drugs showed the most relevant synergism on the evidence of the graphs obtained.



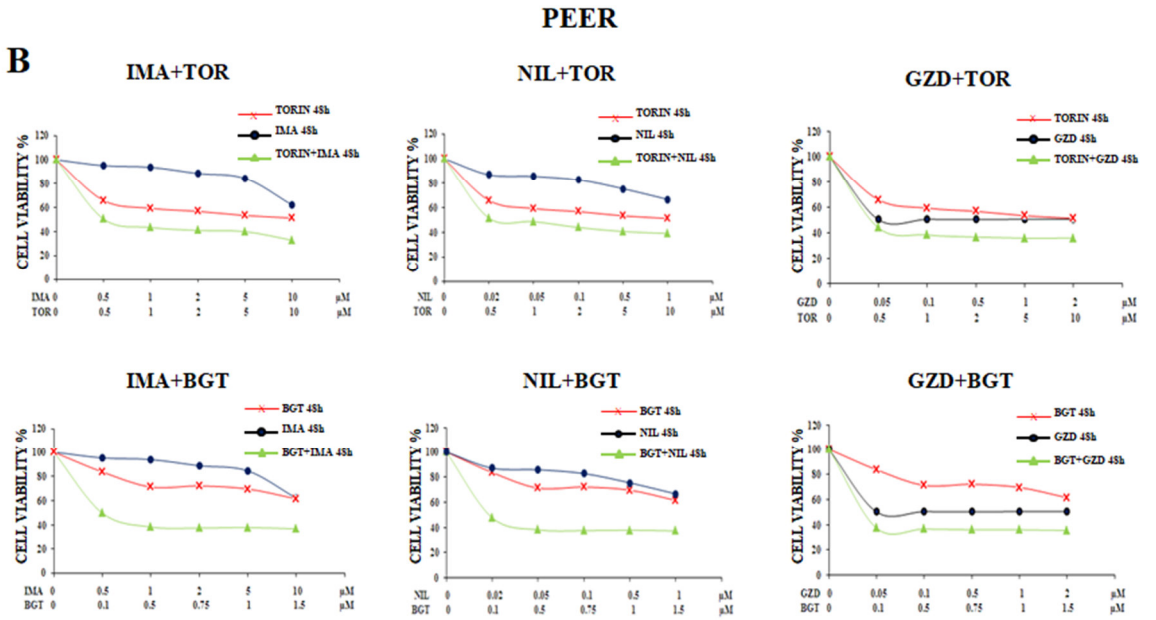
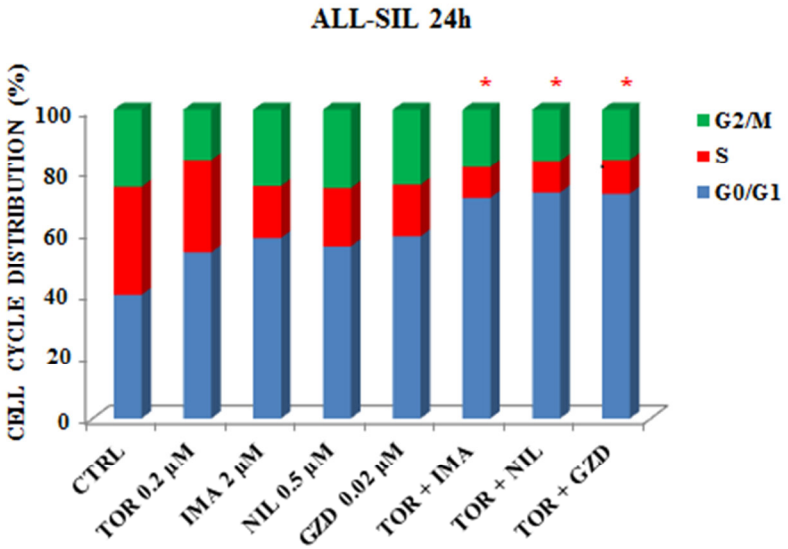


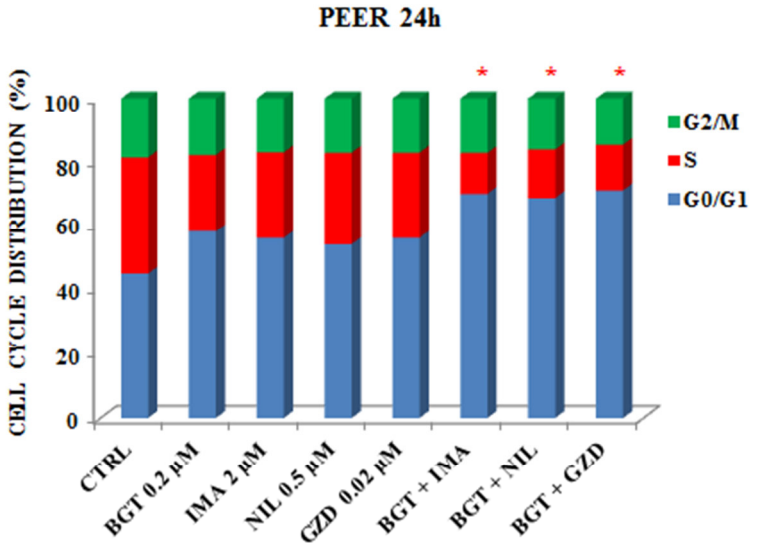
Figure 21: Synergism of Imatinib, Nilotinib and GZD824 with NVP-BGT226, GSK690693, ZSTK474 and Torin-2 in ALLSIL and PEER cells. (A) MTS assays of ALL-SIL cell lines treated for 48 h with increasing concentrations of Imatinib, Nilotinib and GZD inhibitors in combination with BGT226, GSK690693, ZSTK474 and Torin-2 for 48 h. (B) MTS assays of PEER cell lines treated for 48 h with increasing concentrations of Nilotinib, Imatinib and GZD824 inhibitors either in combination with NVP-BGT226 and Torin-2 for 48 h. Concentration of each drug is reported under the graphs. SD was less than 8%. One representative experiment of three is shown. Imatinib, Nilotinib, GZD824, NVP-BGT226, Torin-2, ZSTK474 and GSK690693 were abbreviated in IMA, NIL, GZD, BGT, TOR, ZSTK and GSK.

4.15 Increased cell cycle arrest and programmed cell death by the synergism of Bcr-Abl1 and PI3K/Akt/mTOR inhibitors when compared with single administration of drugs

To evaluate whether the drugs could influence cell cycle progression, flow cytometric analysis was performed. Imatinib, Nilotinib and GZD824 were administered alone and in combination with Torin-2 and NVP-BGT226 drugs for 24 h. These combinations augmented the G0/G1 cell cycle phase in both ALL-SIL and PEER cells, with a parallel decrease mainly in the S phase (Fig. 22A). To further analyze the mechanism of action of these drugs, Annexin-V-FITC staining was performed in all the three cell lines. Flow cytometric analysis showed that dual treatments induced a more important, statistically relevant, increase in apoptosis when compared to single drugs, with an evident synergistic effect. BE-13 cells displayed the lowest sensitivity to the drug combinations (Fig. 22B).

A


ALL-SIL 24h
CELL CYCLE DISTRIBUTION (%)
100
80
60
40
20
0
G2/M
S
G0/G1
*
*
*
CTRL
TOR 0.2 µM
IMA 2 µM
NIL 0.5 µM
GZD 0.02 µM
TOR + IMA
TOR + NIL
TOR + GZD


PEER 24h
CELL CYCLE DISTRIBUTION (%)
100
80
60
40
20
0
G2/M
S
G0/G1
*
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CTRL
BGT 0.2 µM
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NIL 0.5 µM
GZD 0.02 µM
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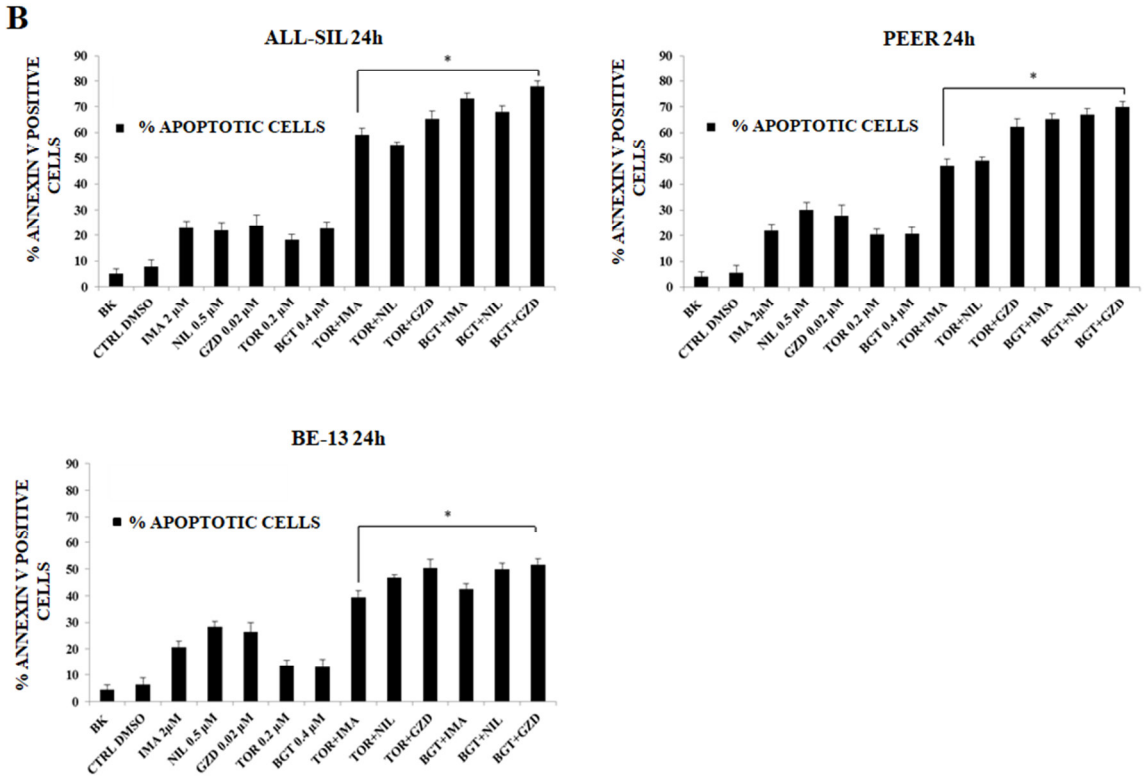


Figure 22: Imatinib, Nilotinib and GZD824 with NVP-BGT226 or Torin-2 induced cell cycle arrest and apoptosis in Nup214-Abl1 positive T-ALL cell lines. (A) Flow cytometric analysis of PI-stained samples in ALL-SIL and PEER cells treated with combined administration of Imatinib, Nilotinib, GZD824 with NVP-BGT226 or Torin-2 for 24 h. CTRL, control (untreated) cells. SD was less than 10%. (B) Analysis of Annexin-V positive ALL-SIL, PEER and BE-13 cells. The analysis was performed after 24 h of treatment with single or combined drugs. Results are the mean of three different experiments ± SD. Asterisks indicate significant differences in comparison to single drug treated samples (*p< 0.05). Imatinib, Nilotinib, GZD824, Torin-2 and NVP-BGT226 were abbreviated in IMA, NIL, GZD, TOR and BGT.

4.16 Nup214-Abl1 and PI3K/Akt/mTOR inhibitors induced autophagy

Bcr-Abl1 is a positive regulator of autophagy and it is deeply involved in the regulation of this process [203, 204]. To determine if the drugs could induce autophagy in Nup214-Abl1 positive leukemia cells, Western blot was performed to analyze the presence 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 of ALL-SIL cells with Imatinib, Nilotinib, GZD824, Torin-2 and NVP-BGT226, it has been detected an increase of LC3A/B II conjugated form (Fig. 23A). To better quantify autophagy induction, the detection of LC3A/B was performed in ALL-SIL and PEER cells by flow cytometry after 24 h of drug treatments. Results showed that all the drugs were able to induce autophagy, with a more evident effect with GZD824. Combined treatments induced a consistent,

statistically important, increase in autophagy when compared to the administration of a drug alone, thus showing a synergistic effect due to the combination of different drugs (Fig. 23B).

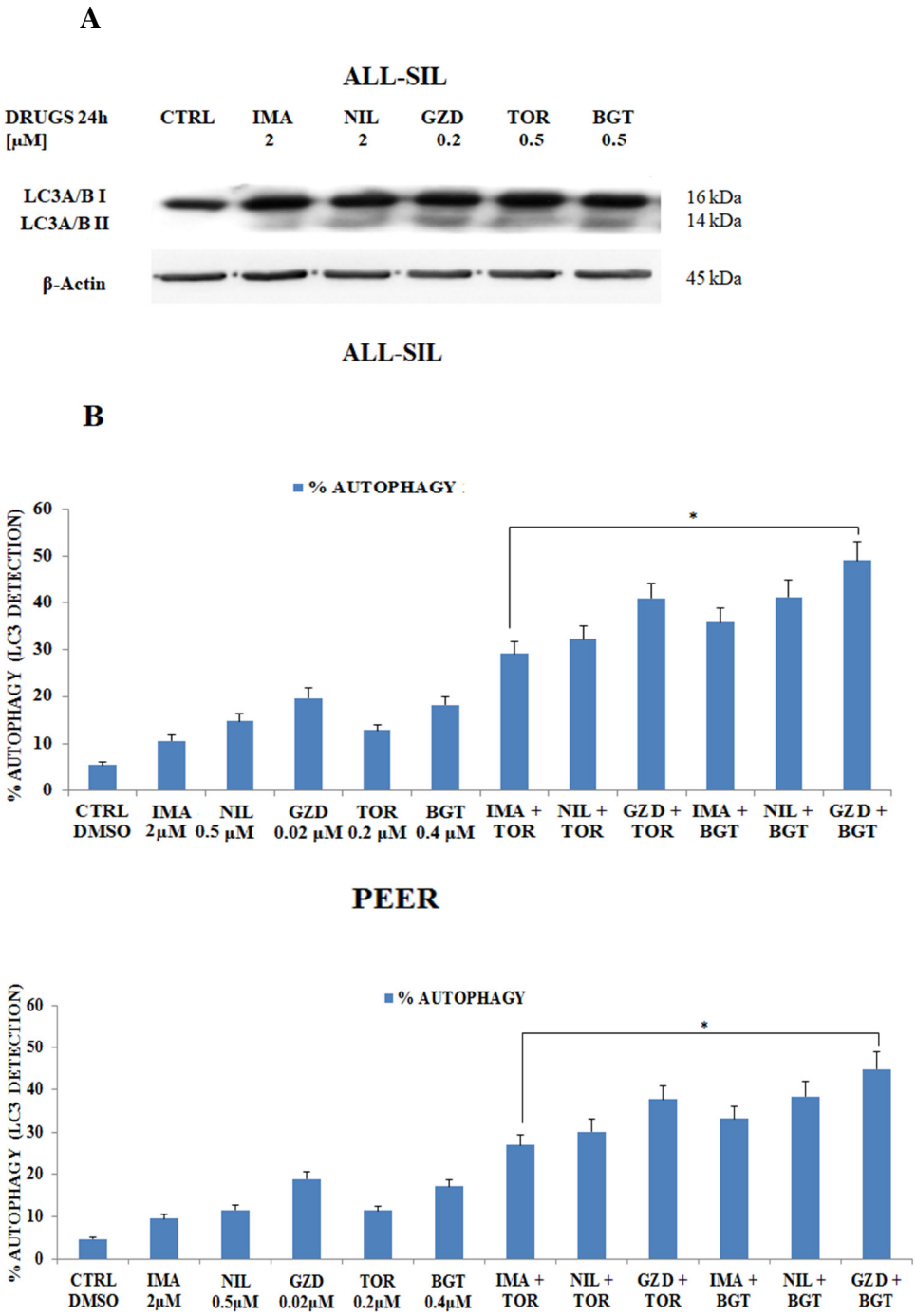


Figure 23: Imatinib, Nilotinib and GZD824 with NVP-BGT226 or Torin-2 induced autophagy in Nup214-Abl1 positive T-ALL cell lines. (A) Western Blot analysis in the ALL-SIL cell line treated with single administration of Imatinib, Nilotinib, GZD824, Torin-2 and NVP-BGT226 for 24 h. An increase of expression of fast-migrating (lipidated) LC3A/B after drug treatments is shown. Twenty-five μg of protein were blotted on each lane. β-Actin documented equal lane loading. (B) Flow cytometric analysis of autophagy in the ALL-SIL and PEER cell lines treated with single or combined administration of Imatinib, Nilotinib, GZD824 with Torin-2 or BGT226 for 24 h. CTRL, control (untreated) cells. Asterisks indicate significant differences in comparison to single drug treated samples (*$p<0.05$). Imatinib, Nilotinib, GZD824, Torin-2 and NVP-BGT226 were abbreviated in IMA, NIL, GZD, TOR and BGT.

4.17 PI3K inhibitors affect cell viability of Ph^+ B-ALL cell lines

Based on data obtained in Nup214-Abl1 positive T-ALL, it has been expanded the research to Ph^+ B-ALL, a subtype that expresses the Bcr-Abl1 fusion protein which represents a negative prognostic factor. Constitutive activation of the PI3K/Akt/mTOR network is a common feature of B-ALL, influencing cell growth and survival.

In order to establish the role of the different PI3K catalytic subunits in sustaining leukemic cells proliferation and survival, it has been investigated by Western blot analysis the baseline expression of Bcr-Abl1 and its substrate CrkL, the PI3K catalytic subunits and the key enzymes of the PI3K/Akt/mTOR network in three pre-B-ALL cell lines (TOM-1, BV-173 and SUP-B15). As shown in Fig. 24, Bcr-Abl1 and CrkL, the p110α, -β, -γ, -δ, PI3K catalytic subunits and the downstream PI3K substrate Akt and the pathway negative regulator PTEN were expressed and/or phosphorylated in all cell lines. For the inhibition of p110α, p110β, p110γ and p110δ, it has been employed ZSTK474, BYL719, TGX221, CAL101 and AS605240, whose selectivity has been reported [110]. Because of the prominent role of p110δ and p110γ isoforms in white blood cells, effects of the γ/δ dual inhibitor IPI145, as well as of a combination consisting of CAL101 and AS605240, were also evaluated. Cell lines were cultured with increasing concentrations of the drugs for 48 h followed by metabolic activity assessment by MTS assay (Fig. 25A). In all cell lines, cell viability decreased after treatment with ZSTK474 with IC50 values close to 0.5 μM. Selective inhibition of p110α, p110β, p110γ and p110δ isoforms had less efficacy, and IC50 values were not attained up to a 10 μM concentration. Longer time points (72 h) did not further affect the metabolic activity of inhibitor-treated cell lines (data not shown). It has been decided to focus the study on ZSTK474 and γ/δ dual inhibitor IPI145 as well as on the p110α inhibitor BYL719, whose role in this type of leukemia has not been thoroughly investigated yet [99, 205, 206]. The PI3K/Akt/mTOR network, its expression and modulation status of key components were assessed to evaluate the efficacy of these inhibitors. Cells were treated with 0.25 μM ZSTK474, 2.5 μM BYL719 or IPI145 and Western blot analysis was performed. These concentrations were chosen as they produced the maximal effects on protein dephosphorylation at the lowest dose. All the PI3K isoform inhibitors decreased the phosphorylation level of the PI3K/Akt/mTOR axis key substrates Akt and S6 ribosomal protein (Fig. 25B).

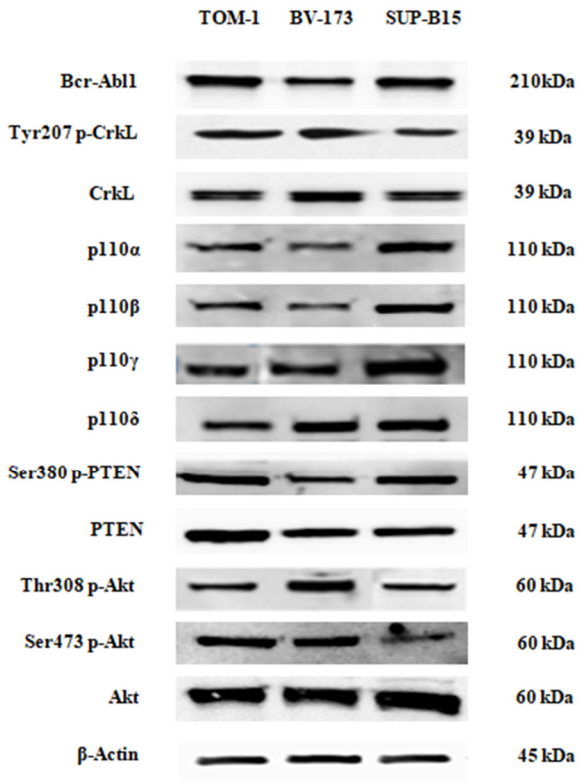
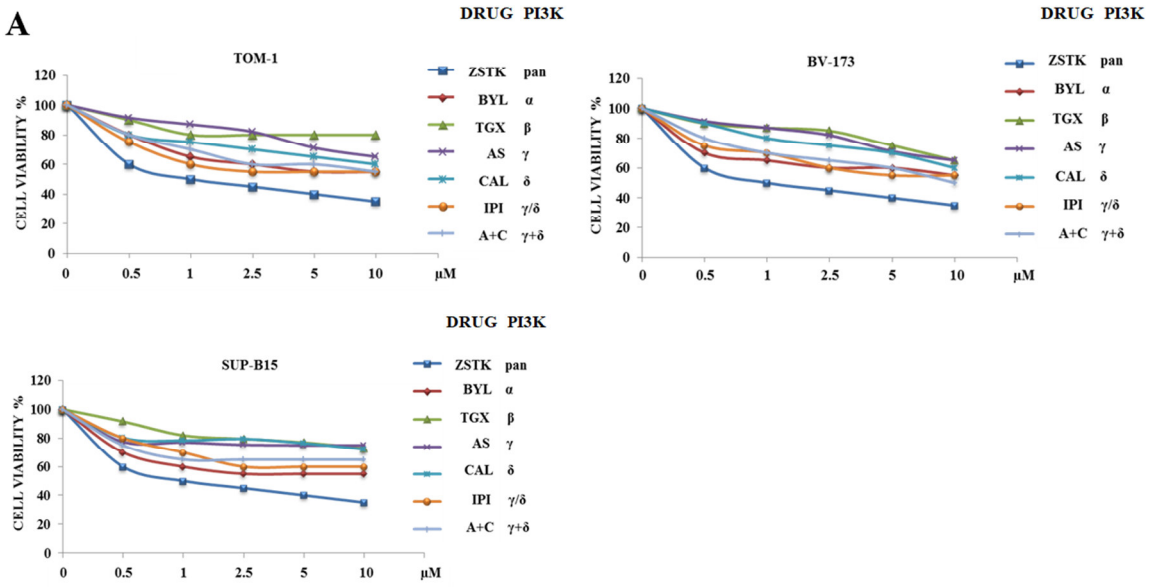


Figure 24: Bcr-Abl1 protein level, PI3K catalytic subunits expression (p110α, p110β, p110γ, p110δ), and phosphorylation level of PI3K/Akt/mTOR pathway key substrates in Ph$^+$ B-ALL cell lines. Western blot analysis of Ph+ B-ALL cell lines to detect the expression of Bcr-Abl1 protein, PI3K catalytic subunits and phosphorylation levels of PTEN, CrkL and Akt protein. Twenty-five µg of protein were blotted on each lane. β-actin was revealed as loading control. One representative of three different blots is shown.



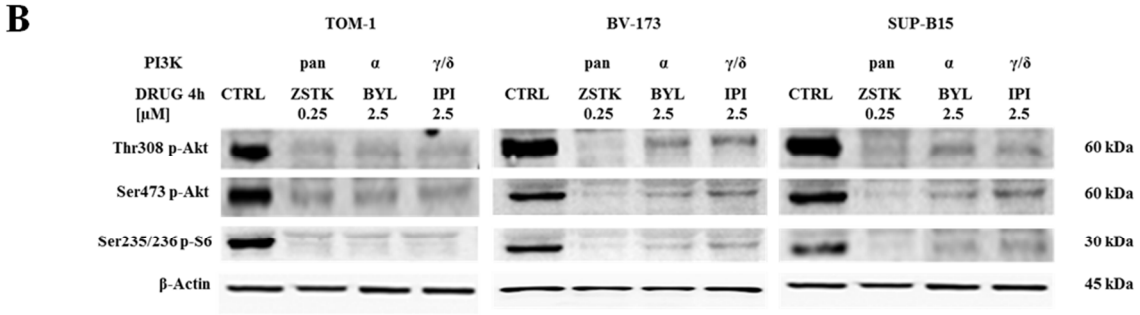
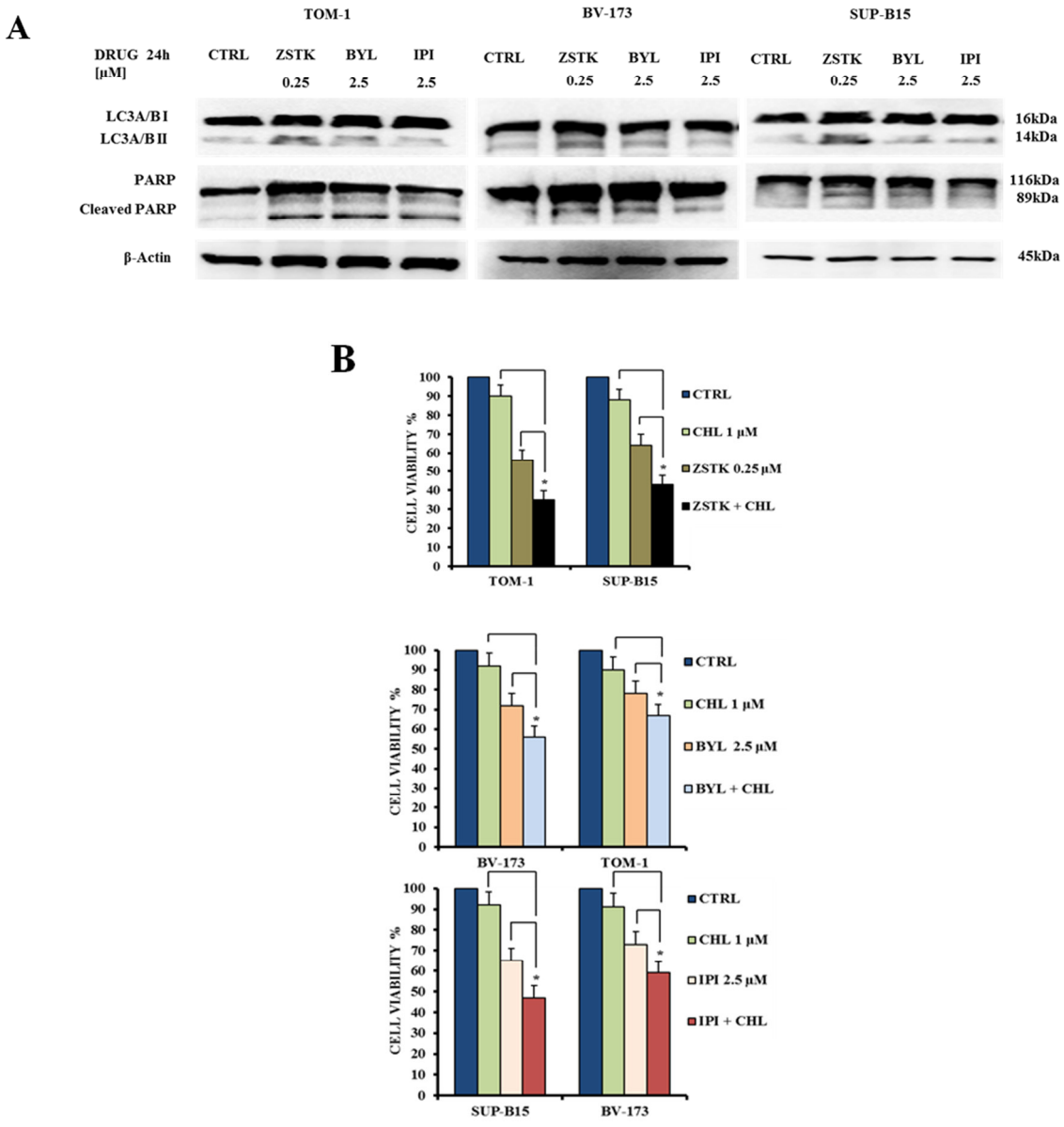


Figure 25: Cytotoxicity of PI3K isoform inhibitors and modulation of PI3K/Akt/mTOR pathway in TOM-1, BV-173 and SUP-B15 cell lines. (A) MTS assays of Ph^+ B-ALL cell lines treated for 48 h with increasing concentrations of ZSTK474, BYL719, TGX221, AS605240, CAL101, IPI145 and AS605240 combined with CAL101. ZSTK474, BYL719, TGX221, AS605240, CAL101 and IPI145 inhibitors were shortened in ZSTK, BYL, TGX, AS, CAL, IPI and A+C respectively. When administered together, AS605240 + CAL101 were used at the same concentrations as specified on the X-axis. Results are the mean of three separate experiments. SD was less than 7%. (B) Western blot analysis of TOM-1, BV-173 and SUP-B15 cell lines treated for 4 h with selected PI3K isoform inhibitors ZSTK474, BYL719 and IPI145 to detect the phosphorylation level expression of Akt and S6 ribosomal protein. Twenty-five μg of protein were blotted on each lane. β-actin was revealed as loading control. Control (untreated cells), ZSTK474, BYL719 and IPI145 inhibitors were abbreviated in CTRL, ZSTK, BYL and IPI respectively. One representative of three different blots is shown.

4.18 Autophagy and apoptosis are induced by PI3K inhibitors in Ph^+ B-ALL cell lines

Autophagic process is an evolutionarily mechanism involved in the degradation of cellular components and is crucial for the maintenance of cell homeostasis [207]. Altered autophagy is related with cancer and has been implicated in the disease development and survival [208-210]. To assess whether the administration of PI3K inhibitors could induce autophagy, it has been analyzed LC3A/B I (non lipidated) and LC3A/B II (lipidated) levels by Western blot in TOM-1, BV-173 and SUP-B15 cell lines after 24 h (Fig. 26A). LC3A/B II expression increased with ZSTK474 pan-inhibitor treatment in all cell lines and to a lower extent with BYL719 and IPI145, indicating the induction of autophagy. To analyze whether the reduced viability was apoptosis-related, cell lines were treated with the inhibitors for 24 h at the same doses used to detect autophagy. Poly (ADP-ribose) polymerase (PARP) cleavage in TOM-1, BV-173 and SUP-B15 cells demonstrated the pro-apoptotic effect induced by the PI3K inhibitors. As demonstrated for the autophagy marker LC3A/B, the expression levels of cleaved PARP increased when cell lines were treated with ZSTK474 pan-inhibitor, or the p110α-selective inhibitor BYL719 and to a lower extent with IPI145 (Fig. 26A). The role of autophagy was further investigated using the lysosomal inhibitor chloroquine at 1 μM. In all cases, chloroquine increased the cytotoxic effects of the PI3K inhibitors, suggesting that autophagy is a protective

mechanism against these drugs (Fig. 26B). Moreover, caspase 3/7 activation was quantified by measuring the enzymatic cleavage of the profluorescent component rhodamine 110, bis-N-CBZL-aspartyl-L-glutamyl- L-valyl-L-aspartic acid amide (Z-DEVD-R110), with release of the strongly fluorescent rhodamine 110-cleaving group. The data on caspase 3/7 activity were in agreement with those of Western blot on PARP cleavage (Fig. 26C).



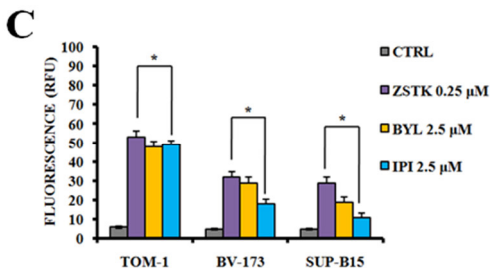


Figure 26: Selected PI3K isoform inhibitors induced autophagy and apoptosis in Ph$^+$ B-ALL cell lines. (A) Western blot analysis documenting the increase of expression of fast-migrating (lipidated) LC3A/B and PARP cleavage in TOM-1, BV-173 and SUP-B15 cells treated for 24 h with ZSTK474, BYL719 and IPI145. Twenty-five µg of protein were blotted on each lane. β-actin documented equal lane loading. Control (untreated cells), ZSTK474, BYL719 and IPI145 inhibitors were abbreviated in CTRL, ZSTK, BYL and IPI respectively. One representative of three different blots is shown. (B) MTS assay documenting the effects of the autophagy inhibitor chloroquine (CHL) on the viability of Ph$^+$ B-ALL cell lines treated for 24 h with the indicated drugs. Results are the mean of three different experiments ± SD. Asterisks indicate significant differences with respect to untreated cells (*$p<0.05$). (C) Enzymatic cleavage of the profluorescent substrate Z-DEVD-R110, with release of the intensely fluorescent rhodamine 110-cleaving group, after PI3K inhibitor treatment. The analysis was performed after 24 h of treatment with the drugs. Results are the mean of three different experiments ± SD. Asterisks indicate significant differences with respect to untreated cells (*$p<0.05$).

4.19 Bcr-Abl1 inhibitors decreased cell viability in Ph$^+$ B-ALL cell lines

It has been examined by MTS assay the IC50 value of each Bcr-Abl1 inhibitor in the three cell lines. After 48 h of drug administration, the cells showed different drug sensitivity. Imatinib attained an IC50 of 1.1 µM in BV-173 cells whereas in TOM-1 and SUP-B15 cell lines IC50 was not attained up to a concentration of 20 µM and 10 µM, respectively. Nilotinib IC50 ranged from 0.1 µM in BV-173 cells to 7.2 µM in TOM-1 cells. GZD824 showed the greatest potency, with IC50 values ranging from 0.03 µM in BV-173 to 0.2 µM in TOM-1 cells (Fig. 27 and Table 6).

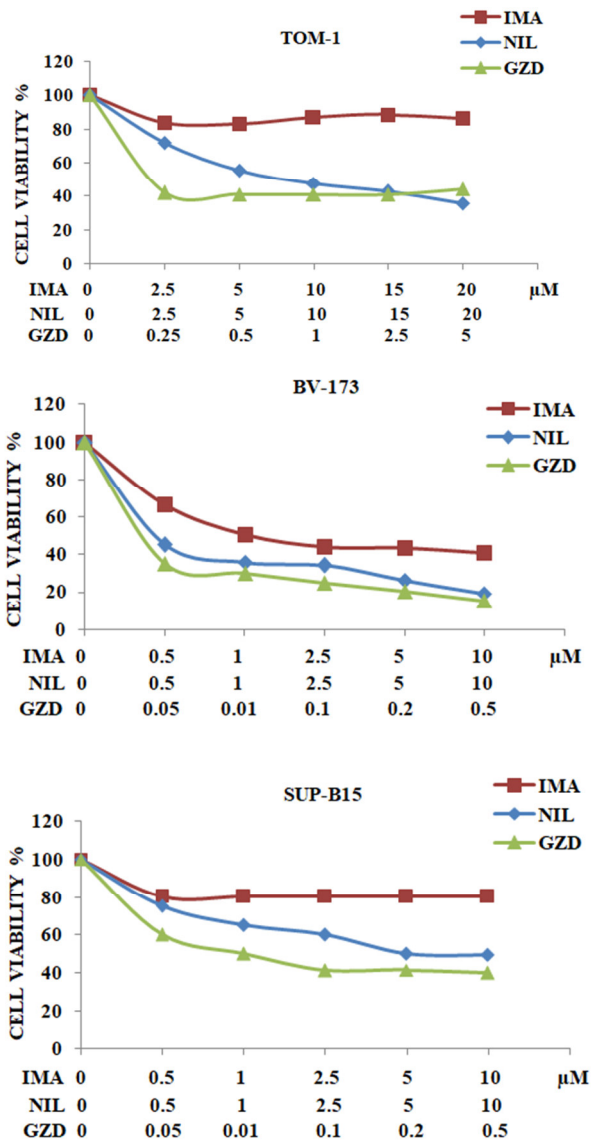


Figure 27: Cytotoxicity of anti Bcr-Abl1 drugs in TOM-1, BV-173 and SUP-B15 cell lines. MTS assays of Ph^+ B-ALL cell lines treated with increasing concentrations of Imatinib, Nilotinib and GZD824 for 48 h. Results are the mean of three separate experiments. SD was less than 10%. Imatinib, Nilotinib and GZD824 drugs were abbreviated in IMA, NIL and GZD respectively.

	IMATINIB	NILOTINIB	GZD824
TOM-1	>10	7.2 ±0.57	0.2 ±0.01
BV-173	1.1 ±0.07	0.1 ±0.01	0.03 ±0.002
SUP-B15	>10	5.8 ±0.46	0.08 ±0.005

Table 6: IC50 and SD values of cells treated for 48 hours with anti Bcr-Abl1 drugs. IC50 values are expressed in μM. Results are the mean of three different experiments ± SD.

4.20 Expression levels of pCrkL protein and PI3K/Akt/mTOR signaling pathway substrates

Requirement for CrkL kinase activity has been demonstrated in a recent report with Bcr-Abl1 transformation and oncogenic signal transduction [211]. As shown in Fig. 28A, cells were exposed for 48 h to Imatinib, Nilotinib or GZD824, and lysates were analyzed by Western blot with an antibody directed to the phosphorylated forms of CrkL and S6 ribosomal protein. All anti Bcr-Abl1 drugs decreased the levels of phosphorylated CrkL with little or no effects on S6 ribosomal protein. In contrast, cell treatment with 0.25 μM ZSTK474 or 2.5 μM BYL719 or IPI145 for 48 h did not affect the phosphorylation levels of pCrkL, while the expression of phosphorylated S6 ribosomal protein was abolished (Fig. 28B).

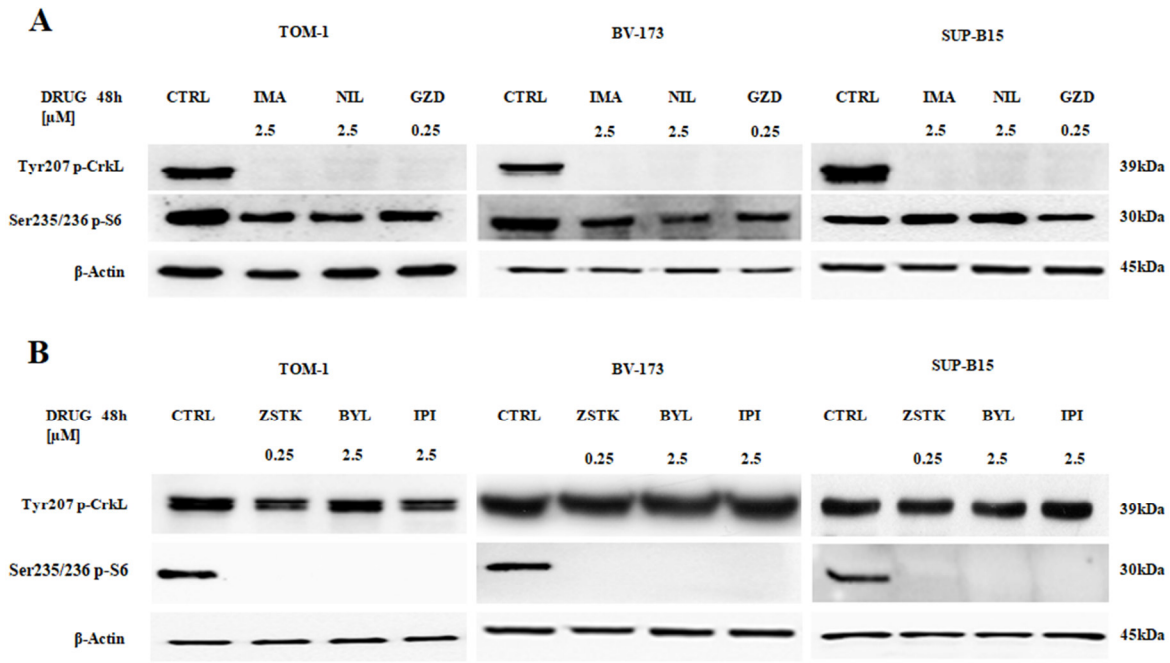


Figure 28: Expression and phosphorylation status of CrkL and S6 ribosomal protein in Ph$^+$ B-ALL cell lines. (A) Western blot analysis of TOM-1, BV-173 and SUP-B15 cells treated for 48 h with 2.5 µM of Imatinib, Nilotinib and 0.25 µM of GZD824. A decrease of the phosphorylated expression of tyrosine kinase CrkL and a slight decrease of the phosphorylated S6 ribosomal protein in all cell lines is shown. Twenty-five µg of protein were blotted on each lane. β-actin documented equal lane loading. One representative of three different blots is shown. Control (untreated cells) was abbreviated in CTRL. Imatinib, Nilotinib and GZD824 drugs were abbreviated in IMA, NIL and GZD, respectively. (B) Western blot analysis of TOM-1, BV-173 and SUP-B15 cells treated for 48 h with 0.25 µM of ZSTK474 and 2.5 µM of BYL719 and IPI145. Twenty-five µg of protein were blotted on each lane. β-actin documented equal lane loading. One representative of three different blots is shown. Control (untreated cells) was abbreviated in CTRL. ZSTK474, BYL719 and IPI145 inhibitors were abbreviated in ZSTK, BYL and IPI, respectively.

4.21 Synergistic cytotoxic effects combining selected PI3K isoform inhibitors with anti Bcr-Abl1 drugs

It has been analyzed whether the combination of ZSTK474, BYL719 or IPI145 with Imatinib, Nilotinib or GZD824 could increase cytotoxic effects on Ph$^+$ B-ALL cells. Therefore, MTS assays were performed in SUP-B15 cells treated with the different drugs administered together for 48 h. As shown in Fig. 29, SUP-B15 were responsive and showed a good, synergistic cytotoxicity as demonstrated by the CIs. As representative of the combined treatments, MTS assays on TOM-1 and BV-173 cell lines were also performed with ZSTK474/Imatinib, BYL719/Nilotinib or IPI145/GZD824. As expected, the combination of the drugs resulted in a potent synergism also at low concentrations of each inhibitor in these cell lines (Fig. 30A and 30B).

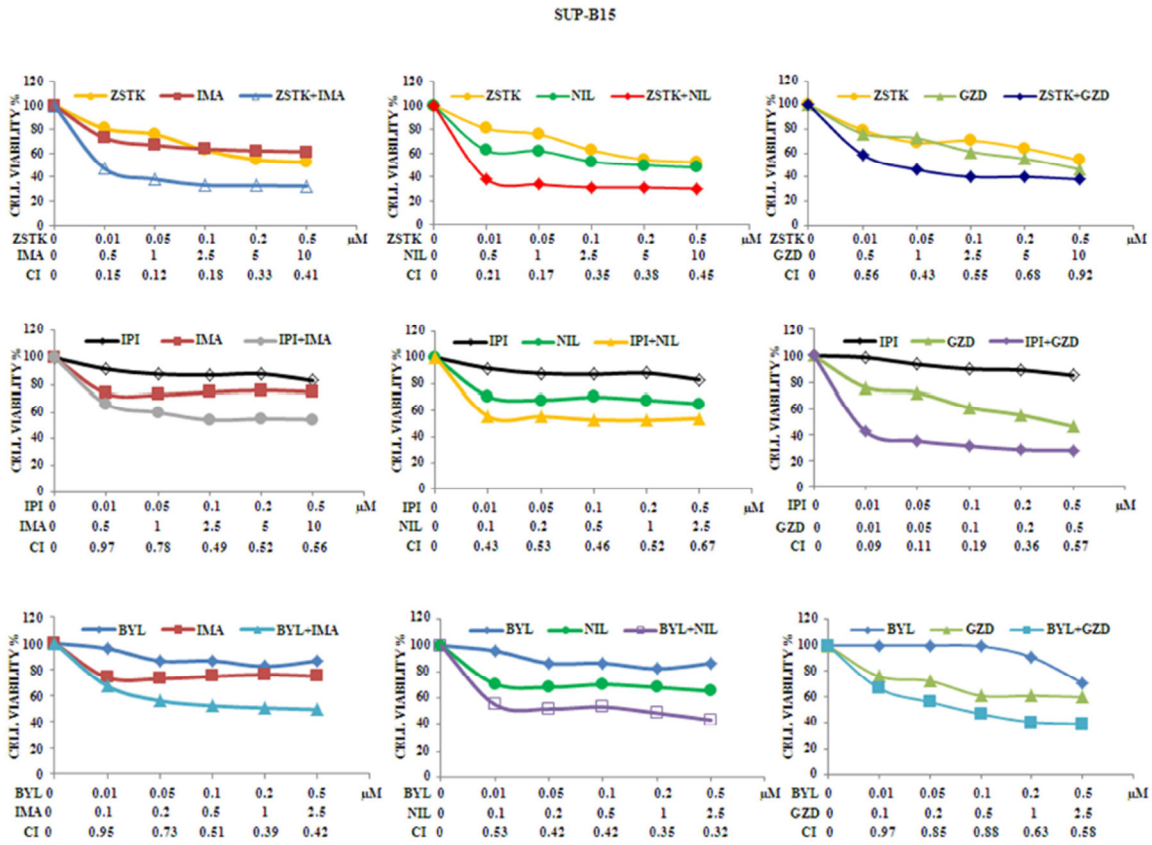


Figure 29: Cytotoxicity and synergism of selected PI3K isoform inhibitors combined with anti Bcr-Abl1 drugs in SUP-B15 cell line. MTS assays of SUP-B15 cells treated with ZSTK474, BYL719, IPI145, Imatinib, Nilotinib and GZD824 alone or with the combinations indicated in the graph legends. The analysis was performed after 48 h of treatment. Results are the mean of three separate experiments. SD was less than 7%. ZSTK474, BYL719, IPI145, Imatinib, Nilotinib and GZD824 inhibitors were abbreviated in ZSTK, BYL, IPI, IMA, NIL and GZD, respectively.

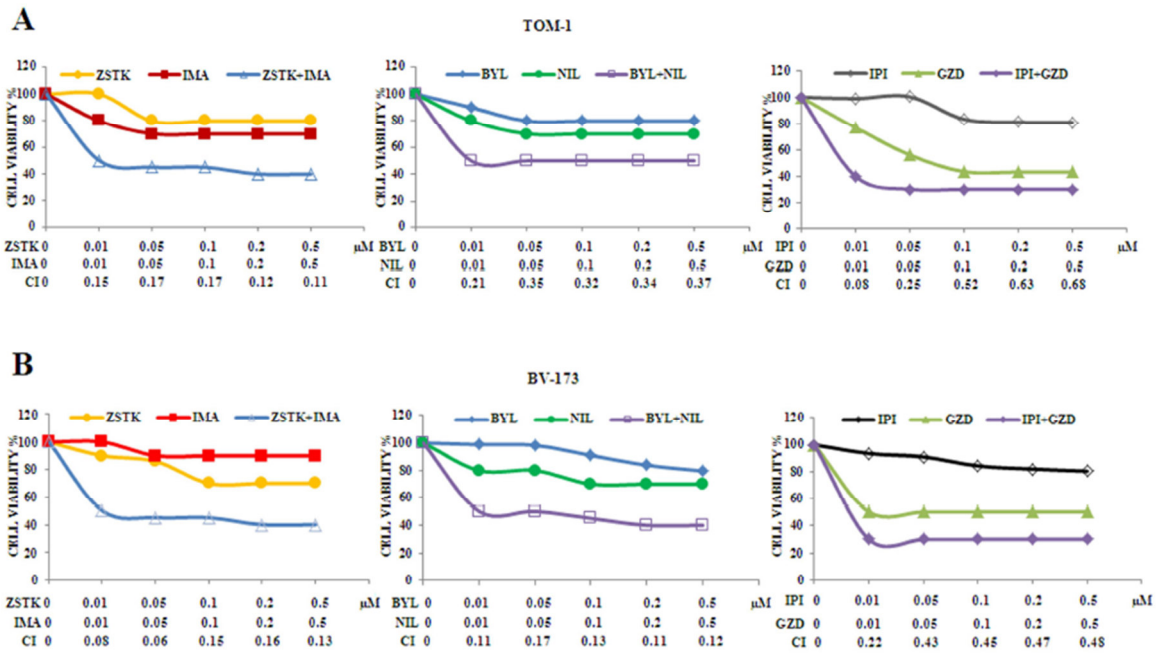


Figure 30: Cytotoxicity and synergism of selected PI3K isoform inhibitors combined with anti Bcr-Abl1 drugs in TOM-1 and BV-173 cell lines. (A) MTS assays of TOM-1 cells treated with ZSTK474, BYL719, IPI145, Imatinib, Nilotinib and GZD824 alone or with the combinations indicated in the graph legends. The analysis was performed after 48 h. Results are the mean of three separate experiments. SD was less than 10%. (B) MTS assays of BV-173 cells treated with ZSTK474, BYL719, IPI145, Imatinib, Nilotinib and GZD824 alone or with the combinations indicated in the graph legends. The analysis was performed after 48 h of treatment. Results are the mean of three separate experiments. SD was less than 7%. ZSTK474, BYL719, IPI145, Imatinib, Nilotinib and GZD824 inhibitors were abbreviated in ZSTK, BYL, IPI, IMA, NIL and GZD, respectively.

4.22 PI3K isoforms inhibition combined with anti Bcr-Abl1 drugs increased apoptotic effects in Ph$^+$ B-ALL cells

The apoptotic effects of the single and combined drug administration compared to the control in the three cell lines was then evaluated (Fig. 31A–31C). The analysis was performed by Annexin-V-FITC staining and PI in flow cytometry after 24 h of drug treatments. The drug concentrations used were 0.25 µM for ZSTK474 and GZD824, and 2.5 µM for the other inhibitors. The combined administration of the PI3K inhibitors with anti Bcr-Abl1 drugs showed a significant increase in the percentage of apoptotic cells.

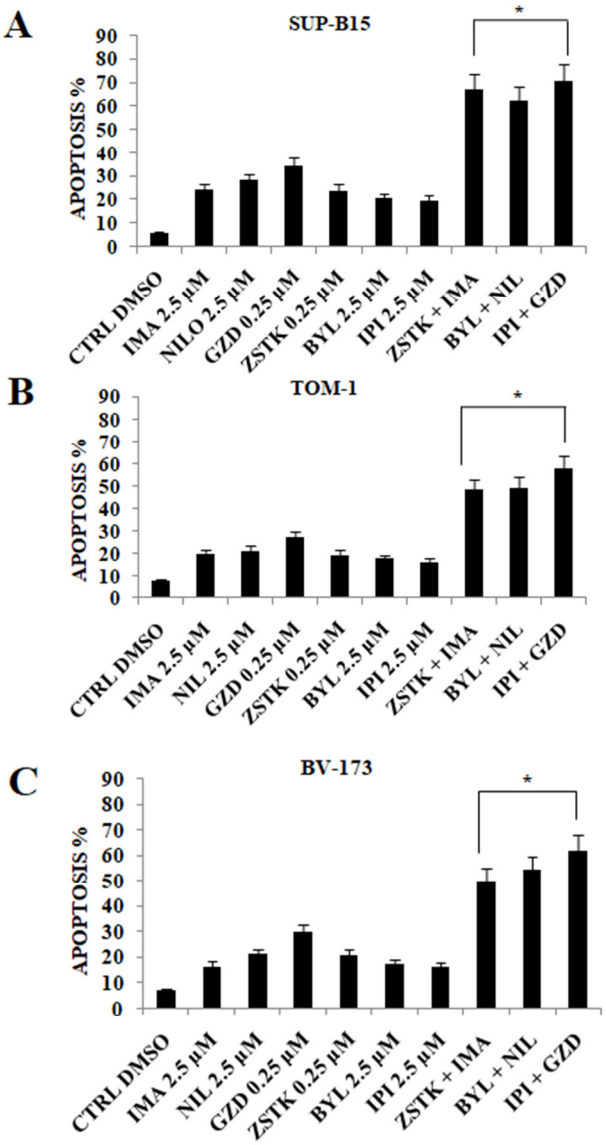


Figure 31: Flow cytometric analysis of drug-induced apoptosis by using selected PI3K isoform inhibitors in combination with anti Bcr-Abl1 drugs in Ph$^+$ B-ALL cell lines. (A) Analysis of Annexin-V positive cells after ZSTK474, BYL719, IPI145 and Imatinib, Nilotinib, GZD824 treatment alone or in combination in SUP-B15 cells. The analysis was performed after 24 h of treatment with 2.5 µM Imatinib, Nilotinib, BYL719 and IPI145 inhibitors, and 0.25 µM GZD824 and ZSTK474 drugs. The data are representative of three experiments ± SD. (B) Analysis of Annexin-V positive cells after ZSTK474, BYL719, IPI145 and Imatinib, Nilotinib, GZD824 treatment alone or in combination in TOM-1 cells. The analysis was performed after 24 h of treatment with 2.5 µM Imatinib, Nilotinib, BYL719 and IPI145 inhibitors and 0.25 µM GZD824 and ZSTK474 drugs. The data are representative of three experiments ± SD.
(C) Analysis of Annexin-V positive cells after ZSTK474, BYL719, IPI145 and Imatinib, Nilotinib, GZD824 treatment alone or in combination in BV-173 cells. The analysis was performed after 24 h of treatment with 2.5 µM Imatinib, Nilotinib, BYL719 and IPI145 inhibitors, and 0.25 µM GZD824 and ZSTK474 drugs. All samples were analyzed by the Muse™ Cell Analyzer. Results are the mean of three different experiments ± SD. In all figures asterisks indicate significant differences compared to control and single treatments (*$p < 0.05$). In all figures Control (untreated cells), ZSTK474, BYL719, IPI145, Imatinib, Nilotinib and GZD824 inhibitors were abbreviated in CTRL, ZSTK, BYL, IPI, IMA, NIL and GZD, respectively.

4.23 PI3K isoforms inhibition combined with anti Bcr-Abl1 drugs induced increased autophagy in Ph^+ B-ALL cells

To better quantify autophagy, the detection of LC3A/B was performed in all cell lines by flow cytometry. After a 24 h incubation, a statistically relevant increase in autophagy was induced by combined treatment of TKIs and PI3K inhibitors when compared to single administrations (Fig. 32A–32C).

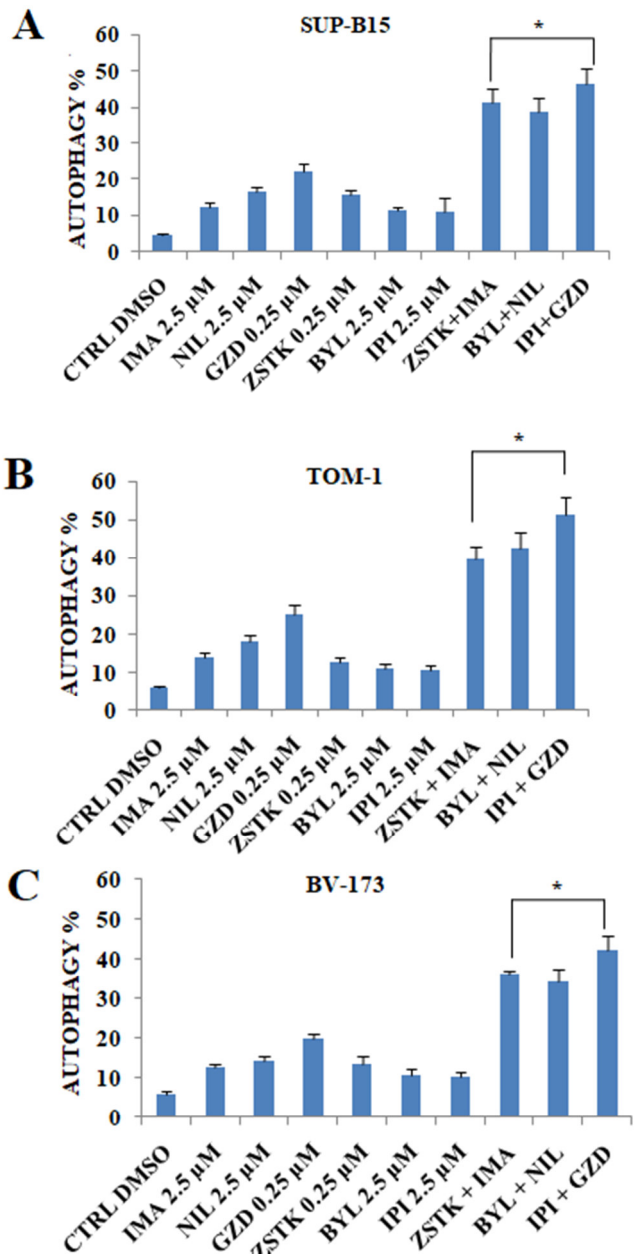


Figure 32: Flow cytometric analysis of drug-induced autophagy by using selected PI3K isoform inhibitors in combination with anti Bcr-Abl1 drugs in Ph$^+$ B-ALL cell lines. (A) Flow cytometric analysis of autophagy LC3 marker in SUP-B15 cell line treated with single drugs or combined administration of Imatinib, Nilotinib, GZD824 with ZSTK474, BYL719 and IPI145. The analysis was performed after 24 h of treatment with 2.5 µM Imatinib, Nilotinib, BYL719 and IPI145 inhibitors and 0.25 µM GZD824 and ZSTK474 drugs. (B) Flow cytometric analysis of autophagy LC3 marker in TOM-1 cell line treated with single drugs or combined administration of Imatinib, Nilotinib, GZD824 with ZSTK474, BYL719 and IPI145. The analysis was performed after 24 h of treatment with 2.5 µM Imatinib, Nilotinib, BYL719 and IPI145 inhibitors and 0.25 µM GZD824 and ZSTK474 drugs. (C) Flow cytometric analysis of autophagy LC3 antibody in BV-173 cell line treated with single and combined administration of Imatinib, Nilotinib, GZD824 with ZSTK474, BYL719 and IPI145. The analysis was performed after 24 h of treatment with 2.5 µM Imatinib, Nilotinib, BYL719 and IPI145 inhibitors and 0.25 µM GZD824 and ZSTK474 drugs. All samples were analyzed by the Muse^{TM} Cell Analyzer. In A-C results are the mean of three different experiments ± SD. In all figures asterisks indicate significant differences compared to control and single treatments (*$p < 0.05$). In A–C control (untreated cells), ZSTK474, BYL719, IPI145, Imatinib, Nilotinib and GZD824 inhibitors were abbreviated in CTRL, ZSTK, BYL, IPI, IMA, NIL and GZD, respectively.

5. DISCUSSION

PI3K/Akt/mTOR signaling upregulation is very common in T-ALL, being detectable in 70–85% of the patients and portends a poorer prognosis [62]. Although the survival of younger patients with acute leukemia has improved in the early 21st century, mutation events can occur at any stage of the disease and negatively influence the response to therapeutic treatments because they can lead to resistance to therapy. A major challenge remains the lifelong morbidity suffered by patients treated with current chemotherapy regimens. For long-term survivors, acute and lasting toxicities remain important issues underlining the critical need of more effective and selective personalized and targeted therapies and treatment strategies.

Although preclinical studies have demonstrated that inhibition of PI3K/Akt/mTOR axis could be an effective strategy for targeted therapy of T-ALL [212] it is still unclear which is the best target in this highly complex and branched signaling network. Pharmaceutical companies have synthesized a wide array of drugs, that target different components of this signaling parthway [213]. This allows to envisage different strategies based on one side on the use of selective inhibitors displaying reduced adverse effects and on the other side pan-inhibitors expected to exert a greater efficacy.

It has been recently documented that PI3K inhibitors were the most effective in inhibiting leukemic cell proliferation and survival in a panel of T-ALL human cell lines, irrespectively of PTEN status. These findings strongly supporting clinical application of class I PI3K pan-inhibitors rather than dual γ/δ or single-isoform inhibitors for treatment of the majority of T-ALL patients [110]. Nevertheless, the functions of the immune system cells could be impaired by these drugs. First of all, it has been evaluated the effects on human healthy $CD4^+$ T-cells of four PI3K/Akt/mTOR inhibitors used as anticancer agents, to explore how these treatments could affect the patient immunological status. No cytotoxic effects were shown by the drugs on heathy T-cells, whereas all the inhibitors and particularly NVP-BGT226 and Torin-2 reached IC50 values in the nanomolar range in T-ALL cells. As to the cell cycle, since the unstimulated $CD4^+$ T lymphocytes are mostly in a quiescent status, the drugs did not exert any effect, thus demonstrating the selectivity of the inhibitors. In contrast, in either stimulated $CD4^+$ T-lymphocytes or MOLT4 and JURKAT cell lines, NVP-BGT226 and Torin-2 induced a concentration-dependent accumulation in the G0/G1 phase, and a concomitant decrease in S and G2-M phases, as reported by others [186, 214, 215]. As shown by Western blot, Torin-2 and NVP-BGT226 effectively blocked the phosphorylation of Akt Ser473 and Thr308, GSK3β Ser21/9, mTORC1 Ser2448 and

mTORC2 Ser2481. Furthermore, a mTORC1 downstream target, S6 protein, was fully dephosphorylated in both stimulated T lymphocytes and T-ALL cells. Healthy unstimulated $CD4^+$ T lymphocytes did not display induction of autophagy as documented by Western blot analysis of LC3A/B. Stimulated T lymphocytes appeared more sensitive to induction of autophagy when compared to unstimulated ones. It has also documented that in leukemic cells the induction of autophagy was a protective mechanism, since cells incubated with autophagy inhibitors were less viable. Similar results were obtained in stimulated T lymphocytes. The drugs also induced apoptosis in both stimulated T lymphocytes and leukemic cells. On the contrary, resting T lymphocytes did not undergo apoptosis. The apoptotic cell death was dependent on caspase activity. Therefore, apoptosis is one of the mechanisms that explain the cytotoxicity of these drugs. It is now assumed that tumor growth and survival can be either promoted or restrained by immune system cells [216, 217]. Therefore, it is critical to define whether novel anti-tumor drugs may impact on cells of the immune system. The ideal targeted therapy should specifically hit cancer cells and enhance anti-tumor immunity while preserving patient immunity. It has been demonstrated that lymphocyte functions were minimally affected by p110α inhibition both *in vitro* and *in vivo*. Although p110α inhibition partially diminished Akt activation, it is likely that selective p110α inhibitors will be less immunosuppressive *in vivo* than p110δ or pan PI3K class I inhibitors [188]. Natural killer cell-mediated cytotoxicity as well as antibody dependent cellular cytotoxicity against tumor cells were significantly impaired by pan class I PI3K inhibitors, whereas p110α selective drugs had no effect [110, 218]. Other authors have shown recently that single inhibitors of class I PI3K isoforms in T-lymphocytes exerted a less potent impairment of T-cell activation than simultaneous inhibition of two or more isoforms [186]. These results suggested that complete blockade of class I PI3K activity strongly impairs T lymphocyte proliferation and activation *in vitro*. In addition, it has been demonstrated that two ATP competitive PI3K pan-class I inhibitors, PX-866 and BKM120, showed differences in their ability to block T-lymphocytes proliferation and IL-2 secretion [186]. Nevertheless, these investigations were restricted only to pan class I PI3K isoform inhibitors and did not explore other drugs, such as Torin-2 and BGT226, which target to additional components of the PI3K/Akt/mTOR axis. Therefore these data expanded the concept that targeted therapies, using different drugs against molecules at different levels of the PI3K/Akt/mTOR cascade, may be effective against tumor cells harboring aberrant upregulation of this signaling network, without affecting at the same time the immune system. Given the commonly observed dysregulation of PI3K/Akt/mTOR pathway in T-ALL, the various types of single or dual

pathway inhibitors under development might be effective in T-ALL treatment, provided that they do not affect the immune system. Therefore, the study of the effects of PI3K/Akt/mTOR inhibition not only in tumor cells, but in immune cells as well, may lead to selection of treatments that, while efficiently targeting deregulated PI3K/Akt/mTOR signals in tumor cells, preserve normal immune function, for appropriately tuning of personalized cancer therapy.

A large variety of inhibitors have been widely used both *in vitro* and *in vivo* in preclinical settings of acute leukemias, where they blocked cell proliferation and induced, sometimes, apoptosis and/or autophagy [53, 219-221].

Several studies have highlighted that both PI3K and mTOR modulators could synergize with a wide range of drugs that are currently in use for treating acute leukemias, including chemotherapeutic drugs [222]. Drugs dual targeting PI3K/Akt/mTOR pathway at various points of the signaling cascade are under evaluation in preclinical models and clinical trials, but the observation that not even the combined inhibition of Akt and mTOR is enough to completely turn off the pathway during chronic treatment is very intriguing [223].

Despite several chemotherapy combinations were tested *in vitro* and are in clinical trials for the treatment of ALL, a new and promising innovative idea, for the individualization of the therapies, could be represented by hitting the same target with multiple specific drugs with different mechanisms of action. After the assessment of drug effect on healthy T-lymphocytes, it has been demonstrated the efficacy of multi-inhibition of the same target, i.e. the Akt protein as a pivotal molecule of the PI3K/Akt/mTOR signaling pathway with three drugs with a totally different mechanism of action in T-ALL cell lines. The triple administration of GSK690693, MK-2206 and Perifosine in T-cells was cytotoxic and synergic at lower doses when compared with the IC50 values. It has been showed the relevance of a fine tuning of the single drug concentration to obtain the best synergistic effect. Single inhibition of Akt was lower when compared with every dual inhibition, which in turn was lower than the triple one. It has also demonstrated the importance of two issues: the compound concentration and the timing of drugs administration. About the first one, two of the drugs, MK-2206 and GSK690693 were very efficient acting synergistically even at low doses, whereas Perifosine administered together did not add its efficacy in the 10–20 μM range. The second issue disclosed that MK-2206 and GSK690693 may act very rapidly (minutes), but Perifosine requires a longer period of time (hours), to enter in action and to really synergize with MK-2206 and GSK690693. These findings indicate that Akt inhibition mechanism may be compatible with a fine tuning of the concentrations of the

single drugs, that may help with low doses to reduce the side effect of the therapy, and with crucial time points in which administer the drugs, that otherwise may improve the efficacy of the therapy. Compensatory upregulation of parallel signaling through the MEK/ERK1/2 pathway in response to PI3K/Akt inhibition, is an emerging theme in cancer cell signal transduction, because it potentially mediates resistance to drug-induced growth inhibition [198]. Indeed, several recent reports have highlighted the importance of functional crosstalks between the MEK/ERK1/2 and PI3K/Akt signaling networks, in response to individual pathway inhibitors [51, 224-226]. The MEK/ERK1/2 pathway often reflects the rebound of Akt inhibition, developing or amplifying hyperactivation. Unexpectedly, these results displayed an inhibition of ERK activation in correspondence of multiple drugs administration. It is tempting to speculate that the multi-Akt inhibition could represent an effective treatment to block crosstalk between PI3K/Akt/mTOR and Raf/MEK/ERK reducing tumor growth and cells proliferation. In conclusion, these preclinical findings strongly demonstrated that a multiple inhibition of Akt could represent a new promising therapeutic strategy to overcome relapse or resistance in the treatment of T- ALL patients.

miRNAs are an evolutionarily conserved class of small, regulatory non-coding RNAs that negatively regulate protein coding gene and other non-coding transcripts expression. Further, widespread deregulation of miRNAs have been reported in several cancers, with several miRNAs playing oncogenic and tumor suppressive roles. In addition, they can be used as prognostic biomarkers and as targets for novel therapies against leukemia. A single miRNA targets many genes and it this matches the question whether or not targeting a single or multiple genes leads to hematological malignancies, as well as interfering with several pathways which are involved in disease development.

The interest on miRNA studies aimed to analyze drug effects on miRNA expression. Therefore, these data by qRT-PCR have shown that these targeted therapies used in this research could affect the expression of miRNA involved in PI3K activation and ALL disease. Indeed, it was very interesting to verify that the drugs upregulated tumor suppressor miR-150 expression, whereas they downregulated oncogenic miR-210 and miR-221 expression, but further experiments are necessary to confirm these data. The main goal of this research could be focused on study the role of miRNAs in *in vivo* models (mouse model) and analyze how the miRNA expression could be manipulate by the drugs as a new therapeutic approach. It is likely that in the next few years, new miRNA-based diagnostic, prognostic and predictive kits will be available for clinicians and miRNA-

derived treatments will begin their clinical trial journey towards the development of new clinical agents.

Ph^+ ALL accounts for 25–30% of adult ALL and its incidence increases with age in adults >40 years old. Irrespective of age, the Abl1 fusion genes are markers of poor prognosis and amplification of the Nup214-Abl1 oncogene can be detected mainly in patients with T-ALL. T cell malignancies harboring the Abl1 fusion genes are sensitive to many cytotoxic agents, but up to date complete remissions have not been achieved.

The Nup214-Abl1 fusion gene has been described in about 6% of patients with T-ALL [227, 228]. Nup214-Abl1 is the most frequent and highly specific for T-ALL whereas Bcr-Abl1 and ETV6-ABL1 are very uncommon in T-ALL and are more frequently associated with other hematologic malignancies [48]. Abl1 is reported to be fused to the Bcr gene in CML and in pre-B ALL as a consequence of the Philadelphia translocation t(9;22) (q34;q11). Abl1 fusion proteins are involved in the pathogenesis of T-ALL despite the fact that they are infrequent in this hematological malignancy [229]. Nup214-Abl1 discovery has emphasized the T-ALL genetic heterogeneity, but more relevant, has revealed new perspectives for targeted therapies using TKIs [230]. However, it is emerging that resistance to TKIs could develop due to activation of other signaling pathways such as the PI3K/Akt/mTOR axis [137]. T-ALL cells frequently display aberrant activation of this signaling pathway, which is due to several causes. It has been used ALL-SIL, PEER and BE-13 T-ALL lines harboring Nup214-Abl1 fusion gene. All these cell lines express PTEN [231, 232] in its phosphorylated form, hence inactivated [61] (data not shown). With the above in mind, it has been decided to investigate whether selective inhibitors of the PI3K/Akt/mTOR could synergize with TKIs in T-ALL cells lines displaying Nup214-Abl1 fusion protein. GSK690693, NVP-BGT226, ZSTK474 and Torin-2 were used to better define their synergism with Imatinib, Nilotinib and GZD824, in terms of viability, apoptosis and cell cycle progression. These data showed that the TKIs used inhibited the proliferation of the Nup214-Abl1-positive ALL-SIL, PEER and BE-13 cells. The third generation TKI GZD824 exhibited a stronger anti-proliferative activity than either Imatinib or Nilotinib. All the TKIs fully abrogated CrkL phosphorylation at Tyr207 in Nup214-Abl1-positive cell lines. The TKIs barely inhibited the PI3K/Akt/mTOR pathway. This finding suggested that this signaling cascade is almost independent from Nup214-Abl1. Similarly, the drugs against PI3K/Akt/mTOR signaling did not affect CrkL phosphorylation. Given the independence of these two signaling pathway, it is noteworthy that it has been found a synergistic effect between TKIs and SMIs that increased the efficacy of single drug administration. Abl1 fusion protein have been often described as a

cause of resistance to therapy, also related to the development of mutant clones in relapsed hematologic malignancies [233]. Bcr-Abl1 independent PI3K activation has been reported as the cause of Imatinib resistance. In agreement with these data, these authors showed that PI3K/Akt/mTOR activity was unaffected after treatment with Imatinib [137]. In Ph^+ leukemic cell lines Nilotinib resistance was overcome by the blockade of PI3K/mTOR by using PI3K/mTOR inhibitor BEZ235 and through translational down regulation of MDM2 [234]. These observations were carried out mainly on CML cells that express the Bcr-Abl1 fusion protein. Currently, the addition TKIs to cytotoxic agents constitutes the therapeutic mainstay for Bcr-Abl1-positive B-ALL patients [49]. It has been extended these studies to a different Abl1 fusion protein, Nup214-Abl1 and it has been used a third generation TKI combined with a large panel of inhibitors of the PI3K/Akt/mTOR axis. The mechanism of resistance to Abl1 inhibitors could be observed also in Nup214-Abl1 positive T-ALL [201]. Indeed, it has been very recently reported two cases with refractory B-ALL and Nup214-Abl1 fusion [232, 235]. On the basis of the results obtained, it is justified to hypothesize that a combined therapy consisting of Abl1 TKIs and PI3K/Akt/mTOR inhibitors may offer a new therapeutic option for T-ALL patients carrying Nup214-Abl1 fusion kinase to overcome the resistance to TKIs. It has been demonstrated here that autophagy is increased when Nup214-Abl1 harboring cells are treated with TKIs or SMIs, alone or in combination. Combining autophagy inhibitors with different drugs for the treatment of Ph^+ leukemias may result in synergistic responses and may open new therapeutic options [236]. TKI therapy renders treatable Nup214-Abl1 subgroup of patients with T cell malignancies [201]. The TKIs investigated in this study showed different levels of efficacy with the Nup214-Abl1 ALL cell lines used: efficacy increased from first (Imatinib) to second (Nilotinib) to third (GZD824) generation. More relevant, TKIs administration when associated with SMIs against PI3K/Akt/mTOR showed a marked synergistic effect. This association might form a novel scheme to treat TKI-resistant Nup214-Abl1 T-ALL patients.

It has been reported that targeted cancer therapy could be more effective and less harmful than conventional chemotherapy [237]. PI3K plays a key role in regulating cell cycle, survival and metabolism, and the PI3K signaling cascade is a very frequently altered axis in human tumors [238]. Different compounds targeting members of the PI3K have been synthesized and are now in clinical trials. Better understanding of the PI3K pathway modulation have led to a rational development and utilization of PI3K inhibitors in patients with leukemias. The pan-PI3K ZSTK474 inhibitor and the dual γ/δ IPI145 inhibitor are undergoing analysis and are already demonstrating increasing preclinical and clinical

results [96, 239]. For example, ZSTK474 inhibitor displayed a potent anticancer activity in human tumor xenografts [240] while BYL719 has shown synergistic antineoplastic efficacy when used in endocrine cure against ER^+/PIK3Cα mutated breast tumor cells [241]. In Ph^+ B-ALL cells, relapses are very frequent, especially in adults, with a very poor prognosis, highlighting the need for new therapeutic strategies [242]. Moreover, PI3K network has been strongly involved either in malignant transformation and in the development of TKI-resistance in Ph^+ B-ALL [243]. It has been explored the therapeutic potential of the PI3K pan-inhibitor ZSTK474, the p110α inhibitor BYL719 and dual γ/δ inhibitor IPI145 in a set of Ph^+ B-ALL cells. It is worth noting that these cell lines showed an hyperphosphorylation of PTEN, that results in its inactivation and hyperactivation of PI3K/Akt/mTOR signaling network [61]. It has been reported that ZSTK474 reduced cell viability and caused both autophagy and apoptosis in Ph^+ B-ALL cells. ZSTK474, when administered in the micromolar range, synergized in all cell lines with either Imatinib, Nilotinib or GZD824. In this study, it has been analyzed for the first time the efficacy of PI3K isoform-selective inhibitors in Ph^+ B-ALL. BYL719 and IPI145 were cytotoxic in the micromolar range to Ph^+ B-ALL cell lines, as shown by MTS and Annexin V-stained samples analysis. However, ZSTK474 was more cytotoxic than BYL719 and IPI145, as it displayed an IC50 value of 0.5 μM. The phosphorylation level of the key components of the PI3K/Akt/mTOR axis, analyzed by Western blot, showed the same sensitivity to ZSTK474 inhibition in all cell lines. It should be highlighted that another recent study has demonstrated the efficacy of the pan-PI3K inhibitor, NVP-BKM120, in pre-clinical models of Ph^+ B-ALL. Interestingly, this inhibitor displayed an IC50 of 0.5 μM which is the same as that of ZSTK474. However, it should be considered that NVPBKM120 displays considerable off-target effects [244] which so far have not been reported for ZSTK474. A greater efficacy was also evident in case of GZD824, a third generation anti Bcr-Abl1 drug, which showed an increased anti-leukemic activity in comparison to first and second generation inhibitors in Ph^+ B-ALL cells. When cell lines were treated with anti Bcr-Abl1 drugs the phosphorylated form of CrkL protein was abolished, whereas the phosphorylated S6 ribosomal protein was minimally affected. Interestingly, these findings were in agreement with those of others who documented in their study that Akt activity was surprisingly not inhibited by Imatinib administration of primary CML-cells [245]. This demonstrated the specificity of these inhibitors against the Bcr-Abl1 fusion protein. In contrast, cell samples treated with selected PI3K isoform inhibitors showed an unchanged expression level of phosphorylated CrkL protein and a strong decrease in the phosphorylated S6 ribosomal protein. Overall, the PI3K isoform inhibitors used in this

study displayed increased potency when combined with anti Bcr-Abl1 drugs in terms of cell viability reduction as well as, apoptosis and autophagy induction. Future studies should address the issue of how these drug combinations increase apoptosis and autophagy. In conclusion, these data showed that investigational PI3K inhibitors can block the growth of Ph^+ B-ALL cell lines but are likely to be most effective when used in combination with Bcr-Abl1 inhibitors and provide an important preclinical rationale for future clinical applications.

All these findings strongly demonstrated that the inhibition of the PI3K/Akt/mTOR signaling pathway could represent a new promising therapeutic strategy to improve current treatments against ALL disease.

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